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ORIGINAL RESEARCH

Cytotoxicity against cholangiocarcinoma and HepG2 cell lines of lignan derivatives from *Hernandia nymphaeifolia*

Jittra Suthiwong[1] · Jaroon Wandee[2] · Siripit Pitchuanchom[3] · Punchapat Sojikul[4] · Veerapol Kukongviriyapan[2] · Chavi Yenjai[1]

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Abstract

Twelve lignan derivatives were synthesized from deoxypodophyllotoxin isolated from *Hernandia nymphaeifolia*. Cytotoxicity evaluation against cholangiocarcinoma, KKU-100, and HepG2 cell lines showed that compounds **3**, **9**, **10**, and **13** exhibited stronger cytotoxicity than the starting material, **1**, with IC_{50} ranging from 0.42 to 2.01 μM. Compound **10** displayed interesting activity by showing IC_{50} values of 0.75 and 0.46 μM against KKU-100 and HepG2 cell lines, respectively. From these observation, **10** seems to be useful as a lead compound for the development of anticancer agents.

Keywords *Hernandia nymphaeifolia* · Deoxypodophyllotoxin · KKU-100 · HepG2

Introduction

Hernandia nymphaeifolia is a coastal tree that grows throughout the tropical and subtropical areas. It is found in the southern part of Thailand and called Pho Kra Ding. Its seed is used as a cathartic (Kan 1970). This plant mainly contains lignans and aporphine alkaloids (Chen et al. 1996; Chao et al. 2002; Suthiwong et al. 2018). Several compounds from this plant have shown antiplatelet aggregation and cytotoxicity (Chen et al. 2000). It has been reported that some compounds exhibit anticancer activity against murine P388 lymphocytic leukemia and human cancer, KB16, A549, and HT-29 cell lines (Chen et al. 1997). Many lignans from medicinal plants have shown anti-oxidant, anti-estrogenic, anti-mitotic, and anti-viral activities (Pettit et al. 2004). Because of this pharmacological information, we are interested in the structural modification of a major lignan, deoxypodophyllotoxin, from the seeds of *H. nymphaeifolia* for cancer therapy, especially for cholangiocarcinoma. Deoxypodophyllotoxin has shown cytotoxicity against several cancer cell lines such as KB16, A549, HT-29, Colo205, k562, LNCaP, and PC-3 (Bogucki and Charlton 1995; Wickramaratne et al. 1995; Lim et al. 1999; Jiang et al. 2007). In addition, this compound inhibits 12-O-tetradecanoylphorbol 13-acetate-induced ornithine decarboxylase in cultured mouse epidermal cells (Chang et al. 2000).

In Thailand, cholangiocarcinoma is the cancer of most interest, due to its being one of the major health problems, especially in the northeastern area (Sripa and Pairojkul 2008). It is believed that infection by the liver fluke, *Opisthorchis viverrini*, is the cause of the disease (McGlynn et al. 2006). In this country, this cancer has been found in men more than women because of the high prevalence of liver fluke infections. There is no effective chemotherapy treatment with advanced cholangiocarcinoma patients (Sampson et al. 1997). Surgical resection is the best treatment and is a potentially curative therapy for this cancer. Thus, effective therapeutic agents from natural sources are still needed. It has been reported that KKU-100 was the

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✉ Chavi Yenjai
chayen@kku.ac.th

[1] Department of Chemistry, Faculty of Science, Natural Products Research Unit, The Center of Excellence for Innovation in Chemistry, Khon Kaen University, Khon Kaen 40002, Thailand

[2] Department of Pharmacology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

[3] Department of Chemistry, Faculty of Science, Natural Products Research Unit, The Center of Excellence for Innovation in Chemistry, Mahasarakham University, Mahasarakham 44150, Thailand

[4] Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

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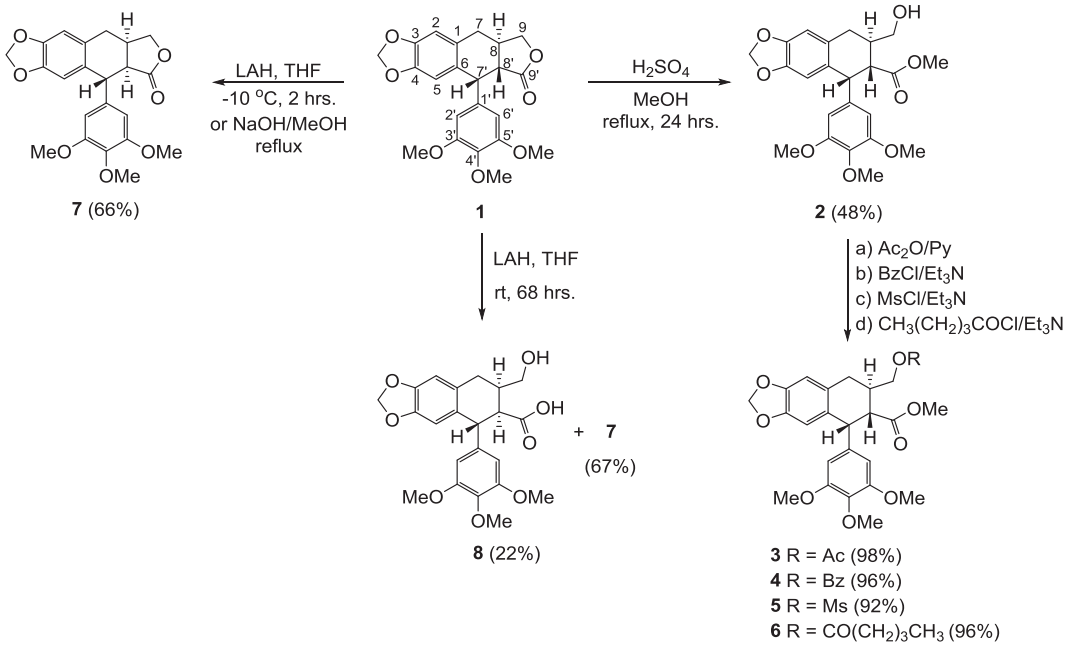


