

Synthesis of phthalates of betulinic acid and betulin with cytotoxic activity

Miroslav Kvasnica,^a Jan Sarek,^{a,*} Eva Klinotova,^a Petr Dzubak^b and Marian Hajduch^b

^aDepartment of Organic and Nuclear Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, 128 43 Prague 2, Czech Republic

^bLaboratory of Experimental Medicine, Departments of Pediatrics and Oncology, Faculty of Medicine, Palacky University in Olomouc, Puskinoва 6, 775 20 Olomouc, Czech Republic

Received 23 November 2004; revised 23 February 2005; accepted 1 March 2005
Available online 30 March 2005

Abstract—Synthesis of 3 β -*O*-phthalic esters from betulinic acid and its esters and synthesis of phthalic esters from betulin and its monoacetates using classical acylation procedure with phthalic anhydride. The evaluation of cytotoxicity of the prepared compounds was using numbers of tumor cell lines in MTT test. It was discovered that hemiphthalic esters had better cytotoxicity than starting compounds as betulinic acid or quite inactive betulin.

© 2005 Elsevier Ltd. All rights reserved.

1. Introduction

Betulinic acid (**1**) (3 β -hydroxy-lup-20(29)-en-28-oic acid), pentacyclic lupane triterpene, is a known natural compound with various biological effects.^{1,2} Cytotoxic activity against various malignant versus nonmalignant cell lines belongs to most intensively studied³ effects of these compounds. Betulinic acid (**1**) demonstrated selective anticancer activity against neuroectodermal tumors,⁴ including human melanoma, neuroblastoma, and Ewing sarcomas. Therefore a lot of effort is focused on modification^{5,6} of betulinic acid in order to increase and broaden its biological activity against tumor cells of various histogenetic origin despite the fact that its mechanism of effect is still being studied⁷ and is not clear enough. In contrast to betulinic acid (**1**), betulin (**5**) (lup-20(29)-en-3 β ,28-diol) has no significant cytotoxic activities. However, based on structural similarity with betulinic acid (**1**), one could expect that appropriate derivatization could increase its cytotoxic potency.

In this paper we performed synthesis of new triterpenic compounds, particularly those derivatized on hydroxy groups (mainly 3 β - and 28-hydroxy groups).⁸ Since derivatization of hydroxy group of betulinic acid (**1**) with

lipophylic groups (e.g., acetates) lead to decrease of biologic activity,⁹ right choice of appropriate substitution is critical for improvement of pharmacological properties of new compounds. In this respect we have mainly paid attention to the synthesis of hemiesters of lower diacids using competent anhydride.¹⁰ This procedure can lead to acquirement of large groups of new polar derivatives with increased cytotoxic activity. Here we report our results on synthesis of betulinic acid and betulin phthalate esters and the effect of this substitution on biological activity of new compounds. Methyl- and ethyl-phthalates were also prepared within the structure–activity relationship study.

2. Chemistry

Betulinic acid (**1**) was isolated from bark of plane trees *Platanus acerifolius* using extraction with MeOH according to literature.⁵ Treatment of acid **1** with etherical solution of diazomethane, resp. diazoethane, gave methyl **2**, resp. ethyl ester **3**, in quantitative yield. Reaction of acid **1** with benzyl bromide in the presence of DBU afforded benzyl ester **4** in the yield 85%.

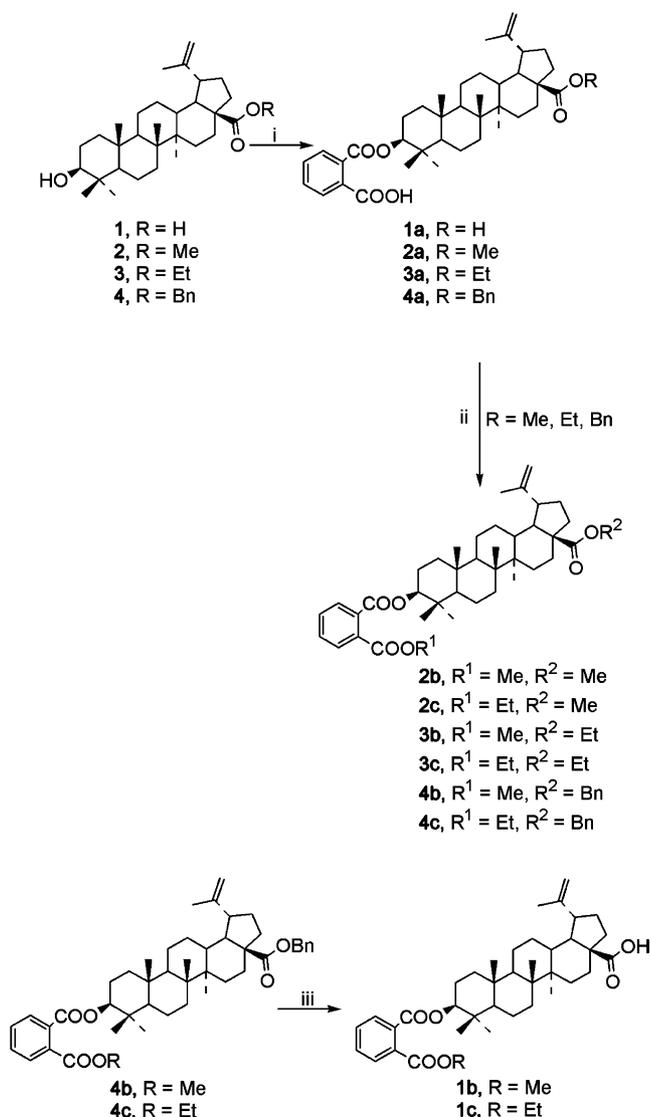
Betulin (**5**) was isolated from the birch bark (*Betula pendula*) using the known extraction procedure.¹¹ Monoacetate **6** was prepared using mild basic hydrolysis¹² of betulin diacetate with calcium hydroxide (5 equiv) at room temperature in yield 65%. The monoacetate **7**

Keywords: Triterpenes; Betulin; Phthalate; Cytotoxicity.

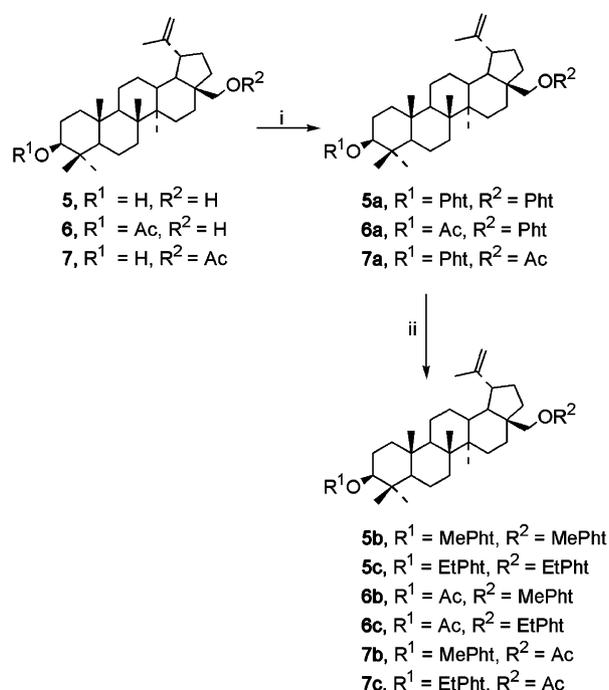
*Corresponding author. Tel./fax: +420 221 951 332; e-mail: jan.sarek@volny.cz

was prepared using selective acetylation of primary hydroxy group of betulin (5) with acetic anhydride in the presence of imidazole¹³ in yield 85%.

