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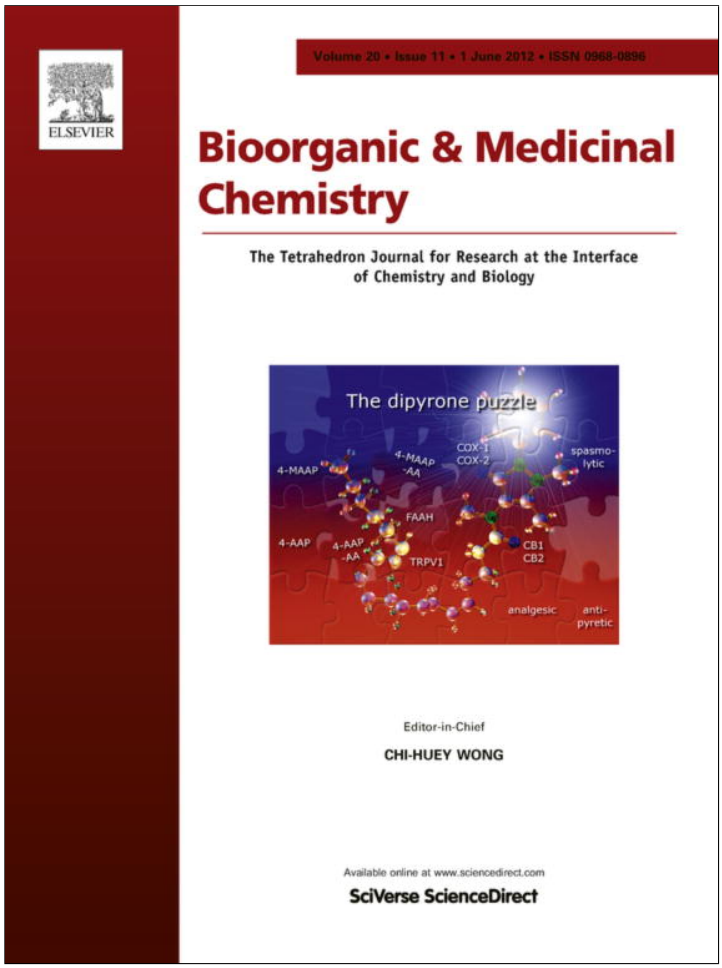

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Cytotoxic heterocyclic triterpenoids derived from betulin and betulinic acid

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ABSTRACT

The aim of this work was to synthesize a set of heterocyclic derivatives of lupane, lup-20(29)-ene, and 18α-oleanane, and to investigate their cytotoxic activities. Some of those heterocycles were previously known in the oleanane (allobetulin) group; however, to our knowledge the syntheses and biological activities of lupane heterocycles have not been reported before. Starting from betulin (**1**) and betulinic acid (**2**), we prepared 3-oxo compounds and 2-bromo-3-oxo compounds **3**–**10**, 2-hydroxymethylene-3-oxo compounds **11**–**13** and β-oxo esters **14**–**16**. Condensation of these intermediates with hydrazine, phenylhydrazine, hydroxylamine, or thiourea yielded the pyrazole and phenylpyrazole derivatives **17**–**22**, pyrazolones **23**–**25**, isoxazoles **26** and **27**, and thiazoles **28**–**31**. Fifteen compounds (**14**–**16**, **18**–**25**, and **29**–**32**) have not been reported before. The cytotoxicity was measured using panel of seven cancer cell lines with/without MDR phenotype and non tumor MRC-5 and BJ fibroblasts. The preferential cytotoxicity to cancer cell lines, particularly to hematological tumors was observed, the bromo acids **5**, **6** showed highest activity and selectivity against tumor cells.

1. Introduction

Triterpenoids are a large group of natural compounds that are found in numerous living organisms, and are particularly prevalent in plants. They often have a variety of biological activities.[1,2] Betulinic acid (**2**) has strong anti-HIV[3–5] and anti-cancer[6–8] activities and belongs to a group of the most interesting lupane derivatives. Zuco et al. were the first to prove[9] that the cytotoxic activity of **2** is selective against cancer cells, without affecting normal cells. Since then, many research groups have tried to find new triterpenoids with improved pharmacological properties that would make them useful candidates for HIV and cancer treatment. These studies have either explored new plant species or modified the structures of known active compounds. Among hundreds of new compounds, we have synthesized several derivatives of lupane that are highly cytotoxic in a variety of cancer cell lines in vitro, including those that are resistant to current chemotherapeutic agents.[10–17] Previously, we described the partial synthesis of triterpenic heterocycles,[14] in which a pyrazine ring was connected to the A-ring or the E-ring of a variety of lupane derivatives. Within this set of pyrazines, three compounds were significantly cytotoxic in vitro to warrant the extension of the in vitro studies to in vivo testing in mice. All of the active compounds have a single pyrazine ring that is connected to the A-ring of **2**, whereas, the other derivatives, in which the pyrazine cycle is connected to a different ring or those derivatives with a quinoxaline system instead of pyrazine, were inactive. These findings suggest that a simple prediction of the structure–activity relationship is difficult, especially with compounds that contain more complicated structures, such as triterpenes. There are many examples in the literature in which a small modification of a barely active triterpene tremendously increases the biological activity, and there are also instances where highly active compounds are rendered less active by a small modification. Examples with anti-HIV activity have been previously reported.[3,18,19] The cytotoxic activity of some triterpenic compounds was also successfully increased by using heterocyclic modifications.[20–22]