Scheme 1 Derivatives of deoxypodophyllotoxin

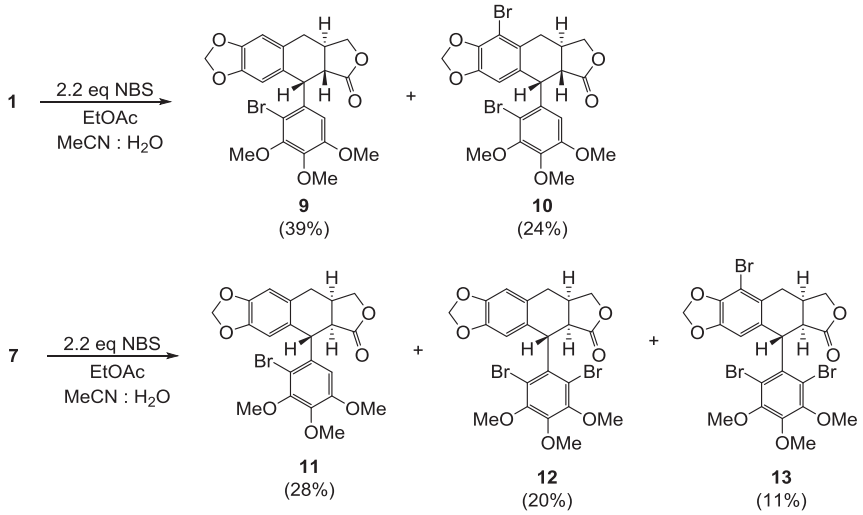
least sensitive cell line which showed the highest IC_{50} value of 25.21 μM to ellipticine, thus this cell line was selected for the study (Songsiang et al. 2010).

Results and discussion

Chemistry

Deoxypodophyllotoxin (**1**), a lignan derivative, was isolated using a chromatographic method from the EtOAc extract of the seed of *H. nymphaeifolia* with a good yield (Suthiwong et al. 2018). In our cytotoxicity screening, this compound showed cytotoxicity against cholangiocarcinoma (KKU-M100) and HepG2 cell lines, with IC_{50} values of 25.78 μM and 8.10 μM, respectively. To explore the structure-cytotoxicity relationship, a series of deoxypodophyllotoxin derivatives have been synthesized and evaluated for cytotoxicity to find novel compounds for cholangiocarcinoma and HepG2 treatment. Twelve lignan derivatives were successfully synthesized using simple organic reactions. Treatment of **1** with conc. sulfuric acid in methanol at room temperature yields methyl ester **2** (48%) (Scheme 1). The stereochemistry at C-8′ is still the same as the starting material by showing a coupling constant of *cis* configuration, $J_{7'/8'} = 5.7$ Hz, while *trans* configuration showed $J_{7'/8'} = 6.4$ Hz. (Gordaliza et al. 1997). The ^{1}H NMR spectrum of **2** showed a singlet signal at δ 3.53, indicating the presence of a methyl ester group. Alcohol **2** was further treated with acetic anhydride in pyridine affording **3** (98%). Lignan derivatives **4**, **5**, and **6** were synthesized by treatment of **2** with benzoyl chloride, methanesulfonyl chloride and valeroyl chloride, respectively, in the presence of Et_3N (Scheme 1). Compounds **3**–**6** showed the coupling constant of the *cis* configuration as $J_{7'/8'} = 5.7$–5.9 Hz. After treatment of **1** with LAH in THF at −10 °C, deoxypicropodophyllotoxin (**7**) was obtained with 66% yield, and no reduced product was obtained. This result indicates that LAH acts as a base and abstracted acidic proton at the *alpha* position leads to the epimerization of the starting material. The ^{1}H NMR spectrum displayed a double of doublet signal of H-8′ at δ 3.34 ($J = 9.6$, 3.0 Hz) where **1** showed a multiplet signal at δ 2.73. In addition, compound **7** showed a doublet signal of H-7′ at δ 4.37 ($J = 3.0$ Hz) where **1** exhibited at δ 4.60. Further treatment of **1** with LAH at room temperature for 68 h, interestingly, carboxylic acid derivative **8** (22%) and epimerized product **7** (67%) were observed. The ^{13}C NMR spectrum of **8** displayed signals at δ 31.4 (C-7) and δ 63.8 (C-9), where **1** showed at δ 33.3 (C-7) and δ 72.2 (C-9). Bromination of **1** with NBS in the presence of aqueous acetonitrile at room temperature yields monobromolignan **9** (39%) and dibromolignan **10** (24%) (Scheme 2). The effect of high electron density at C-2′ (and C-6′) in **1** led to bromination at the C-2′ position. The ^{13}C NMR data of **9** at C-2′ (C-Br) showed a signal at δ_C 114.6 where **1** showed at δ_C 108.5 and the proton signal of H-6′ changed from δ_H 6.34 to δ_H 6.12 (Feliciano et al. 1993). It was found that the addition of the second bromine atom at C-2 in **10** may be due to steric hindrance at C-6′. The HMBC spectrum of dibromolignan **10** showed

Scheme 2 Bromolignan derivatives of deoxypodophyllotoxin



correlations between H-7 (δ_{7a} 3.31 and δ_{7b} 2.56) and C-2 (δ 103.2). The reaction of **7** with NBS in the same condition gave monobromolignan **11** (28%), dibromolignan **12** (20%) and tribromolignan **13** (11%). The ^{13}C NMR signal of **11** at C-2′ changed from δ_C 105.3 to δ_C 110.9 where the signal of H-6′ changed from δ_H 6.33 to δ_H 6.31. In the case of **7**, the bromination of the second bromine atom occurring at C-6′ may be due to this compound being less steric than **10**. The spectroscopic data of dibromolignan **12** exhibited the containing of bromine atoms at C-2′ and C-6′ positions, which were different from **10**. The ^{1}H and ^{13}C NMR of **12** showed signals at δ_H/δ_C 6.69/108.7 (H-2) and δ_H/δ_C 6.06/106.5 (H-5), which correlated with C-3 (δ 146.1) and C-4 (δ 146.9) in the HMBC experiment. In the case of tribromolignan **13**, it showed only one aromatic proton at δ_H/δ_C 6.04/105.8 of the H-5 position. Correlations between H-5 and C-3 (δ 144.8), C-4 (δ 146.6), C-1 (δ 127.3) and C7′ (δ 45.1) were observed in the HMBC spectrum. In this spectrum, the correlations between H-7 and C-2 (δ 103.0), C-6 (δ 131.7) were also observed in the HMBC spectrum.