Synthesis of phthalates of betulinic acid (1) and its derivatives is described in Scheme 1, synthesis of phthalates of betulin (5) and its monoacetates in Scheme 2. The acylation reaction was always carried out by reflux about 24 h. It should be noted that treatment of triterpenes with phthalic anhydride requires reagent in at least 5–10 times molar excess for completion of reaction. After usual working up the crude products were separated by chromatography over silica gel (EtOAc in hexane, 10% → 75%) or chromatographed using HPLC. Products were then crystallized from the solvents mentioned in experimental section, if not otherwise stated. The yields after crystallization were usually about 75–85%. All methyl- and ethyl-phthalates, except 1b and 1c, were prepared using esterification with diazomethane, resp. diazoethane in quantitative yields. Esters



Scheme 1. Reagents and conditions for betulinic acid derivatives: (i) phthalic anhydride/DMAP/Py; (ii) CH₂N₂ (resp. CH₃CHN₂)/Et₂O/CHCl₃; (iii) 1,4-cyclohexadiene/Pd-C/THF/EtOH.



Scheme 2. Reagents and conditions for betulin derivatives: (i) phthalic anhydride/DMAP/Py; (ii) CH₂N₂ (resp. CH₃CHN₂)/Et₂O/CHCl₃. Pht = hemiphthaloyl, MePht = methyl-phthaloyl, EtPht = ethyl-phthaloyl.

1b and **1c** were prepared from esters **4b** and **4c** by transfer-hydrogenolysis¹⁴ with 1,4-cyclohexadiene on Pd/C, as described in Scheme 1, also in good yields.

3. Results and discussion

Our preliminary investigation showed that betulinic acid (**1**) and betulin (**5**) derivatives are potential lead compounds for new anti-tumor agents. Majority of hemiphthalates showed increased cytotoxic activity judged against starting compounds. The lowest effect of hemiphthalates was found in the case of bishemiphthalate **5a**, since activity of those substances increased only slightly compared with starting betulin (**5**). On the other side, derivative **6a** was substantially better than the parent compound. Based on our results we can summarize that derivatization of hydroxy groups of triterpenes by phthalic anhydride increases cytotoxic activity. Esterification of hemiphthalates using diazomethane (resp. diazoethane) did not lead to cytotoxic derivatives, since nearly all methyl- and ethyl-phthalates were found quite inactive (IC₅₀ > 250 μmol/L). There were only two exceptions from this rule. Methyl- **1b** and ethyl-phthalates **1c** of betulinic acid (**1**) showed significant biological activity. Compound **1b** was also the most active derivative prepared in this work. It was also found that active compounds showed cytotoxic activity against broader spectrum of tumors from different histogenetic origin, for example, mesenchymal (CEM, K562, K562-tax), epithelial (HT29, PC-3), and neuroektodermal (SK-MEL2) tumors. Interestingly, introduction of methyl-phthalate **1b** into the structure of betulinic acid (**1**) conferred drug resistance, since this compound showed much lower

Table 1. Cytotoxic activity of compounds **1–7** and **1a,b,c–7a,b,c** against CEM, K562, HT 29, PC-3, and SK MEL2 cells

Compound	IC ₅₀ (μmol/L ^a)					
	CEM	K562	K562-tax	HT29	PC-3	SK MEL2
1	27.5 (±2.7)	54.8 (±5.6)	108.2 (±12.1)	84.5 (±6.6)	94.1 (±8.7)	23.2 (±1.5)
1a	54.4 (±29.4)	29.4 (±4.8)	26.8 (±3.2)	29.9 (±4.2)	32.5 (±3.4)	60.3 (±17.4)
1b	5.7 (±3.0)	8.8 (±1.5)	47.8 (±19.1)	7.5 (±2.0)	18.9 (±9.0)	13.4 (±4.9)
1c	30.4 (±18.9)	44.5 (±12.0)	71.1 (±28.5)	40.7 (±10.3)	98.5 (±31.3)	183.9 (±43.8)
2	155.1 (±17.1)	68.5 (±10.7)	67.2 (±9.9)	65.5 (±15.2)	117.7 (±11.9)	186.3 (±22.4)
2a	29.8 (±5.2)	30.7 (±4.9)	23.1 (±4.6)	18.1 (±4.6)	24.6 (±7.8)	42.1 (±0.6)
2b	>250	>250	>250	>250	>250	>250
2c	>250	>250	>250	>250	>250	>250
3	132.4 (±14.4)	114.0 (±20.2)	122.6 (±37.6)	121.3 (±28.1)	>250	217.9 (±51.7)
3a	13.0 (±3.2)	17.2 (±6.8)	8.8 (±1.1)	9.9 (±1.8)	10.5 (±1.0)	25.8 (±10.3)
3b	>250	>250	>250	>250	>250	>250
3c	>250	>250	>250	>250	>250	>250
4	242.4 (±13.7)	>250	191.7 (±50.4)	248.9 (±2.0)	>250	>250
4a	10.0 (±0.8)	10.1 (±1.1)	6.2 (±1.1)	8.9 (±1.3)	10.9 (±1.3)	15.3 (±3.7)
4b	>250	>250	>250	>250	>250	>250
4c	>250	>250	>250	>250	>250	>250
5	>250	>250	>250	>250	>250	>250
5a	184.5 (±18.9)	149.6 (±8.5)	110.1 (±35.2)	129.1 (±18.4)	134.3 (±10.4)	204.2 (±24.5)
5b	>250	>250	>250	>250	>250	>250
5c	>250	>250	>250	>250	>250	>250
6	96.4 (±36.4)	147.4 (±37.7)	165.6 (±38.3)	154.3 (±14.2)	248.6 (±3.5)	247.0 (±4.7)
6a	13.6 (±5.8)	18.1 (±5.7)	8.3 (±4.6)	13.6 (±5.7)	20.1 (±8.7)	24.9 (±9.9)
6b	>250	>250	>250	>250	>250	>250
6c	>250	>250	>250	>250	>250	>250
7	110.1 (±17.5)	93.5 (±31.1)	72.4 (±27.3)	76.7 (±18.6)	150.1 (±20.0)	223.7 (±23.7)
7a	42.4 (±7.6)	37.8 (±2.2)	34.2 (±6.4)	36.1 (±2.7)	35.2 (±11.2)	90.0 (±46.7)
7b	>250	>250	247.3 (±5.6)	248.3 (±4.3)	>250	>250
7c	>250	>250	>250	>250	>250	>250

Value >250 μmol/L means that compound is not active.

