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Synthesis of selectively deuterated and tritiated lupane derivatives with cytotoxic activity

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Abstract The aim of this work was to synthesize deuterated and tritiated analogues of highly oxidized lupane derivatives known from our group. We selected compounds that previously showed very high cytotoxic activity on multiple cancer cell lines in order to further investigate the mechanism of their action. From starting material (compounds **1**–**4**), we obtained benzyl platanate (**5**) and its reaction with deuteromethyltriphenylphosphonium iodide gave deuterated compound **6**. Following benzyl deprotection gave free acid **7** and oxidation with SeO_2 gave 30-oxo-[29-$^2H_2$]lup-20(29)-en-28-oic acid (**8**), which is one of the most active compounds synthesized in our group to date (IC_{50} 6 μmol/L on CEM cell line). The alkylation of benzyl 2-hydroxy-3-oxolupa-1,20(29)-dien-28-oate (**9**) with methyliodide or deuteromethyliodide followed by a series of deprotection and hydrogenation steps gave compounds **10**–**14**, where 2β-[31-$^2H_3$]methoxy-3-oxolupan-20(29)-en-28-oic acid (**13**) is especially interesting, it showed lower activity on CEM cell line (IC_{50} 10 μmol/L) however, it was very active against Ph1—positive human leukemia BV-173 (IC_{50} 0.91 μmol/L) and against human myelogenous leukemia K562 (IC_{50} 0.52 μmol/L). Selectively labelled [3α-^{2}H] and [3α-^{3}H] methyl 3β-acetoxy-21,22-dioxolup-18-en-28-oates **24**, **25** were prepared in three steps by reduction of corresponding 3-oxo derivatives and they showed moderate activity on CEM cell line (IC_{50} 10 μmol/L). In total, 11 labelled compounds (**6**–**8**, **11**, **14**, **18**, **19**, **21**, **22**, **24** and **25**) have not been reported before.

Keywords Isotopic labelling · Tritium · Deuterium · Betulin · Betulinic acid · Cytotoxicity

Introduction

Lupane derivatives have been studied for a long time especially for a wide variety of their biological activities [1–6] including antitumor, -viral, -inflammatory, -microbial as well as hepato- and cardioprotective effects. Derivatives of betulin (**1**) and betulinic acid (**2**) were tested on various tumor cell lines for their cytotoxic activity [1, 5, 6]. In our research group, we modified **1** and **2** and obtained a large group of active compounds called betulinines [7–9]. The group contains highly oxidized lupane, des-E-lupane, oleanane and other derivatives, which showed very strong cytotoxic and antiviral activities [7–9]. During our research, we were able to demonstrate that betulinines have multispectral cytotoxic activity on various cancer cell lines of different histopathogenetical origin, including those with multidrug resistance [10–12]. For this study, compounds with high cytotoxicity were chosen and their deuterated and tritiated analogues have been synthesized in order to study their mechanism of action in the future. In this paper, we only describe their synthesis.

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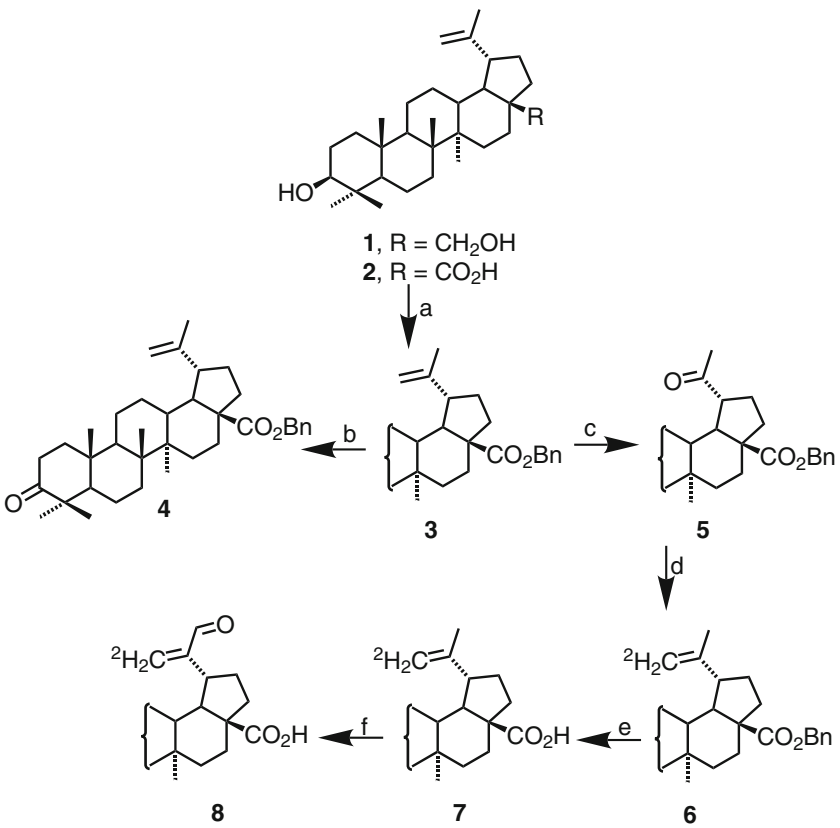
Results and discussion