The promising cytotoxicity of triterpenic pyrazines,[14] nonterpenic heterocycles,[23,24] and pyrrole and indole derivatives of **2**[25] encouraged us to synthesize several different types of heterocycles. Except for a few cases,[25] most efforts to modify **2** have focused on

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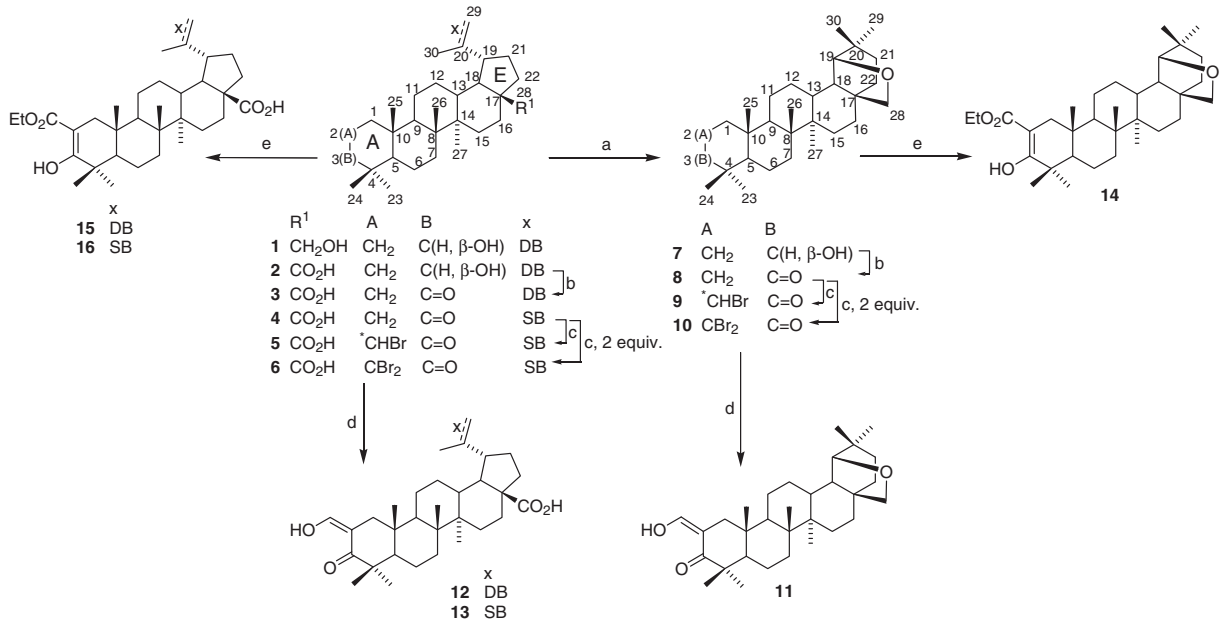
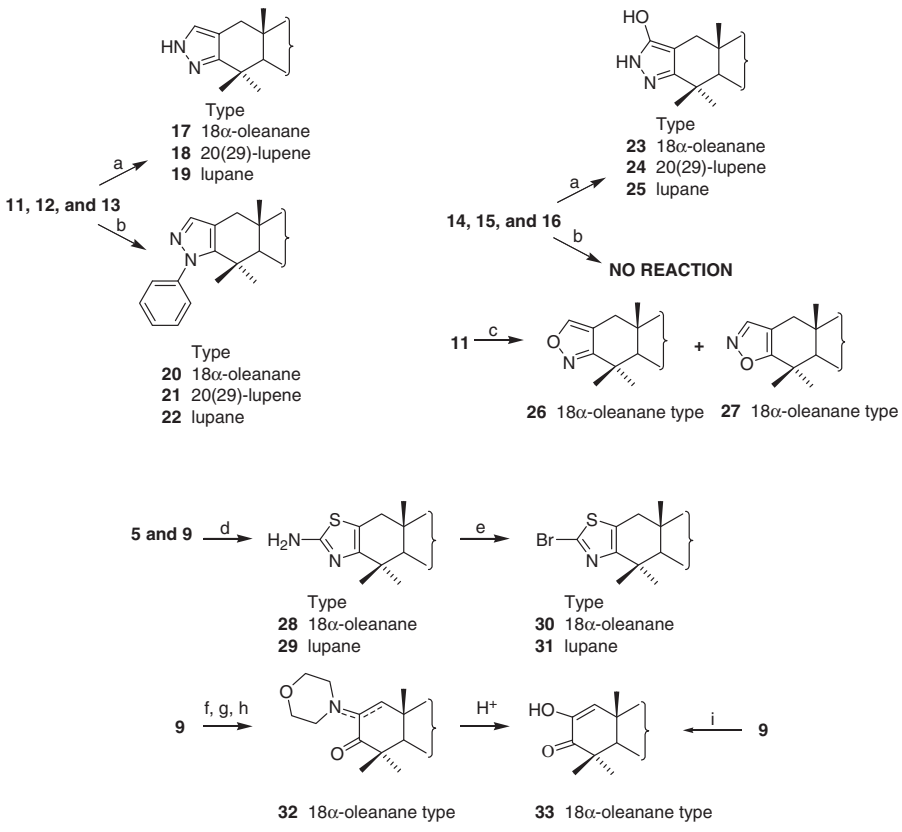
heterocycles, wherein, a heterocycle is condensed with either the carboxylic group at C-28 or to the C-30 position.[20,21,26,27] To our knowledge, pyrrazoles, imidazoles, pyrazolons, or thiazoles have not been condensed with the A-ring of acid **2** or dihydrobetulonic acid (**4**), while several 18α-oleanane heterocycles (such as pyrrazole, methylpyrrazole, oxazole, *N*-methylpyrrole, and aminothiazole) were synthesized by Kim et al.[28–31] along with a number of 5-membered heterocycles that are connected to glycyrrhetic acid.