^a The lowest concentration that kills 50% of tumor cells.

activity on P-glycoprotein overexpressing cell line K562-tax¹⁵ compared to sensitive K562 leukemia cells. The lowest cytotoxicity of synthesized phthalates was observed on human melanoma SK MEL2 cells as summarized in Table 1.

4. Conclusion

Within the worldwide research of betulinic acid (**1**) and betulin (**5**) in the field of anti-tumor agents, number of structural modifications, and derivatizations were studied. Our study demonstrates that derivatization of 3β-hydroxy group of these triterpenoids with lower diacids can result in compounds with higher cytotoxic activity against cell lines of different histogenetic origin, including drug resistant tumors. Even derivatization of quite inactive betulin (**1**) can lead to compounds with higher cytotoxicity as shown in case of **6a** or **7a**. Prepared hemiphthalates are also highly polar compounds (stated according to the solubility in polar solvents, e.g., alcohols), which is very useful in MTT screening, where 10% DMSO in water is used, or in in vivo screenings, where using of polar solvents is necessary. Another advantage of phthalates is also possible of using of many substituted phthalic anhydrides (e.g., by halogen, nitro group) for derivatization reaction in order to modify the properties of prepared

phthalates. For this reason hemiphthalates of triterpenoids represent interesting class of compounds for further studies and/or development.

5. Experimental

Melting points were determined using a Kofler block and are uncorrected. Optical rotations were measured using CHCl₃ solutions (unless otherwise stated) on an Autopol III (Rudolph Research, Flanders, NJ) polarimeter, with an accuracy of ±2°. NMR spectra were recorded on a Varian UNITY INOVA 400 instrument (¹H NMR spectra at 399.95 MHz) using CDCl₃ solutions (unless otherwise stated), with SiMe₄ as an internal standard. Skeletal signals in the region approx. 0–2 ppm were not recorded, except methyl group. EIMS spectra were recorded on an INCOS 50 (Finigan MAT) spectrometer at 70 eV and an ion source temperature of 150 °C. The samples were introduced from a direct exposure probe at a heating rate of 10 mA/s. Relative abundances stated are related to the most abundant ion in the region of *m/z* > 180. TLC was carried out using silica gel 60 F₂₅₄, detection was by spraying with 10% aq H₂SO₄ and heating to 150–200 °C. Column chromatography was performed using silica gel 60 (Merck 7734). Used HPLC system consisted of High

Pressure Pump Gilson (model 361), Inject Valve Rheodyne, Preparative Column (25 × 250 mm) with filling Si gel (Biospher 7 μm), Differential-Refractometrical Detector (Laboratorní přístroje, Praha, CZ) connected with PC (software Chromulan) and Automatic Fraction Collector Gilson (model 246). Mixture of ethyl acetate and hexane was used as the mobile phase, its composition is in each experiment. TLC was carried out on Kiesel gel 60 F₂₅₄ plates (Merck).

5.1. General procedure for 3-*O*-acyl derivatives of hydroxy-compounds with phthalic anhydrides

Phthalic anhydride (4 equiv) and DMAP (4 equiv) was added to a solution of hydroxy-compound (1 mmol) in pyridine (10 mL). After being refluxed for 30 h, the reaction mixture was diluted with ethyl acetate and washed twice with aqueous solution of HCl and twice with water. The organic layer was dried over MgSO₄ and concentrated under reduced pressure. The residue was chromatographed with gradient on silica gel (hexane–ethyl acetate, 9:1–5:1).

5.2. Hemiphthalate of betulinic acid (1a)

Starting with compound **1** (500 mg, 1.09 mmol), chromatography and crystallization from the mixture of acetonitrile gave a white powder of **1a** (490 mg, 74%), mp 259–260 °C; [α]_D +17 (*c* 0.35, THF). IR ν (CHCl₃) cm⁻¹: 3511, 1699 sh, 1641, 1600, 1580, 1291. ¹H NMR (400 MHz): δ 0.88, 0.89, 0.95, 0.96, 1.00, 1.70, (each 3H, s, 6 × CH₃), 1.91–2.00 (2H, m), 2.23–2.29 (2H, m), 3.02 (1H, td, *J* = 11.0, 4.6 Hz), 4.61 (1H, m), 4.69–4.74 (2H, m), 7.52–7.54 (2H, m), 7.68 (1H, m), 7.75 (1H, m). MS *m/z* (rel. intensity): 604 (1, M⁺), 589 (1), 558 (1), 543 (1), 519 (1), 456 (11), 438 (36), 423 (15), 409 (6), 395 (22), 369 (6), 327 (5), 302 (5), 287 (4), 259 (10), 248 (25), 234 (11), 220 (12), 203 (29), 189 (100). Anal. Calcd for C₃₈H₅₂O₆: C, 75.46; H, 8.67. Found: C, 75.70; H, 8.81.

5.3. Hemiphthalate of methyl-betulinatate (2a)

Starting with compound **2** (500 mg, 1.06 mmol), chromatography and crystallization from the mixture of acetonitrile–water gave colorless needles of **2a** (505 mg, 77%), mp 223–225 °C; [α]_D +25 (*c* 0.42). IR ν (CHCl₃) cm⁻¹: 3508, 1717, 1704, 1641, 1600, 1581, 1290. ¹H NMR (400 MHz): δ 0.84, 0.88, 0.91, 0.94, 0.97, 1.69 (each 3H, s, 6 × CH₃), 1.84–1.92 (2H, m), 2.16–2.25 (3H, m), 3.00 (1H, td, *J* = 10.6, 4.1 Hz), 3.67 (3H, s, CH₃), 4.61 (1H, m), 4.72–4.77 (2H, m), 7.54–7.61 (2H, m), 7.72 (1H, m), 7.89 (1H, m). MS *m/z* (rel. intensity): 618 (5, M⁺), 603 (1), 558 (6), 543 (1), 515 (1), 470 (2), 452 (100), 437 (32), 409 (17), 393 (12), 377 (5), 355 (5), 316 (3), 283 (2), 273 (6), 262 (22), 257 (6), 248 (13), 233 (10), 215 (12), 203 (29), 189 (91). Anal. Calcd for C₃₉H₅₄O₆: C, 75.69; H, 8.80. Found: C, 75.62; H, 8.92.