Table 1 Cytotoxicity of all compounds (IC_{50}, μM)*

Compound	KKU-100	HepG2
1	25.78 ± 1.78	8.10 ± 2.00
2	1.63 ± 0.79	6.73 ± 1.37
3	2.01 ± 0.74	1.94 ± 1.12
4	14.96 ± 7.85	3.55 ± 1.44
5	0.84 ± 0.47	16.12 ± 4.32
6	4.47 ± 1.94	41.39 ± 10.49
7	9.04 ± 5.70	1.50 ± 0.53
8	34.58 ± 10.80	14.65 ± 7.20
9	1.84 ± 0.54	1.90 ± 0.77
10	0.75 ± 0.52	0.46 ± 0.19
11	4.82 ± 2.09	5.86 ± 1.88
12	22.6 ± 203	14.92 ± 7.37
13	1.01 ± 0.39	0.42 ± 0.29
Ellipticine	25.21 ± 0.20	
Cisplatin		2.2 ± 0.70

*Data shown are from triplicate experiments

Biological activity

Natural lignan from *H. nymphaeifolia*, deoxypodophyllotoxin, was used as the starting material and 12 derivatives were synthesized. All compounds were evaluated for cytotoxicity against cholangiocarcinoma cells (KKU-M100, poorly-differentiated adenocarcinoma) and HepG2 cell lines (Tusskorn et al. 2013). KKU-100 cell is a poorly differentiated adenocarcinoma and is the least sensitive among cholangiocarcinoma cells. Most lignan derivatives displayed stronger cytotoxicity against two cell lines, KKU-100 and HepG2 cells, except compounds **8** and **12** (Table 1). In cases of KKU-100 cells, methyl ester derivatives (**2**–**6**) showed cytotoxicity with IC_{50} values ranging from 0.84 to 4.47 μM, except compound **4** (IC_{50} was 14.96 μM), which is about 5–30 fold stronger than the starting material. The results show convincingly that polarity may play an important role in cytotoxicity. Among the ester derivatives, **3** exhibited strong cytotoxicity against both cells with IC_{50} values of 2.01 and 1.94 μM, respectively. Compound **7** displayed cytotoxicity with IC_{50} values of 9.04 and 1.5 μM, respectively, which were 2.8 and 5.4 fold stronger than the starting material. These results suggest that the stereochemistry which led to the molecular form may affect the activity. Comparing between compounds **2** and **8**, carboxylic acid, which is more polar than methyl ester, exhibited weaker cytotoxicity (IC_{50} = 34.58 and 14.65 μM). These results confirm that the polarity of a compound may play an important role in activity. In cases of bromide derivatives, they showed strong cytotoxicity,

with the exception of compound **12**, against KKU-100 and HepG2 cell lines, with IC_{50} values ranging from 0.42 to 5.86 μM. Comparing between compounds **9** and **10**, the presence of a bromine atom at the C-2 position increased cytotoxicity. In cases of compounds **12** and **13**, the bromine atom at the C-2 position also improved the cytotoxicity against the two cell lines. Compound **13** exhibited the activity with IC_{50} values of 1.01 and 0.42 μM to KKU-100 and HepG2 cells, respectively. These results confirm that the bromine atom at the C-2 position can enhance the cytotoxicity. Among all derivatives, **10** is the most active compound, showing IC_{50} values of 0.75 and 0.46 μM against KKU-100 and HepG2 cell lines, respectively.

Conclusion

Lignan derivatives were synthesized by using deoxypodophyllotoxin as the starting material. All compounds were evaluated for cytotoxicity against cholangiocarcinoma cells, KKU-100, and hepatoma carcinoma cells, HepG2. Compounds **3**, **9**, **10**, and **13** exhibited cytotoxicity against the two cell lines. It is believed that polarity of these compounds may play an important role in cytotoxicity. Among bromide derivatives (**9**–**13**), it was found that the bromine atom at the C-2 position was favorable for cytotoxicity. Compound **10** displayed highly potent activity against the two cell lines.

Experimental part

General experimental procedures

All melting points were determined on a SANYO Gallenkamp (UK) melting point apparatus. Optical rotations were identified using a JASCO P–1020 digital polarimeter. UV spectra were recorded using an Agilent 8453 UV-Visible spectrophotometer (Germany). IR spectra were taken as thin films using a Perkin Elmer Spectrum One FT-IR spectrophotometer (UK) ^{1}H NMR spectra were determined with a Varian Mercury plus spectrometer (UK) operating at 400 MHz (^{1}H NMR) and at 100 MHz (^{13}C NMR). Mass spectra were recorded on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, UK). Silica gel 60 (100–200 mesh, Merck) was employed for column chromatography. Preparative TLC was carried out using silica gel 60 GF254. TLC was examined on silica gel 60 F_{254} (Merck) precoated aluminum sheets. Spot on TLC were visualized under UV light and by spraying with acidic anisaldehyde solution followed by heating. All solvents were distilled throughout the separation process.

Extraction and isolation of deoxypodophyllotoxin (1)

Air-dried seed (300 g) of *H. nymphaeifolia* were ground and successively extracted at room temperature with EtOAc (3 × 1.5 L), and MeOH (3 × 1.5 L). After filtration and evaporation, the crude EtOAc (79 g) and MeOH (23 g) extracts were obtained. The crude EtOAc extract was separated by silica gel flash column chromatography (FCC) and eluted with a gradient system of hexane and EtOAc to obtain 4 fractions, F1-F4. Fraction F1 (30 g) was identified as the natural oil. Fraction F2 was purified by silica gel FCC, and 5% EtOAc-hexane was used as an eluent to obtain two subfractions, F2.1 and F2.2. Further purification of subfraction F2.2 by CC and elution with 100% CH_2Cl_2 afforded **1** (321 mg, 0.11%) which was used as the starting material.