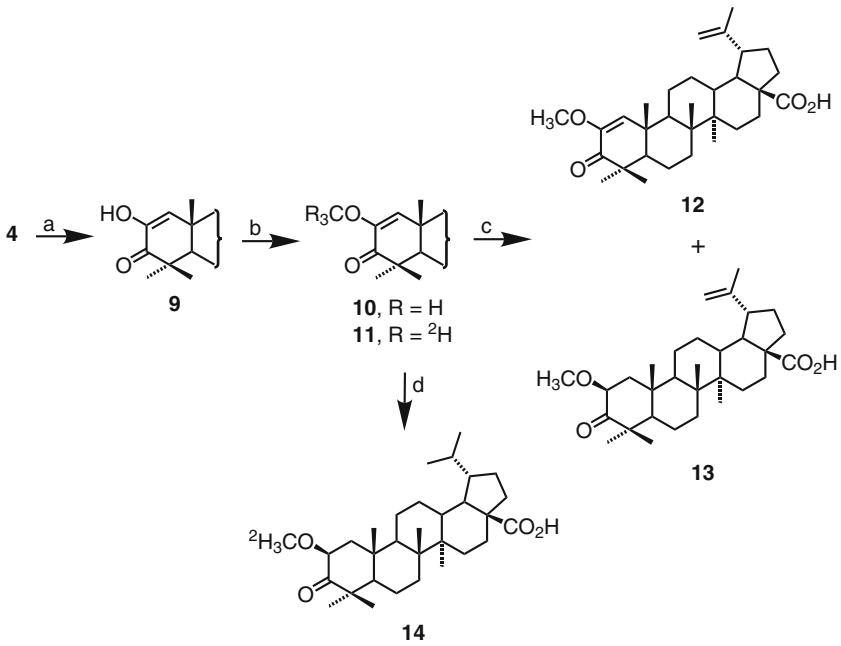
Availability of the key triterpenes from natural sources [13, 14] in large amounts allowed us to synthesize a library of cytotoxic active compounds [6, 7]. Betulin (**1**) was extracted from the outer layers of birch bark [13, 14] (*Betula pendula*). Acid **2** was extracted from bark of sycamore trees [13, 14] (*Platanus hispanica*). Betulinic acid (**2**) may also be synthesized from betulin (**1**) by oxidation of the primary alcoholic function [15, 16]. We synthesized benzyl-platanate (**5**) by introducing ozone made from air in an ozonizer machine into a solution of protected betulinic acid **3** in chloroform at −80 °C. Wittig reaction of substrate **5** with methylidenphosphorane in toluene, previously reported by Tietze et al. [17], gave a very poor yield of benzyl betulinate (**6**) and therefore we had to modify the reaction conditions; replacing toluene with tetrahydrofuran (THF) gave us better yield (80 %). Methyltriphenylphosphonium iodide $C^2H_3[Ph_3P]I$ was prepared in a dry apparatus the day before the experiment was performed [17]. Deprotection of **6** was carried out in a sealed flask with 10 % Pd/C catalyst in a mixture of THF and methanol, hydrogen was introduced via an injection needle. This yielded 29-dideuterated betulinic acid (**7**). Allylic oxidation of olefin **7** by selenium dioxide in 2-methoxyethanol under reflux gave aldehyde **8** in excellent yield [18] (Scheme 1).

Oxidation of benzyl betulinate (**3**) gave benzyl betulonate (**4**). Autooxidation of **4** with dry air in a presence of potassium *tert*-butoxide in *tert*-butanol gave diosphenol **9** [19, 20]. A subsequent alkylation of **9** with methyl iodide followed by hydrogenation at atmospheric pressure yielded a mixture of 2-methoxy-3-oxolupa-1,20(29)-diene-28-oic acid (**12**) and 2β-methoxy-3-oxolup-20(29)-ene-28-oic acid (**13**) [20]. The hydrogenation of a double bond 1(2) was not fully completed under those conditions. Alkylation of **9** with $C[^2H]_3I$ afforded labelled derivative **11** and its hydrogenation in stainless steel autoclave (higher pressure was chosen to ensure the reaction would be fully completed this time) at pressure 8 kPa on 10 % Pd/C gave compound **14** as the only product (Scheme 2).

Compound **15** was obtained by a well-established [8–10] four step synthesis. A mild oxidation of alcohol **15** gave keto ester **16**. Several methods of synthesis of deuterated and tritiated alcohols from corresponding ketones were reported [17, 21, 22], most of them using hydride reagents. Due to the conjugation of 18(19) double bond with 21-oxo group, no product of reduction of an oxo group on ring E was observed. Compounds **17**–**19** were obtained by a reduction of **16** with $NaBH_4$, $NaB[^2H]_4$ or $NaB[^3H]_4$ (molar activity 6.4 Ci/mmol) in a mixture of THF and methanol. Tritium atom in position 3α of **19**, **22** and **25** was confirmed by ^3H NMR with signals at $\delta \approx 3.47$ ppm for



Scheme 1 Reagents and conditions: (*a*) $BnBr/K_2CO_3$, DMF, MeCN, NaI; (*b*) $Na_2Cr_2O_7$/AcONa, AcOH, 1,4-dioxane; (*c*) (*1*) O_3/$CHCl_3$, −80 °C, (*2*) Me_2S; (*d*) (*1*) $C[^2H]_3I$, Ph_3P/THF, −30 °C, (*2*) *t*-BuOK, (*3*) EtOH; (*e*) H_2, 10 % Pd/C/THF, EtOH; (*f*) SeO_2/2-methoxyethanol, refl.



Scheme 2 Reagents and conditions: (*a*) dry air, KOH, *t*-BuOH, 45 °C; (*b*) CH_3I or $C[^2H]_3I$, KOH, 1,4-dioxane, H_2O, 100 °C; (*c*) H_2 (100 kPa), 10 % Pd/C, THF, EtOH; (*d*) H_2 (800 kPa), 10 % Pd/C, THF, EtOH

19 and $\delta \approx 4.47$ ppm for **22** and **25**. Deuterated compounds **18**, **21** and **24** were identified using MS-EI. Derivative **18** was synthesized with a yield of 96 % and with high stereoselectivity. There was no signal of atom H-3α at expected $\delta \approx 3.47$–4.47 ppm [12, 23] in ^1H NMR spectra of compounds **18**, **21** and **24**, which is a proof that all of the prepared molecules contain deuterium at 3α position. Acetylation of hydroxy derivatives **17**–**19** gave acetates **20**–**22** and following oxidation afforded diketones **23**–**25**.