5.4. Hemiphthalate of ethyl-betulinatate (3a)

Starting with compound **3** (500 mg, 1.03 mmol), chromatography and crystallization from the mixture of ace-

tonitrile–water gave colorless needles of **3a** (500 mg, 77%), mp 129–131 °C; [α]_D +26 (*c* 0.45). IR ν (CHCl₃) cm⁻¹: 3618, 1713, 1640, 1600, 1580, 1290. ¹H NMR (400 MHz): δ 0.84, 0.88, 0.92, 0.94, 0.97 (each 3H, s, 5 × CH₃), 1.27 (3H, t, *J* = 7.2 Hz, CH₃), 1.69 (3H, s, CH₃), 1.84–1.94 (3H, m), 2.18–2.26 (2H, m), 3.01 (1H, td, *J* = 11.0, 4.7 Hz), 4.07–4.21 (2H, m), 4.61 (1H, m), 4.73–4.77 (2H, m), 7.54–7.61 (2H, m), 7.73 (1H, m), 7.90 (1H, m). MS *m/z* (rel. intensity): 632 (2, M⁺), 617 (1), 588 (1), 559 (2), 543 (1), 515 (1), 502 (3), 484 (4), 466 (31), 451 (13), 423 (6), 393 (8), 357 (4), 330 (3), 311 (3), 287 (2), 272 (22), 263 (14), 247 (10), 215 (14), 203 (32), 189 (100). Anal. Calcd for C₄₀H₅₆O₆: C, 75.91; H, 8.92. Found: C, 75.80; H, 9.05.

5.5. Hemiphthalate of benzyl-betulinatate (4a)

Starting with compound **4** (700 mg, 1.28 mmol), chromatography and crystallization from the mixture of acetonitrile–water gave colorless needles of **4a** (702 mg, 79%), mp 195–197 °C; [α]_D +30 (*c* 0.37). IR ν (CHCl₃) cm⁻¹: 3508, 1716 sh, 1641, 1600, 1580, 1290. ¹H NMR (400 MHz): δ 0.76, 0.82, 0.87, 0.93, 0.95, 1.68 (each 3H, s, 6 × CH₃), 1.81–1.93 (3H, m), 2.18 (1H, td, *J* = 12.8, 3.7 Hz), 2.28 (1H, br d, *J* = 12.4 Hz), 3.02 (1H, td, *J* = 10.8, 4.6 Hz), 4.60 (1H, m), 4.72–4.76 (2H, m), 5.09 (1H, d, *J* = 12.3 Hz), 5.15 (1H, d, *J* = 12.3 Hz), 7.30–7.38 (5H, m), 7.54–7.62 (2H, m), 7.73 (1H, m), 7.90 (1H, m). MS *m/z* (rel. intensity): 694 (2, M⁺), 679 (1), 666 (1), 603 (1), 575 (1), 559 (6), 546 (4), 528 (38), 514 (14), 485 (11), 455 (2), 437 (100), 423 (5), 409 (12), 395 (11), 377 (2), 338 (9), 325 (6), 301 (1), 279 (2), 257 (5), 247 (13), 233 (10), 215 (7), 203 (12), 189 (44). Anal. Calcd for C₄₅H₅₈O₆: C, 77.77; H, 8.41. Found: C, 77.72; H, 8.52.

5.6. Bis(hemiphthalate) of betulin (5a)

Starting with compound **5** (500 mg, 1.13 mmol), chromatography and crystallization from ethanol gave a white powder of **5a** (633 mg, 76%), mp 178–180 °C; [α]_D +28 (*c* 0.55). IR ν (CHCl₃) cm⁻¹: 3509, 1716, 1641, 1600, 1581, 1291. ¹H NMR (400 MHz): δ 0.89, 0.90, 0.93, 1.02, 1.07, 1.70 (each 3H, s, 6 × CH₃), 2.48 (1H, td, *J* = 11.0, 5.5 Hz), 4.08 (1H, d, *J* = 10.7 Hz), 4.56 (1H, d, *J* = 10.7 Hz), 4.60 (1H, m), 4.70 (1H, br d, *J* = 2.3 Hz), 4.74 (1H, dd, *J* = 11.4, 4.7 Hz), 7.54–7.63 (4H, m), 7.74 (1H, m), 7.77 (1H, m), 7.85–7.88 (2H, m). MS *m/z* (rel. intensity): 738 (1, M⁺), 590 (1), 572 (8), 559 (2), 529 (5), 513 (1), 485 (1), 457 (1), 438 (7), 424 (14), 411 (5), 393 (7), 381 (4), 313 (1), 299 (4), 288 (3), 271 (4), 257 (6), 245 (5), 234 (13), 216 (20), 203 (41), 189 (100). Anal. Calcd for C₄₆H₅₈O₈: C, 74.77; H, 7.91. Found: C, 74.71; H, 7.98.

5.7. Hemiphthalate of 3 β -*O*-acetyl-betulin (6a)

Starting with compound **6** (500 mg, 1.03 mmol), chromatography and crystallization from acetonitrile gave a white powder of **6a** (505 mg, 77%), mp 197–198 °C; [α]_D +20 (*c* 0.50). IR ν (CHCl₃) cm⁻¹: 3512, 1720, 1641, 1600, 1581, 1291. ¹H NMR (400 MHz): δ 0.82 (6H, s, 2 × CH₃), 0.83, 0.96, 1.05, 1.69, 2.05 (each 3H,

s, 5 × CH₃), 2.50 (1H, td, *J* = 11.0, 5.6 Hz), 4.12 (1H, d, *J* = 11.1 Hz), 4.46 (1H, dd, *J* = 10.0, 6.1 Hz), 4.51 (1H, d, *J* = 11.1 Hz), 4.60 (1H, m), 4.70 (1H, br d, *J* = 2.0 Hz), 7.55–7.64 (2H, m), 7.72 (1H, dd, *J* = 7.4, 1.7 Hz), 7.93 (1H, dd, *J* = 7.3, 1.5 Hz). MS *m/z* (rel. intensity): 632 (1, M⁺), 617 (1), 589 (6), 572 (31), 557 (7), 546 (12), 514 (15), 466 (42), 406 (35), 381 (7), 313 (1), 299 (5), 288 (2), 271 (4), 257 (7), 245 (6), 234 (18), 216 (25), 203 (44), 189 (100). Anal. Calcd for C₄₀H₅₆O₆: C, 75.91; H, 8.92. Found: C, 75.87; H, 8.98.

5.8. Hemiphthalate of 28-O-acetyl-betulin (7a)

Starting with compound **7** (500 mg, 1.03 mmol), chromatography and crystallization from the mixture acetonitrile–water gave a white powder of **7a** (530 mg, 80%), mp 192–194 °C; [α]_D+30 (*c* 0.44). IR ν (CHCl₃) cm⁻¹: 3510, 1721, 1641, 1600, 1580, 1290. ¹H NMR (400 MHz): δ 0.85, 0.88, 0.95, 0.99, 1.03, 1.69, 2.08 (each 3H, s, 7 × CH₃), 2.48 (1H, td, *J* = 11.0, 6.0 Hz), 3.86 (1H, d, *J* = 11.3 Hz), 4.25 (1H, d, *J* = 11.3 Hz), 4.60 (1H, m), 4.75 (1H, br d, *J* = 2.3 Hz), 4.46 (1H, dd, *J* = 11.4, 4.4 Hz), 7.54–7.61 (2H, m), 7.72 (1H, m), 7.89 (1H, m). MS *m/z* (rel. intensity): 632 (2, M⁺), 617 (1), 589 (4), 572 (24), 557 (9), 514 (11), 466 (57), 406 (32), 381 (4), 313 (1), 299 (4), 288 (1), 271 (6), 257 (5), 245 (5), 234 (15), 216 (23), 203 (44), 189 (100). Anal. Calcd for C₄₀H₅₆O₆: C, 75.91; H, 8.92. Found: C, 75.52; H, 9.05.