Deoxypodophyllotoxin (1)

White solid; mp 166–168 °C, $[\alpha]^{23}_D$ −54° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2923, 1763, 1588, 1504, 1479, 1459, 1421, 1377, 1336, 1270, 1219, 1121, 1033, 997, 929; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (1H, s, H-2), 6.52 (1H, s, H-5), 6.34 (2H, br s, H-2′,6′), 5.95 (1H, s, –OCH_2O–), 5.92 (1H, s, –OCH_2O–), 4.60 (1H, br s, H-7′), 4.45 (1H, dd, J = 8.3, 5.4 Hz, H-9), 3.92 (1H, t, J = 9.0 Hz, H-9), 3.80 (3H, s, 4′–OCH_3), 3.75 (6H, s, 3′,5′–OCH_3), 3.07 (1H, br d, J = 11.3 Hz, H-7a), 2.73 (3H, m, H-7b, 8, 8′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.0 (C-9′), 152.7 (C-3′, 5′), 147.2 (C-3), 146.9 (C-4), 137.3 (C-4′), 136.4 (C-1′), 130.8 (C-6), 128.4 (C-1), 110.6 (C-5), 108.6 (C-2), 108.5 (C-2′, 6′), 101.3 (–OCH_2O–), 72.2 (C-9), 60.9 (4′-OCH_3), 56.4 (3′, 5′-OCH_3), 47.6 (C-8′), 43.9 (C-7′), 33.3 (C-7), 32.9 (C-8); HRESI-MS *m/z* 421.1277 $[M + Na]^+$ (calcd. for $C_{22}H_{22}O_7$ +Na, 421.1263).

Preparation of lignan derivatives

Delactonization

A solution of compound **1** (172.4 mg, 0.4337 mmol) in MeOH (3 mL) was treated with conc. H_2SO_4 (0.2 mL) at 100 °C for about 24 h. Water was added and the mixture was extracted with EtOAc (2×50 mL). The organic layer was combined, washed with water, brine and dried with anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (50 % EtOAc:hexane) to give a semi-solid of compound **2** (89.1 mg, 48%).

Methyl deoxypodophyllate (2) White solid; mp 154–157 °C, $[\alpha]^{24}_D$ −95° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 3498, 2936, 1734, 1588, 1503, 1483, 1459, 1419, 1328, 1224,

1123, 1035, 1005, 925, 862, 749; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (1H, s, H-2), 6.34 (1H, s, H-5), 6.09 (2H, s, H-2′,6′), 5.83 (2H, br s, –OCH_2O–), 4.32 (1H, d, J = 5.7 Hz, H-7′), 3.74 (3H, s, 4′-OMe), 3.70 (6H, s, 3′ and 5′-OMe), 3.64 (1H, dd, J = 10.9, 3.8 Hz, H-9a), 3.54 (1H, dd overlap, J = 10.9, 5.4 Hz, H-9), 3.53 (3H, s, 9′-OMe), 3.06–2.89 (2H, m, H-8′, 7a), 2.65 (1H, dd, J = 17.1, 10.8 Hz, H-7b), 2.35 (1H, m, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9 (C-9′), 152.7 (C-3′,5′), 146.6 (C-4), 146.2 (C-3), 138.0 (C-4′), 137.1 (C-1′), 130.0 (C-6), 128.5 (C-1), 109.2 (C-5), 108.0 (C-2), 106.8 (C-2′ and 6′), 100.8 (-OCH_2O-), 65.4 (C-9), 60.8 (4′-OMe), 56.1 (3′ and 5′-OMe), 51.5 (9′-OMe), 48.3 (C-8′), 47.8 (C-7′), 32.9 (C-8), 32.1 (C-7); HRESI-MS *m/z* 453.1521 $[M + Na]^+$ (calcd. for $C_{23}H_{26}O_8$+Na, 453.1525).

Acetylation

To a solution of compound **2** (15.3 mg, 0.0355 mmol) in pyridine (1 mL) was added dropwise Ac_2O (excess) at 0 °C, and the reaction mixture was stirred at room temperature for 2 h. The entire reaction mixture was poured into the cold water and extracted with EtOAc (2×20 mL). The organic layers were combined, washed with water, saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated. After recrystallization, compound **3** was obtained as a colorless solid (16.5 mg, 98%).

Methyl acetoxydeoxypodophyllate (3) Viscous oil; $[\alpha]_D^{25}$ −75° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2930, 1738, 1588, 1503, 1484, 1460, 1230, 1127, 1037; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.62 (1H, s, H-2), 6.40 (1H, s, H-5), 6.12 (2H, s, H-2′ and 6′), 5.89 (1H, s, –OCH_2O–), 5.88 (1H, s, –OCH_2O–), 4.37 (1H, d, J = 5.8 Hz, H-7′), 4.21 (1H, dd, J = 11.0, 3.2 Hz, H-9a), 4.13 (1H, dd, J = 11.0, 5.8 Hz, H-9b), 3.79 (3H, s, 4′-OMe), 3.74 (6H, s, 3′ and 6′-OMe), 3.54 (3H, s, 9′-OMe), 3.00 (1H, dd, J = 11.0, 5.8 Hz, H-8′), 2.98 (1H, dd, J = 16.9, 5.8 Hz, H-7a) 2.70 (1H, dd, J = 16.9, 10.8 Hz, H-7b), 2.62–2.50 (1H, m, H-8), 2.05 (3H, s, H-2″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6 (C-9′), 171.2 (C-1″), 152.9 (C-3′ and 5′), 146.8 (C-4), 146.4 (C-3), 137.8 (C-4′), 137.3 (C-1′), 130.0 (C-6), 128.0 (C-1), 109.4 (C-5), 107.9 (C-2), 106.9 (C-2′ and 6′), 101.0 (-OCH_2O-), 66.8 (C-9), 60.9 (4′-OMe), 56.3 (3′ and 5′-OMe), 51.5 (9′-OMe), 47.73 (C-7′), 47.69 (C-8′), 31.9 (C-7), 30.1 (C-8), 21.0 (C-2″); HRESI-MS *m/z* 495.1628 $[M + Na]^+$ (calcd. for $C_{25}H_{28}O_9$+Na, 495.1631).