Experimental

Materials and methods

All reagents and solvents were purchased from Vitrum Praha, s.r.o. and were used without further purification except that THF was distilled prior to use. Sodium [^3H]borohydride 100 mCi (6.4 Ci/mmol) was purchased from MGP Zlin, s.r.o. Sodium [^2H]borohydride and [^2H]methyl iodide were purchased from Sigma Aldrich (^2H enrichment >98 %). Starting compounds **1**–**4**, all in purity 98 %, were obtained from Betulinines Chemical Group. Melting points were determined on Kofler block and are uncorrected. The optical rotatory power was measured on an Autopol III (Rudolph Research, Flanders, NJ) polarimeter as a chloroform solution. The values of optical power are listed [10^{-1} ° cm^2 g^{-1}] and values of concentration are listed [g/100 mL] for each experiment. ^1H NMR spectra were recorded on Varian ^{UNITY}*Inova* 400 (399.95 MHz for ^1H, listed as V 400 MHz) and on Bruker Avance II 300 MHz (299.94 MHz for ^1H, listed as B 300 MHz), using $CDCl_3$ as a solvent. ^1H NMR spectra of tritiated compounds were recorded on spectrometer Bruker Avance II 300 MHz (320 MHz for ^3H), in $CDCl_3$ as a solvent. Chemical shifts are expressed in ppm with TMS as an internal standard for ^1H spectra. ^3H spectra were referenced to the signal of tritiated water at 4.7 ppm taken as an external standard. The type of spectrometer is specified in each experiment. Mass spectra (EI) were recorded on a Shimadzu QP 2010 mass spectrometer at 70 eV and an ion source temperature 200 °C. The samples were introduced via 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 >35. MS-ESI spectra were carried on BRUKER esquire 3000 in methanol solution. Infrared (IR) spectra were recorded as a DRIFTS (KBr) on a NICOLET Impact 400D spectrometer. Wave numbers are given in cm^{-1}. TLC was performed on Kieselgel 60 F_{254} (Merck) sheets or RP-18 F_{254} (Merck), detected by ultraviolet (UV) light 254 nm or spraying 10 % sulfuric acid with heating to 100–250 °C. Column chromatography was performed using silica gel 60 (63–200 μm; Merck 7734). The composition of mobile phases is specified in each experiment. The radio-RP-HPLC system consisted of a Watrex SDS30 high-pressure pump (model SDS 30), a Rheodyne injection valve, RP analytical column C18 (25 × 5 mm) with filling modified silica gel (C18 5 μm), UV detector DeltaChrom^{TM} UV200 connected via RS-232 with PC (SW Clarity). The mixture of acetonitrile and water was used as a mobile phase; its composition is specified in each experiment. Ozone was generated from dry air with Azcozone VMUS-4 ozone generator (AZCO

Industries Limited). Concentration of ozone was 4 g/h at the flow rate of air 5 L/min (contain 0.1 % of ozone in ozonized air). Evaporating of the solvents was performed on Büchi Rotavapor R-200. The reaction at lower temperature was cooled with cryocooler Huber TC 100E. Radioactivity was measured with a Beckmann LS 6000 SE liquid scintillation coulter in Ultima Gold^{TM} cocktail.

*Benzyl-20-oxo-29-norlupan-28-oate (**5**)*

Benzyl betulinate (**3**) (25.0 g; 46 mmol) was dissolved in cooled (−80 °C) chloroform (450 mL) and ozone was introduced from ozonizer until the starting benzyl betulinate (**3**) disappeared. The reaction was monitored by TLC in the mixture toluene/diethylether (5:1). The reaction mixture was worked up by addition of dimethylsulfide (5 mL) and solvents were removed under vacuum. Column chromatography of crude ketone **5** (21 g) on silica gel (150.0 g) eluted with toluene and crystallization from methanol afforded ketone **4** (17.5 g, 80 % yield): R_f 0.26 (toluene/diethylether 5:1); mp 191–193 °C (MeOH); $[\alpha]_D$ +12° (c 0.70); ^{1}H NMR (V 400 MHz): δ 0.73 s, 3H; 0.75 s, 3H; 0.79 s, 3H; 0.95 s, 3H; 0.97 s, 3H; 1.27 s, 3H; (6 × CH_3); 2.15 td, 2H ($J = 2.4$, $J' = 11.2$, $J'' = 22.5$, H-12); 2.26 dt, 1H ($J = 3.4$, $J' = 6.6$, $J'' = 12.9$, H-13β), 3.16 dd, 1H ($J = 4.4$, $J' = 10.9$, H-19); 3.16 m, 1H ($\Sigma J = 26.6$, H-3α); 5.10 q, 2H ($J = 12.3$, $J' = 23.4$, Bn); 7.35–7.37 m, 5H ($\Sigma J = 7.2$, Ph). MS-EI, *m/z* (%): [$C_{36}H_{52}O_4$, M^+ 548], 548 (M^+, 20), 458 (19), 440 (41), 413 (20), 250 (28), 237 (20), 220 (49), 191 (51). IR ν cm^{-1}: 703, 759, 1047, 1186, 1215, 1249, 1377, 1448, 1695, 1713, 2870, 2942, 3547.

*Benzyl-[29-$^2H_2$]betulinate (**6**)*

(a) Methyltriphenylphosphonium salt was prepared by following procedure: triphenylphosphine (4.0 g, 15 mmol) was dissolved in toluene (4 mL) and cooled to −30 °C. Deuteromethyliodide (2 mL, 21 mmol) was added into the stirred mixture. The solution was then left at room temperature. White crystals were removed from the solution by filtration under reduced pressure; they were washed with toluene and dried over phosphorus (+V) oxide at diminished pressure overnight.

(b) Deuteromethyltriphenylphosphonium iodide (4.0 g, 9.9 mmol), anhydrous THF (10 mL), and potassium *tert*-butoxide (840 mg, 7.5 mmol) were added into a dry round bottom double necked flask with a magnetic stir bar. The mixture was stirred for 30 min at 85 °C. A solution of ketone **5** (1.0 g, 2.0 mmol) in THF (10 mL) was added by a syringe into the generated ylide. The mixture was heated under reflux with slight over pressure of argon for 2 h. The reaction was monitored by TLC in toluene:diethylether (5:1). The reaction was quenched after 2 h by addition of ethanol (10 mL) and solvents were removed under vacuum. Crude product was dissolved in ethyl acetate (10 mL) and poured into H_2O (50 mL). The mixture was acidified by diluted HCl and then extracted with ethyl acetate (15 mL, two times). Organic layer was washed with H_2O and dried over $MgSO_4$. Solvents were removed under vacuum. The product (1 g) was chromatographed on silica gel (60.0 g), eluted with toluene and lyophilized from *tert*-butanol to give pure **6** (800 mg, 80 % yield): R_f 0.37 (toluene/diethylether 5:1); mp 192–194 °C (*tert*-butanol); $[\alpha]_D$ +12.7° (c 0.80); ^{1}H NMR (V 400 MHz): δ 0.75 s, 3H; 0.76 s, 3H; 0.79 s, 3H; 0.94 s, 3H; 0.96 s, 3H; 1.56 s, 3H; (6 × CH_3); 2.18 td, 2H ($J = 2.4$, $J' = 12.8$, $J'' = 20.8$, H-12); 2.26 dd, 1H ($J = 3.2$, $J' = 9.6$, H-13β), 2.98 td, 1H ($J = 4.4$, $J' = 10.8$, $J'' = 22$, H-19); 3.16 m, 1H ($\Sigma J = 22.0$, H-3α); 5.07 q, 2H ($J = 12.4$, $J' = 23.2$, Bn); 7.35–7.37 m, 5H ($\Sigma J = 7.6$, Ph). MS-EI, *m/z* (%): [$C_{37}H^2_{52}H_2O_3$, M^+ 548], 548 (M^+, 17), 458 (14), 440 (41), 413 (22), 328 (13), 250 (28), 237 (20), 220 (49), 191 (41). IR ν cm^{-1}: 700, 757, 1070, 1185, 1214, 1289, 1375, 1447, 1691, 2869, 2958, 3534.