5.9. General procedure for esterification of hemiphthalate

A solution of diazomethane (resp. diazoethane) in diethylether was added to a solution of hemiphthalate (1 mmol) in chloroform (5 mL). After 30 min the reaction mixture was evaporated and the residue was chromatographed using preparative-scale HPLC (hexane–ethyl acetate) to afford the product.

5.10. Methyl-phthalate of methyl-betulin (2b)

HPLC (hexane–ethyl acetate; 9:1), mp 224–226 °C (methanol); [α]_D+28 (*c* 0.41). IR ν (CHCl₃) cm⁻¹: 1720, 1641, 1600, 1580, 1292. ¹H NMR (400 MHz): δ 0.87, 0.88, 0.93, 0.94, 0.98, 1.70 (each 3H, s, 6 × CH₃), 2.17–2.25 (2H, m), 3.00 (1H, td, *J* = 11.0, 4.3 Hz), 3.67, 3.90 (each 3H, s, 3 × CH₃), 4.61 (1H, m), 4.72 (1H, dd, *J* = 11.6, 4.6 Hz), 4.74 (1H, br s), 7.50–7.54 (2H, m), 7.68–7.73 (2H, m). MS *m/z* (rel. intensity): 632 (9, M⁺), 617 (1), 572 (4), 452 (100), 437 (25), 409 (14), 393 (8), 371 (5), 316 (3), 283 (1), 273 (3), 262 (16), 257 (2), 248 (9), 233 (3), 215 (7), 203 (17), 189 (61). Anal. Calcd for C₄₀H₅₆O₆: C, 75.91; H, 8.92. Found: C, 75.83; H, 9.01.

5.11. Ethyl-phthalate of methyl-betulin (2c)

HPLC (hexane–ethyl acetate; 7:1), mp 211–213 °C (ethanol); [α]_D+27 (*c* 0.43). IR ν (CHCl₃) cm⁻¹: 1719, 1641, 1600, 1580, 1289. ¹H NMR (400 MHz): δ 0.87, 0.89, 0.93, 0.94, 0.98 (each 3H, s, 5 × CH₃), 1.36 (3H, t, *J* = 7.1, CH₃), 1.70 (3H, s, CH₃), 2.17–2.25 (2H, m),

3.00 (1H, td, *J* = 11.0, 4.3 Hz), 3.67 (3H, s, CH₃), 4.32–4.40 (2H, m), 4.61 (1H, m), 4.72 (1H, dd, *J* = 11.6, 4.6 Hz), 4.74 (1H, br s), 7.49–7.54 (2H, m), 7.67 (1H, m), 7.72 (1H, m). MS *m/z* (rel. intensity): 646 (6, M⁺), 631 (1), 586 (4), 466 (3), 452 (100), 437 (26), 409 (14), 393 (8), 377 (3), 316 (2), 283 (1), 273 (3), 262 (12), 257 (13), 248 (9), 233 (3), 215 (8), 203 (17), 189 (57). Anal. Calcd for C₄₁H₅₈O₆: C, 76.12; H, 9.04. Found: C, 76.10; H, 9.11.

5.12. Methyl-phthalate of ethyl-betulin (3b)

HPLC (hexane–ethyl acetate; 6:1), mp 167–169 °C (methanol); [α]_D+33 (*c* 0.36). IR ν (CHCl₃) cm⁻¹: 1715, 1639, 1600, 1580, 1289. ¹H NMR (400 MHz): δ 0.87, 0.88 (each 3H, s, 2 × CH₃), 0.93 (6H, s, 2 × CH₃), 0.98 (3H, s, CH₃), 1.27 (3H, t, *J* = 7.2 Hz, CH₃), 1.70 (3H, s, CH₃), 1.82–1.94 (3H, m), 2.19–2.26 (2H, m), 3.02 (1H, td, *J* = 11.0, 4.8 Hz), 4.08–4.21 (2H, m), 4.61 (1H, m), 4.70–4.74 (2H, m), 7.50–7.54 (2H, m), 7.69 (1H, m), 7.72 (1H, m). MS *m/z* (rel. intensity): 646 (7, M⁺), 631 (1), 618 (1), 573 (4), 466 (70), 451 (29), 438 (3), 423 (19), 409 (2), 393 (11), 371 (6), 330 (5), 309 (2), 287 (7), 276 (20), 263 (13), 215 (17), 203 (29), 189 (100). Anal. Calcd for C₄₁H₅₈O₆: C, 76.12; H, 9.04. Found: C, 76.00; H, 9.15.

5.13. Ethyl-phthalate of ethyl-betulin (3c)

HPLC (hexane–ethyl acetate; 6:1), mp 172–174 °C (ethanol); [α]_D+27 (*c* 0.41). IR ν (CHCl₃) cm⁻¹: 1715, 1639, 1600, 1580, 1289. ¹H NMR (400 MHz): δ 0.87, 0.89, 0.93, 0.94, 0.98 (each 3H, s, 5 × CH₃), 1.27 (3H, t, *J* = 7.2 Hz, CH₃), 1.36 (3H, t, *J* = 7.2 Hz, CH₃), 1.70 (3H, s, CH₃), 1.82–1.94 (3H, m), 2.19–2.26 (2H, m), 3.02 (1H, td, *J* = 11.0, 4.8 Hz), 4.08–4.22 (2H, m), 4.30–4.42 (2H, m), 4.61 (1H, m), 4.70–4.74 (2H, m), 7.49–7.54 (2H, m), 7.68 (1H, m), 7.72 (1H, m). MS *m/z* (rel. intensity): 660 (7, M⁺), 645 (2), 632 (1), 587 (9), 572 (1), 466 (95), 451 (64), 438 (3), 423 (29), 410 (4), 393 (27), 385 (15), 330 (8), 309 (6), 287 (32), 276 (38), 263 (28), 229 (12), 215 (23), 203 (75), 189 (100). Anal. Calcd for C₄₂H₆₀O₆: C, 76.33; H, 9.15. Found: C, 76.30; H, 9.22.