General procedure to prepare 4–6

To a solution of compound **2** (14.6 mg, 0.034 mmol) in Et_3N (1 mL) was added dropwise BzCl (excess) at 0 °C and the reaction mixture was stirred at room temperature for 30 min. The entire reaction mixture was poured into cold water and extracted with EtOAc (2×20 mL). The organic layer were combined, washed with water, saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to give a crude oil, which was purified by PLC (20 % EtOAc:hexane) to give **4** (17.4 mg, 96%).

The reaction of **2** with MsCl or valerory chloride was examined in the same procedure as described above and then **5** (92%) or **6** (96%) were obtained, respectively.

Methyl benzyloxydeoxypodophyllate (4)

White solid; mp 171–173 °C; $[\alpha]_D^{25}$ −86° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2934, 1718, 1587, 1503, 1483, 1454, 1271, 1222, 1123, 1036, 1005, 938, 749, 710; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (2H, d, J = 7.9 Hz, H-3″), 7.56 (1H, t, J = 7.6 Hz, H-5″), 7.44 (2H, t, J = 7.6 Hz, H-4″), 6.63 (1H, s, H-2), 6.43 (1H, s, H-5), 6.16 (2H, s, H-2′ and 6′), 5.90 (1H, s, –OCH_2O–), 5.88 (1H, s, –OCH_2O–), 4.48 (1H, dd, J = 11.1, 3.1 Hz, H-9a), 4.41 (1H, d, J = 5.7 Hz, H-7′), 4.39 (1H, m overlap, H-9b) 3.81 (3H, s, 4′-OMe), 3.76 (6H, s, 3′,6′-OMe), 3.55 (3H, s, 9′-OMe), 3.14 (1H, dd, J = 11.5, 5.7 Hz, H-8′), 3.09 (1H, dd, J = 16.9, 5.8 Hz, H-7a) 2.84 (1H, dd, J = 16.9, 10.8 Hz, H-7b), 2.77–2.66 (1H, m, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6 (C-9′), 166.6 (C-1″), 152.9 (C-3′,5′), 146.9 (C-4), 146.5 (C-3), 137.8 (C-4′), 137.4 (C-1′), 133.2 (C-5″), 130.2 (C-6), 130.0 (C-2″), 129.7 (C-3″), 128.6 (C-4″), 128.1 (C-1), 109.4 (C-5), 108.0 (C-2), 106.9 (C-2′ and 6′), 101.0 (−OCH_2O−), 67.1 (C-9), 61.0 (4′-OMe), 56.3 (C-3′ and 5′-OMe), 51.6 (9′-OMe), 47.9 (C-8′), 47.8 (C-7′), 32.1 (C-8), 30.4 (C-7); HRESI-MS *m/z* 557.1789 $[M+Na]^+$ (calcd. for $C_{30}H_{30}O_9$ +Na, 557.1788).

Methyl mesyloxydeoxypodophyllate (5)

White solid; mp 175–177 °C; $[\alpha]_D^{25}$ −62° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2935, 1733, 1588, 1503, 1483, 1460, 1420, 1352, 1331, 1223, 1172, 1124, 1035, 926, 752; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.63 (1H, s, H-2), 6.39 (1H, s, H-4), 6.10 (2H, s, H-2′ and 6′), 5.90 (2H, s, −OCH_2O−), 4.42 (1H, d, J = 5.9 Hz, H-7′), 4.40–4.32 (2H, m, H-9), 3.80 (3H, s, 4′-OMe), 3.74 (6H, s, 3′ and 5′-OMe), 3.61 (3H, s, 9′-OMe), 3.10–3.00 (2H, m, H-7a and 8′), 2.98 (3H, s, SO_2Me), 2.85 (1H, dd, J = 17.1, 11.2 Hz, H-7b), 2.68–2.56 (1H, m, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3 (C-9′), 153.0 (C-3′ and 5′), 147.0 (C-4), 146.6 (C-3), 137.4 (C-1′ and 4′), 129.7 (C-6), 127.5 (C-1), 109.3 (C-5), 107.9 (C-2), 106.9 (C-2′ and 6′), 101.1(−OCH_2O−), 72.2 (C-9), 61.0 (4′-OMe), 56.3 (3′ and 5′-OMe), 51.7 (9′-OMe), 47.6 (C-8′), 46.8 (C-7′), 37.2 (SO_2Me), 31.6 (C-8), 30.4 (C-7); HRESI-MS *m/z* 531.1283 $[M + Na]^+$ (calcd. for $C_{24}H_{28}O_{10}$+Na, 531.1301).

Methyl valeroyloxydeoxypodophyllate (6)

White solid; mp 168–169 °C; $[\alpha]_D^{25}$ −52° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2954, 1732, 1587, 1503, 1483, 1459, 1419, 1390, 1330, 1222, 1162, 1124, 1035, 1007, 937, 752; ^1H NMR (400 MHz, $CDCl_3$) δ 6.62 (1H, s, H-2), 6.40 (1H, s, H-5), 6.12 (2H, s, H-2′ and 6′), 5.89 (1H, s, –OCH_2O–), 5.88 (1H, s, –OCH_2O–), 4.37 (1H, d, $J = 5.7$ Hz, H-7′), 4.20 (1H, dd, $J = 11.2$, 3.0 Hz, H-9a), 4.15 (1H, dd, $J = 11.0$, 5.6 Hz, H-9b), 3.80 (3H, s, 4′-OMe), 3.74 (6H, s, 3′ and 5′-OMe), 3.54 (3H, s, 9′-OMe), 3.05–2.95 (2H, m, H-7a and 8′), 2.71 (1H, dd, $J = 16.9$, 10.8 Hz, H-7b), 2.61–2.51 (1H, m, H-8), 2.31 (2H, t, $J = 7.6$ Hz, H-2″), 1.59 (2H, m, H-3″), 1.33 (2H, m, H-4″), 0.91 (3H, t, $J = 7.3$ Hz, H-5″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.9 (C-1″), 172.6 (C-9′), 152.9 (C-3′,5′), 146.8 (C-4), 146.4 (C-3), 137.8 (C-4′), 137.3 (C-1′), 130.0 (C-6), 128.1 (C-1), 109.4 (C-5), 108.0 (C-2), 106.9 (C-2′ and 6′), 101.0 (−OCH_2O−), 66.4 (C-9), 61.0 (4′-OMe), 56.3 (3′ and 5′-OMe), 51.5 (9′-OMe), 47.7 (C-8′), 47.7 (C-7′), 34.1 (C-2″), 32.0 (C-8), 30.2 (C-7), 27.2 (C-3″), 22.4 (C-4″), 13.8 (C-5″); HRESI-MS *m/z* 537.2101 $[M + Na]^+$ (calcd. for $C_{28}H_{34}O_9$+Na, 537.2101).