*[29-$^2H_2$]betulinic acid (**7**)*

Ester (**6**) (700 mg, 1.3 mmol) was dissolved in THF (5 mL) and the 10 % Pd/C (80 mg) was added. The flask was sealed by a septum and slightly evacuated and then hydrogen was introduced via an injection needle. The mixture was vigorously stirred at room temperature for 3 h. The reaction was monitored by TLC in toluene/diethylether (5:1). The catalyst was filtered off and the solvents were removed under vacuum. Crude acid **7** was crystallized from isopropanol to give white crystals of **7** (500 mg, 85 % yield): R_f 0.37 (toluene/diethylether 5:1); mp 297–299 °C (isopropanol); $[\alpha]_D$ +8° (c 0.72); ^{1}H NMR (V 400 MHz): δ 0.75 s, 3H; 0.82 s, 3H; 0.94 s, 3H; 0.96 s, 3H; 0.97 s, 3H; 1.19 s, 3H; 1.20 s, 3H; (7 × CH_3); 1.66 m, 1H ($\Sigma J = 22.0$, H-13β); 1.93 td, 1H ($J = 2.8$, $J' = 5.2$, $J'' = 14.4$, H-18); 2.19 td, 1H ($J = 3.6$, $J' = 12.4$, $J'' = 22$, H-12); 2.98 td, 1H ($J = 4.8$, $J' = 10.8$, $J'' = 16.8$, H-19α); 3.15 m, 1H ($\Sigma J = 16.4$, H-3α). MS-EI, *m/z* (%): [$C_{30}H^2_{46}H_2O_3$, M^+ 458], 458 (M^+, 16), 441 (11), 425 (5), 413 (7), 397 (9), 249 (30), 234 (16), 220 (35), 207 (85), 189 (87). IR ν cm^{-1}: 985, 1043, 1190, 1234, 1375, 1387, 1449, 1682, 2868, 2942, 3245, 3432.

*30-Oxo-[29-$^2H_2$]lup-20(29)-en-28-oic acid (**8**)*

Acid **7** (200 mg, 437 μmol) was dissolved in 2-methoxy-ethanol (5 mL) and powdered selenium dioxide (80 mg, 720 μmol) was added. The mixture was stirred under reflux for 3 h. The reaction was monitored by TLC in toluene/diethylether (10:1). The reaction mixture was filtered to remove precipitated gray selenium and left to crystallize overnight. Yellow crystals of aldehyde **8** (160 mg, 77 % yield) were removed by filtration under reduced pressure, washed with 2-methoxy-ethanol, dried over phosphorus (+V) oxide at diminished pressure and lyophilized from *tert*-butanol. R_f 0.32 (toluene/diethylether 10:1); mp 44–45 °C and decomp. (2-methoxy-ethanol); $[\alpha]_D$ 0° (c 0.70); ^{1}H NMR (V 400 MHz): δ 0.69 s, 3H; 0.77 s, 3H; 0.88 s, 3H; 0.90 s, 3H; 1.32 s, 3H; (5 × CH_3); 2.01 m, 1H (H-16α); 2.16 m, 2H (H-22); 3.12 t, 1H (J = 8, H-3α); 3.34 m (ΣJ = 8.0, H-19, 9.45 s, 1H (COH). MS-EI, *m/z* (%): [$C_{30}H^2_{44}H_2O_4$, M^+ 472], 472 (M^+, 46), 452 (64), 437 (30), 425 (36), 409 (42), 391 (6), 273 (10), 260 (8), 248 (25), 233 (55), 217 (13), 207 (69), 189 (100), 175 (12). IR ν cm^{-1}: 944, 1029, 1044, 1191, 1208, 1224, 1376, 1449, 1618, 1685, 2869, 2942.

*Benzyl-2-hydroxy-3-oxo-lupa-1,20(29)-dien-28-oate (**9**)*

Benzyl betulonate (**4**) (30.0 g, 55 mmol) was dissolved in *tert*-butanol (1.0 L) in a double jacketed reaction vessel (volume 2.2 L) equipped with a mechanical stirrer and a condenser. Potassium *tert*-butoxide (45.0 g, 0.40 mmol) was added into the reaction vessel, the mixture was heated to 45 °C and dry air was bubbled through the reaction mixture using an air pump. The reaction was monitored by ^{1}H NMR (sampling every 20 min) and RP-TLC in THF/acetic acid (1 % in water) 2:1. The reaction mixture was worked up by pouring into water acidified with HCl (10 %) and extracted with diethylether. Organic layer was washed with water, filtered over silica gel (25.0 g) and evaporated under vacuum. The crude diosphenol **9** (25.0 g, 81 % yield) was dried under vacuum and was stored at −20 °C. R_f 0.35 (THF/1 % aq. sol. acetic acid 2:1); mp 83–86 °C (diethylether); $[\alpha]_D$ +17° (c 0.70); ^{1}H NMR (V 400 MHz): δ 0.80 s, 3H; 0.93 s, 3H; 1.00 s, 3H; 1.05 s, 3H; 1.08 s, 3H; 1.17 s, 3H; 1.69 s, 3H; (7 × CH_3); 2.29 m, 1H (ΣJ = 12.9, H-12β); 3.05 td, 1H (J = 4.6, J' = 10.7, J'' = 21.8, H-19β); 4.62 d, 1H (J = 1.6, H-29b); 4.74 d, 1H (J = 1.6, H-29a); 5.1 q, 2H (J = 9.8, J' = 23.2, Bn); 6.45 s, 1H (H-1); 7.33–7.37 m, 5H (ΣJ = 13.7, Ph). MS-EI, *m/z* (%): [$C_{37}H_{50}O_4$, M^+ 558], 558 (M^+, 19), 545 (3), 469 (16), 438 (13), 254 (6), 224 (8), 167 (20). IR ν cm^{-1}: 697, 754, 878, 1039, 1128, 1151, 1211, 1377, 1455, 1644, 1668, 1723, 2869, 2946, 3069, 3436.