5.14. Methyl-phthalate of benzyl-betulin (4b)

HPLC (hexane–ethyl acetate; 9:1), mp 110–112 °C (methanol–chloroform); [α]_D+33 (*c* 0.38). IR ν (CHCl₃) cm⁻¹: 1720, 1640, 1600, 1580, 1292. ¹H NMR (400 MHz): δ 0.77, 0.85, 0.88, 0.93, 0.95, 1.69 (each 3H, s, 6 × CH₃), 1.82–1.94 (3H, m), 2.19 (1H, td, *J* = 12.8, 3.6 Hz), 2.28 (1H, br d, *J* = 12.5 Hz), 3.02 (1H, td, *J* = 10.8, 4.4 Hz), 3.89 (3H, s, CH₃), 4.60 (1H, br s), 4.70–4.73 (2H, m), 5.09 (1H, d, *J* = 12.4 Hz), 5.15 (1H, d, *J* = 12.4 Hz), 7.30–7.38 (5H, m), 7.50–7.54 (2H, m), 7.68–7.73 (2H, m). MS *m/z* (rel. intensity): 708 (4, M⁺), 693 (1), 680 (1), 617 (2), 590 (1), 573 (5), 528 (55), 514 (28), 485 (9), 437 (100), 423 (4), 409 (7), 393 (8), 371 (7), 338 (8), 325 (5), 301 (1), 279 (2), 271 (11), 255 (3), 247 (10), 233 (9), 221 (3), 203 (25), 189 (40). Anal. Calcd for C₄₆H₆₀O₆: C, 77.93; H, 8.53. Found: C, 77.88; H, 8.55.

5.15. Ethyl-phthalate of benzyl-betulinic acid (4c)

HPLC (hexane–ethyl acetate; 9:1), mp 111–112 °C (ethanol); $[\alpha]_D^{25} +28$ (*c* 0.39). IR ν (CHCl₃) cm⁻¹: 1720, 1640, 1600, 1580, 1293. ¹H NMR (400 MHz): δ 0.77, 0.85, 0.87, 0.93, 0.95 (each 3H, s, 5 × CH₃), 1.35 (3H, t, *J* = 7.2 Hz, CH₃), 1.69 (3H, s, CH₃), 1.82–1.94 (3H, m), 2.19 (1H, td, *J* = 12.8, 3.6 Hz), 2.28 (1H, br d, *J* = 12.4 Hz), 3.02 (1H, td, *J* = 10.8, 4.4 Hz), 4.32–4.40 (2H, m), 4.60 (1H, br s), 4.70–4.73 (2H, m), 5.09 (1H, d, *J* = 12.4 Hz), 5.15 (1H, d, *J* = 12.4 Hz), 7.30–7.38 (5H, m), 7.49–7.54 (2H, m), 7.68 (1H, m), 7.72 (1H, m). MS *m/z* (rel. intensity): 722 (5, M⁺), 707 (1), 679 (1), 631 (4), 604 (1), 587 (6), 528 (62), 514 (25), 485 (10), 437 (100), 423 (4), 409 (6), 393 (7), 371 (7), 338 (9), 325 (5), 301 (1), 279 (2), 271 (15), 255 (4), 247 (12), 233 (9), 221 (3), 203 (24), 189 (43). Anal. Calcd for C₄₇H₆₂O₆: C, 78.08; H, 8.64. Found: C, 77.92; H, 8.70.

5.16. Bis(methyl-phthalate) of betulin (5b)

HPLC (hexane–ethyl acetate; 4:1), mp 122–124 °C (methanol); $[\alpha]_D^{25} +30$ (*c* 0.50). IR ν (CHCl₃) cm⁻¹: 1725, 1640, 1600, 1580, 1291. ¹H NMR (400 MHz): δ 0.89 (6H, s, 2 × CH₃), 0.94, 1.00, 1.08, 1.71 (each 3H, s, 4 × CH₃), 2.03 (1H, m), 2.51 (1H, td, *J* = 11.0, 5.8 Hz), 3.90, 3.91 (each 3H, s, 2 × CH₃), 4.11 (1H, d, *J* = 11.2 Hz), 4.50 (1H, dd, *J* = 11.2, 1.2 Hz), 4.61 (1H, m), 4.71–4.75 (2H, m), 7.50–7.56 (4H, m), 7.68–7.77 (4H, m). MS *m/z* (rel. intensity): 766 (1, M⁺), 586 (100), 571 (14), 543 (13), 530 (1), 518 (1), 504 (1), 450 (3), 423 (14), 406 (40), 391 (11), 383 (1), 363 (16), 337 (3), 323 (2), 295 (2), 270 (5), 255 (4), 241 (3), 229 (7), 216 (18), 203 (36), 189 (95). Anal. Calcd for C₄₈H₆₂O₈: C, 75.16; H, 8.15. Found: C, 75.17; H, 8.21.

5.17. Bis(ethyl-phthalate) of betulin (5c)

HPLC (hexane–ethyl acetate; 4:1), mp 175–177 °C (ethanol); $[\alpha]_D^{25} +29$ (*c* 0.43). IR ν (CHCl₃) cm⁻¹: 1725, 1640, 1600, 1580, 1291. ¹H NMR (400 MHz): δ 0.89, 0.90, 0.95, 1.00, 1.08 (each 3H, s, 5 × CH₃), 1.36, 1.38 (each 3H, t, *J* = 7.2 Hz, 2 × CH₃), 1.71 (3H, s, CH₃), 2.51 (1H, td, *J* = 11.1, 6.0 Hz), 4.11 (1H, d, *J* = 11.0 Hz), 4.32–4.41 (4H, m), 4.50 (1H, dd, *J* = 11.2, 1.2 Hz), 4.61 (1H, m), 4.71–4.75 (2H, m), 7.49–7.56 (4H, m), 7.67–7.77 (4H, m). MS *m/z* (rel. intensity): 794 (1, M⁺), 601 (100), 586 (21), 558 (15), 545 (2), 533 (1), 519 (2), 465 (5), 438 (16), 406 (42), 391 (15), 383 (1), 363 (20), 337 (5), 323 (1), 295 (2), 270 (5), 255 (8), 241 (1), 229 (7), 216 (22), 203 (30), 189 (93). Anal. Calcd for C₅₀H₆₆O₈: C, 75.53; H, 8.37. Found: C, 75.28; H, 8.49.

5.18. Methyl-phthalate of 3 β -O-acetyl-betulin (6b)

HPLC (hexane–ethyl acetate; 9:1), mp 182–184 °C (methanol–butanone); $[\alpha]_D^{25} +21$ (*c* 0.48). IR ν (CHCl₃) cm⁻¹: 1724, 1640, 1600, 1580, 1292, 1260. ¹H NMR (400 MHz): δ 0.84, 0.85, 0.86, 0.99, 1.07, 1.70, 2.05 (each 3H, s, 7 × CH₃), 2.50 (1H, td, *J* = 10.8, 5.8 Hz), 3.90 (3H, s, CH₃), 4.10 (1H, d, *J* = 11.0 Hz), 4.45–4.51 (2H, m), 4.60 (1H, m), 4.71 (1H, br d, *J* = 2.0 Hz), 7.53–

7.56 (2H, m), 7.70 (1H, m), 7.75 (1H, m). MS *m/z* (rel. intensity): 646 (1, M⁺), 631 (1), 603 (5), 586 (35), 560 (10), 543 (15), 466 (40), 406 (35), 381 (10), 313 (1), 299 (3), 288 (1), 271 (4), 257 (6), 245 (6), 234 (20), 216 (25), 203 (48), 189 (100). Anal. Calcd for C₄₁H₅₈O₆: C, 76.12; H, 9.04. Found: C, 76.07; H, 9.05.