Epimerization

A solution of compound **1** (46.2 mg, 0.1159 mmol) in THF at −10 °C was treated with LAH (4.5 mmol, 1 M solution in THF) under argon. The reaction mixture was stirred at −10 °C for 2 h. The reaction mixture was quenched with 10% HCl followed by extraction of the aqueous layer with EtOAc (2×20 mL). The combined organic layers were washed with water and brine, dried with Na_2SO_4, and concentrated in vacuo. Chromatographic separation on silica gel with EtOAc-hexane (2:3) as the eluent afforded the epimerized product **7** (30.5 mg, 66%).

Deoxypicropodophyllotoxin (7)

White solid; mp 159–161 °C, $[\alpha]_D^{24}$ +61° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2924, 1761, 1589, 1505, 1478, 1459, 1423, 1325, 1245, 1180, 1121, 1033, 1005, 926, 791; ^1H NMR (400 MHz, $CDCl_3$) δ 6.66 (1H, s, H-2), 6.58 (1H, s, H-5), 6.33 (2H, s, H-2′, 6′), 5.95 (1H, br s, –OCH_2O–), 5.92 (1H, br s, –OCH_2O–), 4.45 (1H, dd, $J = 9.2$, 7.4 Hz, H-9a), 4.37 (1H, d, $J = 3.0$ Hz, H-7′), 3.97 (1H, dd, $J = 9.2$, 3.2 Hz, H-9b), 3.82 (3H, s, 4′-OMe), 3.78 (6H, s, 5′ and 3′-OMe), 3.34 (1H, dd, $J = 9.6$, 3.0 Hz, H-8′), 3.06–2.96 (1H, m, H-8), 2.86 (1H, dd, $J = 15.4$, 6.4 Hz, H-7a), 2.48 (1H, dd, $J = 15.4$, 5.5 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.5 (C-9′), 153.5 (C-3′,5′), 147.0 (C-3), 146.9 (C-4), 138.4 (C-1′), 130.6 (C-6), 128.4 (C-1), 110.0 (C-5), 108.9 (C-2), 105.3 (C-2′), 105.2 (C-6′), 101.1 (−OCH_2O−), 72.9 (C-9), 61.0 (4′-OMe), 56.4 (3′,5′-OMe), 46.5 (C-8′), 45.5 (C-7′), 33.2 (C-8), 32.2 (C-7); HRESI-MS *m/z* 421.1261 $[M + Na]^+$ (calcd. for $C_{22}H_{22}O_7$+Na, 421.1263).

Hydrolysation of 1

A solution of compound **1** (89.7 mg, 0.2251 mmol) in THF at −10 °C was treated with LAH (4.5 mmol, 1 M solution in THF) under argon. The reaction mixture was stirred at −10 °C for 2 h. and then warmed to room temperature until the disappearance of the starting material (68 h) which was determined by TLC. The reaction mixture was quenched with 10% HCl followed by extraction of the aqueous layer with EtOAc (2×20 mL). The combined organic layers were washed with water and brine, dried with Na_2SO_4, and concentrated in vacuo. Chromatography on silica gel with EtOAc-hexanes (2:3) as the eluent afforded products **8** (20.5 mg, 22%) and **7** (60.3 mg, 67%).

Deoxypicropodophyllic acid (8)

White solid; mp 162–165 °C; $[\alpha]_D^{25}$+21° (*c* 0.1 CH_3OH); FT-IR (film) ν_{max} cm^{-1}: 3402, 2918, 1762, 1589, 1504, 1482, 1461, 1424, 1328, 1228, 1128, 1007, 982; ^1H NMR (400 MHz, Methanol-d4) δ 6.62 (1H, s, H-2), 6.38 (2H, s, H-2′ and 6′), 6.30 (1H, s, H-5), 5.86 (1H, s, –OCH_2O–), 5.85 (1H, s, –OCH_2O–), 4.36 (1H, d, $J = 6.1$ Hz, H-7′), 3.75 (6H, s, 3′ and 5′-OMe), 3.73 (3H, s, 4′-OMe), 3.70 (1H, dd, $J = 10.9$, 5.9 Hz, H-9a), 3.55 (1H, dd, $J = 10.9$, 8.3 Hz, H-9b), 3.06 (1H, dd, $J = 6.1$, 3.5 Hz, H-8′), 2.95 (1H, dd, $J = 16.6$, 5.5 Hz, H-7a), 2.84 (1H, dd, $J = 16.6$, 7.8 Hz, H-7b), 2.40–2.31 (1H, m, H-8); ^{13}C NMR (100 MHz, CD_3OD) δ 176.9 (C-9′), 154.3 (C-3′ and 5′), 147.8 (C-4), 147.5 (C-3), 143.4 (C-1′), 137.7 (C-4′), 131.0 (C-6), 129.9 (C-1), 110.4 (C-5), 109.3 (C-2), 107.7 (C-2′ and 6′), 102.0 (-OCH_2O-), 63.8 (C-9), 61.1 (4′-OMe), 56.6 (3′ and 5′-OMe), 50.6 (C-8′), 47.5 (C-7′), 37.0 (C-8), 31.4 (C-7); HRESI-MS *m/z* 439.1372 $[M + Na]^+$ (calcd. for $C_{22}H_{24}O_7$+Na, 439.1369).