*Benzyl-2-methoxy-3-oxolupa-1,20(29)-dien-28-oate (**10**)*

Diosphenol **9** (1.2 g, 1.7 mmol) was dissolved in a mixture of 1,4-dioxane (4 mL), water (2 mL) and potassium hydroxide (500 mg, 9 mmol). The mixture was stirred under reflux. After cooling down, methyliodide (600 μL, 6.3 mmol) was added and the heating continued at 45 °C for 1 h. The reaction was monitored by TLC in toluene/diethylether (5:1). The reaction mixture was poured into water and the product extracted with diethylether. Organic layer was washed with water and evaporated under vacuum. Crude ether **10** was purified by chromatography on silica gel (50.0 g; eluted with toluene); ether **10** (800 mg, 65 % yield) was obtained after lyophilization from *tert*-butanol. R_f 0.24 (toluene/diethylether 5:1); mp 84–86 °C (decomp.); $[\alpha]_D$ +37° (c 0.37); ^{1}H NMR (V 400 MHz): δ 0.82 s, 3H; 0.97 s, 3H; 1.05 s, 3H; 1.11 s, 3H; 1.14 s, 3H; (5 × CH_3); 1.70 m, 3H (H-30); 2.26 td, 1H (J = 13.2, J' = 13.2, J'' = 3); 2.30 dt, 1H (J = 12.8, J' = 4, J'' = 24.4); 3.04 td, 1H (J = 10.8, J' = 10.8, J'' = 4.8, H-19β); 3.54 s, 3H (OCH_3); 4.62 m, 1H (H-29 *pro-E*); 4.75 bd, 1H (J = 2.4, H-29 *pro-Z*); 5.10 d, 1H (J = 12.0, H-31a); 5.17 d, 1H (J = 12.0, H-31b); 6.03 s, 1H (H-1); 7.29–7.40 m, 5H (Bn). MS-EI, *m/z* (%): [$C_{38}H_{52}O_4$, M^+ 572], 572 (M^+, 21), 481 (19), 435 (22), 368 (17), 269 (16), 236 (25), 179 (100). IR ν cm^{-1}: 1720, 1675, 1641, 1621.

*Benzyl-2-[$^2H_3$]methoxy-3-oxolupa-1,20(29)-dien-28-oate (**11**)*

Diosphenol **9** (1.0 g, 1.6 mmol) was dissolved in a mixture of dioxane (4 mL), water (2 mL) and potassium hydroxide (500 mg, 9 mmol). The mixture was stirred and refluxed. Deuteromethyliodide (600 μL, 6.3 mmol) was added to the reaction mixture and heating to 45 °C was continued for 1 h. The reaction was monitored by TLC in toluene/diethylether (5:1). The reaction mixture was poured into water and extracted with diethylether. Organic layer was washed with water and evaporated under vacuum. Column chromatography of crude **11** (900 mg) over silica gel (50.0 g), toluene as mobile phase afforded methoxy derivate **11** (603 mg, 60 % yield) (lyophilized from *tert*-butanol): R_f 0.24 (toluene/diethylether 5:1); mp 109–112 °C and decomp.; $[\alpha]_D$ +37° (c 0.71); ^{1}H NMR (V 400 MHz): δ 0.82 s, 3H; 0.97 s, 3H; 1.05 s, 3H; 1.11 s, 3H; 1.19 s, 3H; 1.69 s, 3H; (7 × CH_3); 2.26 dt, 1H (J = 4.0, J' = 12.8, J'' = 24.4, H-12β); 3.01 td, 1H (J = 4.4, J' = 10.8, J'' = 21.6, H-19β); 4.62 d, 1H (J = 1.6, H-29b); 4.74 d, 1H (J = 1.6, H-29a); 5.11 q, 2H (J = 12.4, J' = 14.4, Bn); 6.03 s, 1H (H-1); 7.33–7.37 m, 5H (ΣJ = 8.4, Ph). MS-EI, *m/z* (%): [$C_{38}H^2_{49}H_3O_4$, M^+ 575], 575 (M^+, 28), 560 (3), 484 (12), 449 (5), 438 (13), 421 (5), 313 (6), 269 (6), 239 (8), 182 (20). IR ν cm^{-1}: 697, 751,

987, 1075, 1116, 1130, 1150, 1171, 1213, 1237, 1382, 1455, 1559, 1618, 1683, 1724, 2067, 2869, 2945, 3065.

*2-Methoxy-3-oxolupa-1,20(29)-diene-28-oic acid (**12**) and 2β-methoxy-3-oxolup-20(29)-ene-28-oic acid (**13**)*

In an autoclave, methoxy derivate **11** (400 mg, 0.7 mmol) was dissolved in a mixture of THF (20 mL) and ethanol (20 mL) and 5 % Pd/C (750 mg) was added. The autoclave was evacuated and then filled with hydrogen (100 kPa). The reaction mixture was stirred for 2 h and then monitored by TLC in toluene/diethylether (5:1). The reaction mixture was filtered under argon atmosphere. The solvents were removed under vacuum. Column chromatography of crude (500 mg) on silica gel (10.0 g) eluted with toluene/diethylether (10:1) afforded acids **12** and **13**.

Diosphenol **12** (83 mg; 25 %): R_f 0.19 (toluene/diethylether 5:1); mp 213–215 °C (methanol); $[\alpha]_D$ +70° (c 0.31); ^{1}H NMR (V 400 MHz): δ 1.01 s, 3H; 1.01 s, 3H; 1.07 s, 3H; 1.16 s, 3H; 1.28 s, 3H; (5 × CH_3); 1.72 m, 3H (H-30), 1.78–1.87 m, 1H (J = 13.3); 1.94–2.08 m, 2H; 2.20–2.36, 3H; 3.03 td, 1H (J = 10.8, J' = 10.8, J'' = 4.8, H-19β); 3.55 s, 1H (OCH_3); 4.54 m, 1H (H-29 *pro-E*); 4.77 bd, 1H (J = 2.3, H-29 *pro-Z*); 6.05 s, 1H (H-1). MS-ESI, *m/z* (%): [$C_{31}H_{46}O_4$, M^+ 482], 483 ([M+H]$^+$, 12), 505 ([M+Na]$^+$, 27), 521 ([M+K]$^+$, 11). IR ν cm^{-1}: 3513, 1721, 1695, 1642.