5.19. Ethyl-phthalate of 3 β -O-acetyl-betulin (6c)

HPLC (hexane–ethyl acetate; 9:1), mp 186–188 °C (ethanol–butanone); $[\alpha]_D^{25} +20$ (*c* 0.44). IR ν (CHCl₃) cm⁻¹: 1720, 1640, 1600, 1580, 1292, 1265. ¹H NMR (400 MHz): δ 0.84, 0.85, 0.86, 0.99, 1.07 (each 3H, s, 5 × CH₃), 1.37 (3H, t, *J* = 7.2 Hz, CH₃), 1.70, 2.05 (each 3H, s, 2 × CH₃), 2.50 (1H, td, *J* = 11.1, 5.8 Hz), 4.11 (1H, d, *J* = 11.0 Hz), 4.37 (2H, q, *J* = 7.2 Hz); 4.45–4.49 (2H, m), 4.60 (1H, m), 4.71 (1H, br d, *J* = 2.3 Hz), 7.52–7.54 (2H, m), 7.70 (1H, m), 7.74 (1H, m). MS *m/z* (rel. intensity): 660 (1, M⁺), 645 (1), 617 (5), 600 (32), 574 (12), 557 (12), 466 (47), 406 (33), 381 (8), 313 (1), 299 (3), 288 (1), 271 (3), 257 (7), 245 (7), 234 (20), 216 (25), 203 (50), 189 (100). Anal. Calcd for C₄₂H₆₀O₆: C, 76.33; H, 9.15. Found: C, 76.30; H, 9.15.

5.20. Methyl-phthalate of 28-O-acetyl-betulin (7b)

HPLC (hexane–ethyl acetate; 9:1), mp 89–91 °C (lyophilization from *tert*-butanol); $[\alpha]_D^{25} +27$ (*c* 0.21). IR ν (CHCl₃) cm⁻¹: 1720, 1640, 1600, 1580, 1290, 1263. ¹H NMR (400 MHz): δ 0.88, 0.89, 0.94, 0.99, 1.04, 1.69, 2.07 (each 3H, s, 7 × CH₃), 2.45 (1H, td, *J* = 11.4, 6.0 Hz), 3.86 (1H, d, *J* = 10.8 Hz), 3.90 (3H, s, CH₃), 4.26 (1H, dd, *J* = 10.8, 1.2 Hz), 4.60 (1H, m), 4.70 (1H, br d, *J* = 2.4 Hz), 4.72 (1H, dd, *J* = 11.8, 5.2 Hz), 7.50–7.54 (2H, m), 7.67–7.74 (2H, m). MS *m/z* (rel. intensity): 646 (1, M⁺), 631 (1), 603 (5), 586 (28), 571 (7), 528 (9), 466 (61), 406 (35), 381 (5), 313 (2), 299 (4), 288 (1), 271 (7), 257 (5), 245 (7), 234 (18), 216 (23), 203 (48), 189 (100). Anal. Calcd for C₄₁H₅₈O₆: C, 76.12; H, 9.04. Found: C, 76.10; H, 9.13.

5.21. Ethyl-phthalate of 28-O-acetyl-betulin (7c)

HPLC (hexane–ethyl acetate; 9:1), mp 96–98 °C (lyophilization from *tert*-butanol); $[\alpha]_D^{25} +28$ (*c* 0.36). IR ν (CHCl₃) cm⁻¹: 1720, 1640, 1600, 1580, 1290, 1263. ¹H NMR (400 MHz): δ 0.88, 0.90, 0.94, 0.99, 1.05 (each 3H, s, 5 × CH₃), 1.36 (3H, t, *J* = 7.2 Hz, CH₃), 1.69, 2.07 (each 3H, s, 2 × CH₃), 2.45 (1H, td, *J* = 11.4, 6.0 Hz), 3.86 (1H, d, *J* = 10.8 Hz), 4.26 (1H, dd, *J* = 10.8, 1.2 Hz), 4.31–4.42 (2H, m), 4.60 (1H, m), 4.70 (1H, br d, *J* = 2.4 Hz), 4.72 (1H, dd, *J* = 11.6, 5.0 Hz), 7.49–7.55 (2H, m), 7.66–7.74 (2H, m). MS *m/z* (rel. intensity): 660 (1, M⁺), 645 (1), 617 (3), 600 (33), 574 (13), 557 (12), 466 (51), 406 (29), 381 (10), 313 (1), 299 (2), 288 (1), 271 (2), 257 (6), 245 (6), 234 (20), 216 (22), 203 (48), 189 (100). Anal. Calcd for C₄₂H₆₀O₆: C, 76.33; H, 9.15. Found: C, 76.28; H, 9.22.

5.22. Methyl-phthalate of betulinic acid (1b)

1,4-*cyclo*-Hexadiene (300 μ L, 3.2 mol) and 10% palladium on charcoal (300 mg) was added to a solution of

4b (300 mg, 0.42 mmol) in THF (20 mL) and anhydrous EtOH (10 mL). After being stirred at 30 °C for 10 h, palladium was filtered off and solvents were evaporated. The residue was crystallized from methanol to afford **1c** as a white powder (228 mg, 88%), mp 250–252 °C; $[\alpha]_D^{25} +32$ (*c* 0.41). IR ν (CHCl₃) cm⁻¹: 3508, 1710 sh, 1690, 1641, 1600, 1580, 1290. ¹H NMR (400 MHz): δ 0.88 (6H, s, 2 × CH₃), 0.94, 0.95, 0.99, 1.71 (each 3H, s, 4 × CH₃), 1.85 (1H, m), 1.95–2.04 (2H, m), 2.19 (1H, td, *J* = 12.4, 5.8 Hz), 2.28 (1H, td, *J* = 12.4, 3.0 Hz), 3.02 (1H, td, *J* = 10.7, 4.5 Hz), 3.89 (3H, s, CH₃), 4.62 (1H, m), 4.71–4.76 (2H, m), 7.50–7.54 (2H, m), 7.67–7.74 (2H, m). MS *m/z* (rel. intensity): 618 (2, M⁺), 603 (1), 588 (1), 572 (1), 528 (1), 438 (58), 423 (22), 395 (37), 369 (4), 287 (1), 269 (2), 259 (4), 248 (5), 232 (4), 215 (2), 202 (9), 189 (31). Anal. Calcd for C₃₉H₅₄O₆: C, 75.69; H, 8.80. Found: C, 75.63; H, 8.90.