Addition with N-Bromosuccinamide

To a solution of **1** (33 mg, 0.0828 mmol) in EtOAc (2 mL) was added dropwise the solution of NBS (2.2 eq.) in MeCN: H_2O (2 mL) at 0 °C and this was stirred at 0 °C for 1 h and kept stirring at room temperature for 1 h. The entire reaction mixture was poured into cold water and extracted with EtOAc (2×20 mL). The organic layers were combined, washed with water, saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to give solid products. After purification by PLC, lignan derivatives **9** (15.5 mg, 39%) and **10** (10.9 mg, 24%) were obtained.

The reaction of **7** (29.9 mg) with NBS was examined in the same procedure as described above and then **11**

(10.2 mg, 28%), **12** (8.4 mg, 20%) and **13** (11.3 mg, 24%) were obtained.

2′-Bromodeoxypodophyllotoxin (9)

White solid; mp 221–223 °C; $[\alpha]_D^{25}$ −93° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2927, 1779, 1563, 1480, 1389, 1334, 1313, 1283, 1224, 1197, 1164, 1103, 1037, 999, 944, 928, 872, 754; ^1H NMR (400 MHz, $CDCl_3$) δ 6.63 (1H, s, H-2), 6.38 (1H, s, H-5), 6.12 (1H, br s, H-6′), 5.90 (2H, s, –OCH_2O–), 5.28 (1H, d, J = 5.9 Hz, H-7′), 4.51 (1H, dd, J = 8.6, 6.7 Hz, H-9a), 3.93 (1H, dd, J = 10.2, 8.6 Hz, H-9b), 3.91 (3H, s, 3′-OMe), 3.86 (3H, s, 4′-OMe), 3.63 (3H, s, 5′-OMe), 3.08 (1H, dd, J = 15.5, 4.7 Hz, H-7a), 3.03–2.89 (1H, m, H-8), 2.83–2.73 (2H, m, H-7b and 8′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.4 (C-9′), 152.3 (C-5′), 151.0 (C-3′), 147.1 (C-4), 147.0 (C-3), 142.7 (C-4′), 136.6 (C-1′), 131.3 (C-6), 127.6 (C-1), 114.6 (C-2′), 110.8 (C-6′), 110.4 (C-5), 108.5 (C-2), 101.3 (–OCH_2O–), 71.7 (C-9), 61.2 (4′-OMe), 61.1 (3′-OMe), 56.5 (5′-OMe), 46.8 (C-8′), 41.5 (C-7′), 34.4 (C-8), 32.9 (C-7); HRESI-MS *m/z* 499.0385, 501.0365 $[M + Na]^+$ (calcd. for $C_{22}H_{21}BrO_7$+Na, 499.0368, 501.0348).

2,2′-Dibromodeoxypodophyllotoxin (10)

White solid; mp 225–227 °C; $[\alpha]_D^{25}$ −84° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2936, 1778, 1563, 1465, 1390, 1334, 1313, 1223, 1198, 1167, 1104, 1040, 1000, 932, 837, 752; ^1H NMR (400 MHz, $CDCl_3$) δ 6.39 (1H, s, H-5), 6.10 (1H, br s, H-6′), 6.01 (1H, s, –OCH_2O–), 5.99 (1H, s, –OCH_2O–), 5.29 (1H, br d, J = 6.4 Hz, H-7′), 4.56 (1H, dd, J = 8.5, 6.8 Hz, H-9a), 3.98 (1H, dd, J = 10.5, 8.5 Hz, H-9b), 3.91 (3H, s, 5′-OMe), 3.86 (3H, s, 4′-OMe), 3.66 (3H, s, 3′-OMe), 3.31 (1H, dd, J = 16.4, 5.0 Hz, H-7a), 3.00–2.86 (1H, m, H-8), 2.75 (1H, dd, J = 14.3, 6.4 Hz, H-8′), 2.56 (1H, dd, J = 16.4, 11.6 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0 (C-9′), 152.4 (C-3′), 151.2 (C-5′), 146.9 (C-4), 145.8 (C-3), 142.9 (C-4′), 135.9 (C-1′), 133.5 (C-6), 127.2 (C-1), 144.7 (C-2′), 110.9 (C-6′), 109.8 (C-5), 103.2 (C-2), 101.7 (–OCH_2O–), 71.7(C-9), 61.2 (4′-OMe), 61.1 (5′-OMe), 56.7 (3′-OMe), 46.4 (C-8′), 42.0 (C-7′), 34.3 (C-8), 32.5 (C-7); HRESI-MS *m/z* 576.9457, 578.9458, 580.9421 $[M + Na]^+$ (calcd. for $C_{22}H_2Br_2O_7$ +Na, 576.9473, 578.9453, 580.9433).

2′-Bromodeoxypicropodophyllotoxin (11)

Viscous oil; $[\alpha]_D^{24}$+34° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2928, 1768, 1566, 1481, 1390, 1328, 1256, 1161, 1104, 1036, 933, 801, 756; ^1H NMR (400 MHz, $CDCl_3$) δ 6.67 (1H, s, H-2), 6.40 (1H, s, H-5), 6.31 (1H, s, H-6′), 5.93 (1H, br s, –OCH_2O–), 5.90 (1H, br s, –OCH_2O–), 4.65 (1H, d, J = 3.8 Hz, H-7′), 4.43 (1H, dd, J = 9.2, 6.3 Hz, H-9a), 4.08 (1H, dd, J = 9.2, 2.0 Hz, H-9b), 3.92 (3H, s, 5′-OMe), 3.89 (3H, s, 4′-OMe), 3.70 (3H, s, 3′-OMe), 3.35 (1H, dd, J = 8.8, 3.8 Hz, H-8′), 2.97 (1H, dd, J = 15.4, 6.5 Hz, H-7a), 2.92–2.82 (1H, m, H-8), 2.60 (1H, dd, J = 15.4, 7.8 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.7 (C-9′), 152.7 (C-3′), 151.6 (C-5′), 147.0 (C-4), 146.9 (C-3), 142.3 (C-4′), 137.1 (C-1′), 129.8 (C-6), 128.4 (C-1), 110.9 (C-2′), 110.1 (C-6′), 109.2 (C-5), 108.5 (C-2), 101.1 (–OCH_2O–), 72.8 (C-9), 61.20 (5′-OMe), 61.16 (4′-OMe), 56.3 (3′-OMe), 45.1 (C-8′), 45.0 (C-7′), 33.3 (C-8), 32.2 (C-7); HRESI-MS *m/z* 499.0376, 501.0353 $[M + Na]^+$ (calcd. for $C_{22}H_{21}BrO_7$ +Na, 499.0368, 501.0348).