Acid **13** (214 mg; 63 %): R_f 0.20 (toluene/diethylether 5:1); mp 221–226 °C (methanol); $[\alpha]_D$ +68° (c 0.29); ^{1}H NMR (V 400 MHz): δ 0.73 s, 3H; 0.91 s, 3H; 1.02 s, 3H; 1.05 s, 3H; 1.12 s, 3H; (5 × CH_3); 1.69 bs, 3H (CH_3–C=C); 1.94–2.05 bm, 2H; 2.14–2.36 bm, 3H (H-1α); 3.00 dt, 1H (J = 4.6, J' = 10.6, J'' = 10.6, H-19β); 3.37 s, (OCH_3); 4.22 dd, 1H (J = 11.3, J' = 8.3, H-2α); 4.62 m, 1H and 4.74 bd, 1H (H-29a and -29b). MS-ESI, *m/z* (%): [$C_{31}H_{48}O_4$, M^+ 484], 485 ([M+H]$^+$, 10), 507 ([M+Na]$^+$, 19), 523 ([M+K]$^+$,10). IR ν cm^{-1}: 3515, 1720, 1694, 1640.

*2β-[$^2H_3$]methoxy-3-oxolupan-28-oic acid (**14**)*

In an autoclave, methoxy derivate **11** (500 mg, 0.9 mmol) was dissolved in a mixture of THF (1 mL), ethanol (1 mL), and 5 % Pd/C (500 mg) catalyst was added. The autoclave was evacuated and then filled with hydrogen (800 kPa). The reaction mixture was stirred for 4 h and then monitored by TLC in toluene/diethylether (5:1). The mixture was filtered under argon atmosphere. The solvents were removed under vacuum. Column chromatography of crude derivate **14** (500 mg) over silica gel (10.0 g), toluene/diethylether (10:1) as a mobile phase, afforded acid **14** (300 mg, 70 % yield) (lyophilized from *tert*-butanol): R_f 0.20 (toluene/diethylether 5:1); mp 221–226 °C; $[\alpha]_D$ +68° (c 0.72); ^{1}H NMR (V 400 MHz): δ 0.75 s, 3H; 0.85 s, 3H; 0.91 s, 3H; 0.99 s, 3H; 1.05 s, 3H; 1.13 s, 3H; 1.42 s, 3H; (7 × CH_3); 2.26 dt, 1H (J = 4.0, J' = 12.8, J'' = 24.4, H-19β); 4.06 t, 1H (J = 8.8, J' = 20.8, H-2). MS-EI, *m/z* (%): [$C_{31}H^2_{47}H_3O_4$, M^+ 489], 489 (M^+, 20), 475 (42), 455 (16), 441 (25), 427 (22), 396 (18), 355 (20), 269 (10), 259 (25), 248 (44), 213 (23), 203 (30), 189 (40). IR ν cm^{-1}: 877, 949, 967, 1105, 1130, 1158, 1367, 1385, 1458, 1696, 1724, 2868, 2954, 3065.

*Methyl-3,21-dioxolup-18-en-28-oate (**16**)*

Ketone **15** (9.30 g, 19 mmol) was oxidized using sodium dichromate (4.7 g, 16 mmol) in a mixture of acetic acid (250 mL) and 1,4-dioxane (250 mL) with addition of sodium acetate (9.30 g, 113 mmol). The mixture was stirred at room temperature for 3 days and was monitored by TLC in toluene/diethylether (5:1). The reaction mixture was poured into water and product was extracted by ethyl acetate. The organic layer was washed with water, filtered through a column of aluminum oxide (30.0 g) and evaporated under vacuum. Crystallization from methanol gave white crystals of diketone **16** (7.80 g, 85 %): R_f 0.30 (toluene/diethylether 5:1); mp 207–209 °C ($CHCl_3$/MeOH); $[\alpha]_D$ −51° ($CHCl_3$); ^{1}H NMR (V 400 MHz): δ 0.95 s, 3H; 0.97 s, 3H; 1.03 s, 3H; 1.06 s, 3H; 1.08 s, 3H; 1.19 d, 3H (J = 3.6); 1.21 s, 3H (J = 3.2); (7 × CH_3); 2.11 dd, 1H (J ~ 1, J' = 18.6, H-22b); 2.45 d, 1H (J = 18.6, H-22a); 2.68 d, 1H (J = 3.2, J' = 12.6, H-13β); 3.20 septet, 1H (ΣJ = 28.1, H-20); 3.70 s, 3H (OCH_3). MS-EI, *m/z* (%): [$C_{31}H_{46}O_4$, M^+ 482], 482 (M^+, 15), 451 (10), 262 (70), 235 (7), 203 (12), 191 (15). IR ν cm^{-1}: 959, 1026, 1107, 1178, 1214, 1260, 1384, 1434, 1455, 1605, 1695, 1710, 1733, 2831, 2870, 2956, 2983.