5.23. Ethyl-phthalate of betulinic acid (**1c**)

Compound **1c** was prepared from **4c** by a procedure similar to that described for **1b**. Crystallization from ethanol gave a white powder of **1c** (220 mg, 85%), mp 247–249 °C; $[\alpha]_D^{25} +25$ (*c* 0.42). IR ν (CHCl₃) cm⁻¹: 3508, 1710 sh, 1691, 1641, 1600, 1580, 1289. ¹H NMR (400 MHz): δ 0.88, 0.89, 0.94, 0.95, 0.99 (each 3H, s, 5 × CH₃), 1.35 (3H, t, *J* = 7.2 Hz, CH₃), 1.71 (3H, s, CH₃), 1.85 (1H, m), 1.95–2.04 (2H, m), 2.19 (1H, td, *J* = 12.4, 5.8 Hz), 2.28 (1H, td, *J* = 12.4, 3.0 Hz), 3.02 (1H, td, *J* = 10.7, 4.5 Hz), 4.31–4.42 (2H, m), 4.62 (1H, m), 4.71–4.76 (2H, m), 7.49–7.54 (2H, m), 7.66–7.74 (2H, m). MS *m/z* (rel. intensity): 632 (4, M⁺), 617 (1), 586 (3), 528 (1), 466 (2), 438 (53), 423 (16), 395 (34), 369 (2), 287 (1), 259 (4), 248 (5), 232 (4), 217 (2), 202 (9), 189 (31). Anal. Calcd for C₄₀H₅₆O₆: C, 75.91; H, 8.92. Found: C, 75.85; H, 8.94.

6. Materials and methods

6.1. Chemicals

Phthalic anhydride, DMAP, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and SDS were purchased from Sigma–Aldrich, s.r.o., Czech Republic.

6.2. Cell lines

Cell lines CEM, HT29, K562, PC-3, and SK MEL2 were purchased from the American Tissue Culture Collection (ATCC). Paclitaxel-resistant subline of K562 cells (K562-tax) was prepared and characterized in our laboratories.¹⁵

6.3. MTT cytotoxicity assay

Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (2500–30,000 cells/well based on cell growth characteristics). Cells were added by pipette (80 μ L) into 96-well microtiter plates. Inoculates were allowed

a pre-incubation period of 24 h at 37 °C and 5% CO₂ for stabilization. Fourfold dilutions, in 20 μ L aliquots, of the intended test concentration were added at time zero to the microtiter plate wells. All test compound (dissolved in 10 μ L of 10% DMSO) concentrations were examined in duplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C, in a 5% CO₂ atmosphere at 100% using MTT. Aliquots (10 μ L) of the MTT stock humidity. At the end of the incubation period, the cells were assayed solution were pipetted into each well and incubated for a further 1–4 h. After this incubation period formazan produced was dissolved by the addition of 100 μ L/well of 10% aq SDS (pH = 5.5), followed by a further incubation at 37 °C overnight. The optical density (OD) was measured at 540 nm with a Labsystem iEMS Reader MF. Inhibition of tumor growth/survival (IC) was calculated using the following equation: IC = (ODdrug-exposed well/mean ODcontrol wells) × 100%. The IC₅₀ value, the drug concentration lethal to 50% of the tumor cells, was calculated from appropriate dose–response curves.

Acknowledgements

This study was supported in part by the Ministry of Education of the Czech Republic (MSM 113100001, 151100001), which paid for instrumental equipment, by the Czech Science Foundation (203/03/D152), from which the chemicals were paid and by MPO project (FT-TA/027) from which HPLC column, silica gel and all other material support were paid. Biological testing was supported by the Czech Science Foundation (301/03/1570). We are grateful to Iva Tislerova for measurement of NMR spectra. Special thanks to Bohunka Sperlichova for measurement of optical rotatory power.

References and notes

- Pisha, E.; Chai, H.; Lee, I. S.; Chagwedera, T. E.; Farnsworth, N. R.; Cordell, G. A.; Beecher, C. W. W.; Fong, H. H. S.; Kinghorn, A. D.; Brown, D. M.; Want, M. C.; Wall, M. E.; Hieken, T. J.; Gupta, T. K. D.; Pezzuto, J. M. *Nat. Med.* **1995**, *1*, 1046–1051.
- Cichewicz, R. H.; Kouzi, S. A. *Med. Res. Rev.* **2004**, *24*, 90–114.
- Zuco, V.; Supino, R.; Righetti, S. C.; Cleris, L.; Marchesi, E.; Gambacorti-Passerini, C.; Formelli, F. *Cancer Lett.* **2002**, *175*, 17–25.
- Kim, D. S. H. L.; Pezzuto, J. M.; Pisha, E. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1707–1712.
- Urban, M.; Sarek, J.; Klinot, J.; Korinkova, G.; Hajduch, M. *J. Nat. Prod.* **2004**, *67*, 1100–1105.
- Mukherjee, R.; Jaggi, M.; Rajedran, P.; Siddiqui, M. J. A.; Srivastara, S. K.; Vardhan, A.; Burman, A. C. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2181–2184.
- Fulda, S.; Jeremias, I.; Debatin, K. M. *Oncogene* **2004**, *23*, 7611–7620.
- Hajduch, M.; Sarek, J.; Triterpenoid derivatives. PCT Int. Patent Appl. WO0190046, 23 May 2001.
- Kim, J. Y.; Koo, H. M.; Kim, D. S. H. L. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2405–2408.

10. Hashimoto, F.; Kashiwada, Y.; Cosentino, L. M.; Chen, C.-H.; Garrett, P. E.; Lee, K.-H. *Bioorg. Med. Chem.* **1997**, *5*, 2133–2143.
11. Šarek, J.; Klinot, J.; Bražinová, S.; Džubák, P.; Klinotová, E.; Nosková, V.; Křeček, V.; Kořínková, G.; Thomson, J. O.; Janošťáková, A.; Wang, S.; Parsons, S.; Fischer, P. M.; Zhelev, N. Z.; Hajdúch, M. *J. Med. Chem.* **2003**, *46*, 5402–5415.
12. Li, T.-S.; Wang, J.-X.; Zheng, X.-J. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3957–3965.
13. Tietze, L. F.; Heinzen, H.; Moyna, P.; Rischer, M.; Neunaber, H. *Liebigs Ann. Chem.* **1991**, 1245–1249.
14. Felix, A. M.; Heimer, E. P.; Lambros, T. J.; Tzougraki, C.; Meienhofer, J. *J. Org. Chem.* **1978**, *43*, 4194–4196.
15. Noskova, V.; Dzubak, P.; Kuzmina, G.; Ludkova, A.; Stehlik, D.; Trojanec, R.; Janostakova, A.; Korinkova, G.; Mihal, V.; Hajduch, M. *Neoplasma* **2002**, *49*, 418–425.