2′,6′-Dibromodeoxypicropodophyllotoxin (12)

White solid; mp 220–223 °C $[\alpha]_D^{24}$+23° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2925, 1772, 1503, 1481, 1464, 1407, 1388, 1334, 1242, 1215, 1159, 1126, 1088, 1038, 1009, 929, 837, 754; ^1H NMR (400 MHz, $CDCl_3$) δ 6.69 (1H, s, H-2), 6.06 (1H, s, H-5), 5.91 (1H, br s, –OCH_2O–), 5.87 (1H, br s, –OCH_2O–), 5.16 (1H, d, J = 6.4 Hz, H-7′), 4.51 (1H, dd, J = 9.3, 5.6 Hz, H-9a), 4.26 (1H, d, J = 9.3 Hz, H-9b), 3.98 (3H, s, 4′-OMe), 3.96 (3H, s, 5′-OMe), 3.89 (3H, s, 3′-OMe), 3.43 (1H, t, J = 7.3 Hz, H-8′), 2.90–2.80 (2H, m, H-7a, 8), 2.62 (1H, t, J = 14.5 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.6 (C-9′), 151.6 (C-3′), 151.1 (C-5′), 146.9 (C-4), 146.9 (C-4′), 146.1 (C-3), 136.3 (C-1′), 129.8 (C-6), 128.2 (C-1), 119.5 (C-2′), 112.7 (C-6′), 108.7 (C-2), 106.5 (C-5), 101.0 (–OCH_2O–), 71.9 (C-9), 61.4 (4′-OMe), 61.2 (5′-OMe), 61.2 (3′-OMe), 46.0 (C-8′), 44.6 (C-7′), 35.9 (C-8), 32.8 (C-7); HRESI-MS *m/z* 576.9452, 578.9453, 580.9416 $[M + Na]^+$ (calcd. for $C_{22}H_{20}Br_2O_7$ +Na, 576.9473, 578.9453, 580.9433).

2,2′,6′-Tribromodeoxypicropodophyllotoxin (13)

White solid; mp 233–235 °C $[\alpha]_D^{24}$+53° (*c* 0.1 $CHCl_3$); FT-IR (film) ν_{max} cm^{-1}: 2924, 1774, 1607, 1499, 1465, 1406, 1388, 1316, 1238, 1160, 1086, 1041, 1009, 930, 837, 754; ^1H NMR (400 MHz, $CDCl_3$) δ 6.04 (1H, s, H-5), 5.99 (1H, br s, –OCH_2O–), 5.97 (1H, br s, –OCH_2O–), 5.16 (1H, d, J = 6.5 Hz,, H-7′), 4.54 (1H, dd, J = 9.4, 5.9 Hz, H-9a), 4.32 (1H, d, J = 9.4 Hz, H-9b), 3.98 (3H, s, 4′-OMe), 3.96 (3H, s, 3′-OMe), 3.89 (3H, s, 5′-OMe), 3.46–3.37 (2H, m, H-7a, 8′), 2.87–2.78 (1H, m, H-8), 2.42 (1H, dd, J = 15.1, 13.0 Hz, H-7b); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.2 (C-9′), 151.6 (C-3′), 151.1 (C-5′), 147.0 (C-4′), 146.6 (C-4), 144.8 (C-3), 135.8 (C-1′), 131.7 (C-6), 127.3 (C-1), 119.5 (C-2′), 114.8 (C-6′), 105.8 (C-5), 103.0 (C-2), 101.4 (-OCH_2O-), 71.9 (C-9), 61.4 (4′-OMe), 61.3 (3′-OMe), 61.2 (5′-OMe), 45.9 (C-8′), 45.1 (C-7′), 35.5 (C-8), 30.6 (C-7); HRESI-MS *m/z* 654.8581, 656.8575, 658.8549, 660.8506

$[M + Na]^+$ (calcd. for $C_{22}H_{19}Br_3O_7$+Na, 654.8518, 656.8558, 658.8538, 660.8518).

Cell culture

The human cholangiocarcinoma (CCA) cell lines; KKU-M100 cells were routinely cultured in Ham's F12, supplemented with 10% fetal bovine serum, 12.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.3), 100 U/ml penicillin G and 100 µg/ml gentamicin. Human hepatoma cell line, HepG2 cells, from the American Type Culture Collection (ATCC HB 8065), were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (1%), MEM nonessential amino acids (Gibco), 12.5 mM HEPES, pH 7.3, 100 U/ml penicillin and 100 µg/ml gentamicin. Cultured cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were subcultured every 2–3 days before cultured cell confluence using 0.25% trypsin–EDTA, and medium was changed after an overnight incubation.

Cytotoxicity assay

KKU-M100 and HepG2 cells were seeded onto 96-well plates at a density of 7.5×10^3 and 1.5×10^4 cells/well, respectively. After an overnight incubation, cultured media were changed to serum-free media. Test compounds were dissolved in DMSO and diluted with medium to various concentrations before use. The compounds were added into cultured cells and incubated for 24 h. The cytotoxicity was assessed by the sulphorhodamine B (SRB) assay as previously described (Tusskorn et al. 2013). In brief, cultured cells were fixed with 15% trichloroacetic acid and stained with 0.4% SRB. The protein-bound dye was solubilized with 10 mM Tris-base solution for determination of the absorbance at 540 nm with a microplate reader. The cytotoxicity was calculated as percent absorbance of controls. The IC_{50} value was calculated by a non-linear curve-fitting program.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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