*Methyl-3β-hydroxy-21-oxo[3α-^2H]lup-18-en-28-oate (**18**)*

Diketone **16** (2.0 g, 4 mmol) was dissolved in a mixture of THF (20 mL) and methanol (20 mL). The mixture was cooled to −10 °C while being stirred and then sodium borodeuteride (1.0 g, 23 mmol) was added. The reaction was monitored by TLC in toluene/diethylether (5:1). After completion, the reaction mixture was poured into water acidified with 1 mL of 10 % H_2SO_4, product was extracted with ethyl acetate, organic layer was washed with water and dried over anhydrous $MgSO_4$. Lyophilization from *tert*-butanol afforded ester **18** (1.9 g, 96 % yield): R_f 0.30 (toluene/diethylether 5:1); mp 177–183 °C; $[\alpha]_D$ −54° (c 0.72); ^{1}H NMR (V 400 MHz): δ 0.77 s, 3H; 0.89 s, 3H; 0.94 s, 3H; 0.98 s, 3H; 1.03 s, 3H; 1.21 d, 3H (J = 3.6); 1.22 s, 3H (J = 4.0); (7 × CH_3); 2.09 d, 1H (J = 18.6, H-22b); 2.15 d, 1H (J = 18.6, H-22a); 2.46 m, 1H (H-16β); 2.66 dd, 1H (J = 3.6, J' = 13.2, H-13β); 3.20 septet, 1H (ΣJ = 9.6, H-20); 3.70 s, 3H (OCH_3). MS-EI, *m/z* (%):

[$C_{31}H^2_{47}HO_4$, M^+ 485], 485 (M^+, 15), 442 (5), 424 (7), 276 (12), 263 (73), 249 (7), 203 (12), 191 (19). IR ν cm^{-1}: 968, 993, 1035, 1116, 1176, 1258, 1313, 1379, 1455, 1611, 1701, 1734, 2872, 2949, 3436.

*Methyl-3β-hydroxy-21-oxo[3α-^{3}H]lup-18-en-28-oate (**19**)*

Ketone **16** (10 mg, 15 μmol) was dissolved in a mixture of THF (500 μL) and methanol (100 μL). The mixture was introduced into a vial with sodium borotritide (molar activity 6.4 Ci/mmol) cooled to −10 °C. The mixture was then stirred for 3 h at room temperature. The reaction was monitored by TLC in toluene/diethylether (5:1, det. LSC). The reaction was worked up by addition of water (1 mL) acidified with 100 μL of 10 % H_2SO_4 followed by the extraction with ethyl acetate. The organic layer was washed with water and the solvents were evaporated under vacuum. The preparative TLC in toluene/diethylether (5:1) afforded product **19** (30 mCi, 30 % radiochemical yield): radio-RP-HPLC in MeCN/H_2O (8:2), t_r 15.0 min; ^{1}H NMR (B 300 MHz): δ 0.77 s, 3H; 0.89 s, 3H; 0.94 s, 3H; 0.98 s, 3H; 1.03 s, 3H; 1.21 d, 3H (J = 4.4); 1.22 d, 3H (J = 4.0); (7 × CH_3); 2.03 m, 1H (H-16α); 2.15 d, 1H (J = 15, J' ∼ 1, H-22b); 2.45 m, 1H (H-16β); 2.50 d, 1H (J = 15, H-22a); 2.65 dd, 1H (J = 6.0, J' = 15.0, H-13β); 3.20 septet, 1H (ΣJ = 9.6, H-20); 3.70 s, 3H (OCH_3). ^{3}H NMR (B 300 MHz): δ 3.47 s, (^{3}H-3α).

*Methyl-3β-acetoxy-21-oxo[3α-^{2}H]lup-18-en-28-oate (**21**)*

Acetic anhydride (3 mL) was added into a stirred solution of ketone **18** (1.0 g, 2 mmol) in pyridine (4 mL) at room temperature. The reaction was monitored by TLC in toluene/diethylether (5:1). The reaction mixture was poured into water acidified with 1 mL of 10 % HCl, product was extracted by ethyl acetate and the organic layer was washed with water and solvents were evaporated under vacuum. Crystallization of the crude material from methanol afforded ester **21** (800 mg, 73 % yield): R_f 0.36; mp 220–222 °C; $[\alpha]_D$ −33° (c 0.71); ^{1}H NMR (V 400 MHz): δ 0.84 s, 3H; 0.85 s, 3H; 0.91 s, 3H; 0.93 s, 3H; 1.03 s, 3H; 1.20 s, 3H; 1.22 s, 3H; (7 × CH_3); 2.05 s, 3H (OAc); 2.10 d, 1H (J = 18.7, H-22a); 2.46 d, 1H (J = 18.7, H-22b); 2.48 dd, 1H (J = 13.5, J' = 3.9, H-16β); 2.64 dd, 1H (J = 12.7, J' = 3.2, H-13β); 3.20 septet, 1H (ΣJ = 7.0, H-20); 3.70 s, 3H (OCH_3). MS-EI, *m/z* (%): [$C_{33}H^2_{49}HO_5$, M^+ 527], 527 (M^+, 20), 484 (5), 467 (13), 424 (17), 276 (5), 263 (100), 205 (14), 191 (16). IR ν cm^{-1}: 854, 992, 1049, 1154, 1167, 1211, 1250, 1276, 1362, 1438, 1611, 1707, 1727, 1739, 2827, 2852, 2875, 2952, 2992, 3436, 3685.

*Methyl-3β-acetoxy-21-oxo[3α-^{3}H]lup-18-en-28-oate (**22**)*

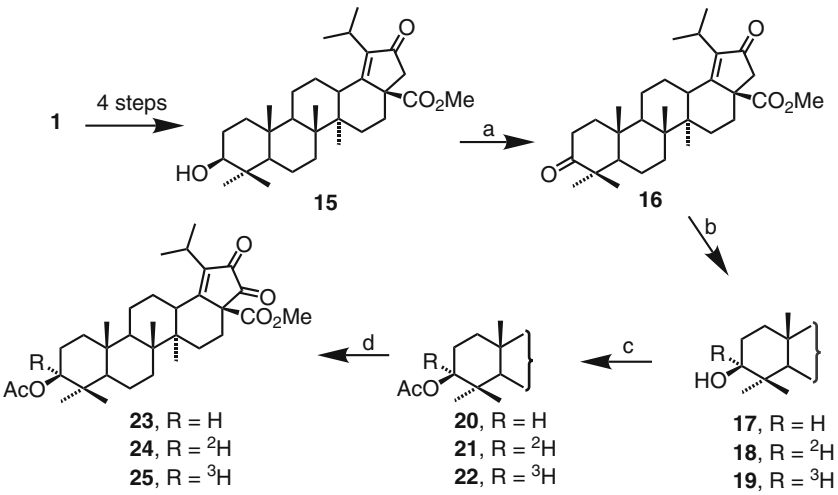
Acetic anhydride (30 μL) was added into a solution of ketone **19** (30 mCi, 20 μmol) in pyridine (40 μL). The mixture was stirred overnight. The reaction was monitored by TLC in toluene/diethylether (5:1, det. LSC). The reaction mixture was worked up by pouring into water acidified with 1 mL of 10 % HCl, product was extracted into ethyl acetate, the organic layer was washed with water and the solvents were evaporated under vacuum. The preparative TLC in toluene/diethylether (5:1) afforded product **22** (28 mCi, 94 % radiochemical yield): radio-RP-HPLC in MeOH/H_2O (7:3), t_r 11.3 min; ^{1}H NMR (B 300 MHz): δ 0.84 s, 3H; 0.85 s, 3H; 0.91 s, 3H; 0.93 s, 3H; 1.03 s, 3H; 1.20 s, 3H; 1.23 s, 3H; (7 × CH_3); 2.05 s, 3H (OAc); 2.13 d, 1H (J = 15.0, H-22a); 2.46 d, 1H (J = 15.0, H-22b); 2.48 dd, 1H (J = 13.5, J' = 3.9, H-16β); 2.64 dd, 1H (J = 12.7, J' = 3.2, H-13β); 3.20 septet, 1H (ΣJ = 7.0, H-20); 3.70 s, 3H (OCH_3). ^{3}H NMR (B 300 MHz): δ 4.47 s, (^{3}H-3α).

*Methyl-3β-acetoxy-21,22-dioxo[3α-^{2}H]lup-18-en-28-oate (**24**)*

Ketone **21** (600 mg, 1 mmol) was dissolved in a mixture of 1,4-dioxane (2.3 mL), acetic acid (1.2 mL), acetic anhydride (120 μL) and selenium dioxide (180 mg, 1.6 mmol) was added. The mixture was stirred and heated under reflux in argon atmosphere for 3 h. The reaction was monitored by TLC in toluene/diethylether (5:1). The reaction mixture was diluted with ethyl acetate (10 mL) and filtered through silica gel (5.0 g). The organic layer was washed with water, dried over anhydrous $MgSO_4$ and solvents were evaporated under vacuum. HPLC in hexane/ethyl acetate (8:2) afforded diketone **24** (550 mg, 89 % yield) white (lyophilized from *tert*-butanol): R_f 0.40 (toluene/diethylether 5:1); mp 228–234 °C and decomp. (*tert*-butanol); $[\alpha]_D$ −100° (c 0.71); ^{1}H NMR (V 400 MHz): δ 0.85 s, 3H; 0.86 s, 3H; 0.92 s, 3H; 0.97 s, 3H; 1.06 s, 3H; 1.26 d, 3H (J = 7.0); 1.34 s, 3H; (7 × CH_3); 2.05 s, 3H (OAc); 2.54 ddd, 1H (J = 13.5, J' = 4.4, J'' = 2.2, H-16β); 2.76 dd, 1H (J = 12.7, J' = 3.3, H-13β); 3.36 septet, 1H (ΣJ = 7.0, H-20); 3.72 s, 3H (OCH_3). MS-ESI, *m/z* (%): [$C_{33}H^2_{47}HO_6$, M^+ 541], 541 (M^+, 40). IR ν cm^{-1}: 855, 990, 1050, 1156, 1167, 1211, 1255, 1275, 1362, 1440, 1611, 1711, 1735, 1769, 2829, 2856, 2875, 2952, 2992.

*Methyl-3β-acetoxy-21,22-dioxo[3α-^{3}H]lup-18-en-28-oate (**25**)*

Ketone **22** (28 mCi, 18 μmol) was dissolved in a mixture of 1,4-dioxane (120 μL), acetic acid (60 μL), acetic anhydride (8 μL) and selenium dioxide (15 mg, 133 μmol) was added.



Scheme 3 Reagents and conditions: (*a*) $Na_2Cr_2O_7$/AcOH, 1,4-dioxane, AcONa; (*b*) $NaBH_4$ or $Na[B^2H_4]$ or $Na[B^3H_4]$/THF, MeOH; (*c*) Ac_2O/pyridine; (*d*) SeO_2/1,4-dioxane, AcOH, Ac_2O

The mixture was stirred and heated to reflux under argon atmosphere for 3 h. The reaction was monitored by TLC in toluene/diethylether (5:1). The reaction was diluted with ethyl acetate (2 mL) and filtered over silica gel (500 mg). Organic layer was washed with water and solvents were evaporated under vacuum. Preparative TLC in toluene/diethylether (5:1) afforded product **25** (25 mCi, 89 % radiochemical yield): radio-RP-HPLC in MeOH/H_2O (6:4), t_r 12.0 min; ^{1}H NMR (B 300 MHz): δ 0.85 s, 3H; 0.86 s, 3H; 0.92 s, 3H; 0.97 s, 3H; 1.06 s, 3H; 1.26 d, 3H ($J = 4.4$); 1.29 d, 3H ($J = 4.4$); (7 × CH_3); 2.05 s, 3H (OAc); 3.48 s, 3H (OCH_3); 4.20 septet, 1H ($\Sigma J = 4.4$, H-20). ^{3}H NMR (B 300 MHz): δ 4.47 s, (^{3}H-3α).

Conclusion

The main aim of this work was to synthesize a set of deuterated and tritiated derivatives, as summarized in Schemes 1, 2, 3. In total, 11 new labelled analogues of some of the most cytotoxic compounds previously found in our research group were synthesized (**6–8**, **11**, **14**, **18**, **19**, **21**, **22**, **24** and **25**). All deuterated compounds were prepared with deuterium degree of incorporation higher than 98 % (MS).

Reaction of ketone **16** with sodium borohydride afforded hydroxy derivative **17**. A similar procedure was employed to prepare **18** using $NaB[^2H]_4$, and **19** using $NaB[^3H]_4$. The position of ^{3}H-3α label in compounds **19**, **22** and **25** was confirmed by ^{3}H NMR. Radiochemical yield of **19** was 30 % and of **22** and **25** was about 90 %. Deuterated methoxy derivate **14** was synthesized in three steps from benzyl betulonate (**4**). Compound **13** shows interesting cytotoxic activity in the model of human T-lymphoblastic leukemia CEM IC_{50} 10.9 μmol/L, on Ph1—positive human leukemia BV-173 IC_{50} 0.91 μmol/L and IC_{50} 0.52 μmol/L on human myelogenous leukemia K562. Therefore, unlabelled analogue of **13** entered in vivo experiments in hollow fibers mice model. Deuterated acid **8** was prepared from betulinic acid (**1**) in five steps in the total yield 42 %. All new compounds and labelled intermediates were characterized by available spectral data.

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