Scheme 1. Preparation of starting material for the synthesis of heterocycles. Reagents and conditions: (a) Montmorillonite K10, $CHCl_3$, reflux; (b) $Na_2Cr_2O_7$, AcONa, AcOH, dioxane, rt; (c) Br_2, AcOH, AcONa; (d) HCO_2Et, NaH, dioxane, reflux; (f) Et_2CO_3, NaH, dioxane, reflux.



Scheme 2. Reagents and conditions: (a) $N_2H_4{\cdot}H_2O$, dioxane, reflux; (b) phenylhydrazine, AcOH, reflux; (c) $NH_2OH.HCl$, EtOH, pyridine, reflux; (d) thiourea, morfoline, reflux; (e) isoamylnitrite, TBAB, $CHCl_3$, reflux; (f) urea or (g) guanidine or (h) acetamide, all refluxed in morpholine; (i) urea, reflux in pyridine or DMF or DMSO or *N*-methylpyrrolidone.

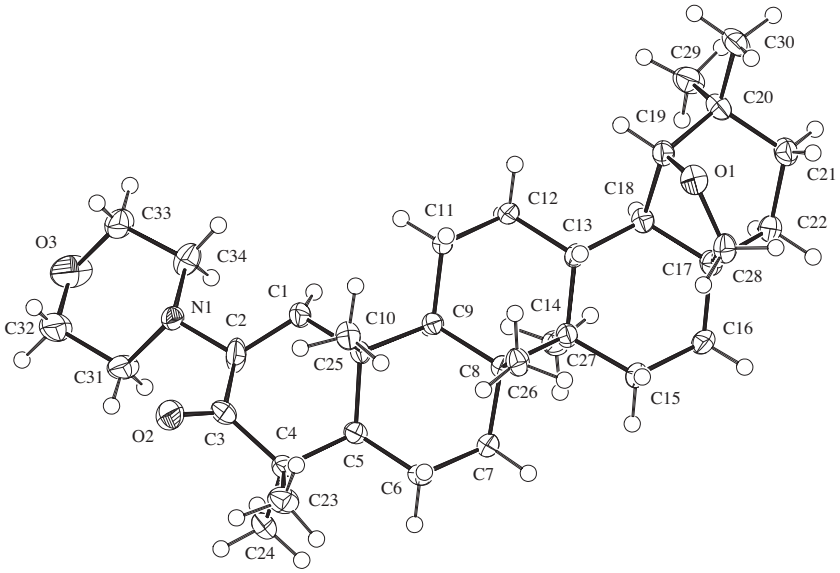


Figure 1. Ortep representation of morpholino derivative **32** with the atom numbering scheme. Thermal ellipsoids are drawn at the 50% probability level.[41]

Table 1
Cytotoxic activity of compounds **1**–**33** against CEM leukemia cell line

Compound	IC_{50}[a] (μmol/L) CEM	Compound	IC_{50}[a] (μmol/L) CEM
1	250	**18**	3
2	30	**19**	3
3	20	**20**	250
4	4	**21**	31
5	1	**22**	45
6	1	**23**	33
7	250	**24**	22
8	250	**25**	123
9	250	**26**	250
10	250	**27**	250
11	250	**28**	85
12	4	**29**	3
13	61	**30**	150
14	250	**31**	11
15	161	**32**	226
16	142	**33**	25
17	210	–	–

[a] The lowest concentration that kills 50% of tumor cells. The standard deviation in cytotoxicity assays is typically up to 10% of the average value.

Imidazoles **26** and **27** were previously studied.[32,33] However, only Kumar et al.[25] have reported on anti-cancer properties of their products.

In this study, we prepared triterpenes with various 5-membered heterocycles condensed to the A-ring of betulonic acid (**3**), dihydrobetulonic acid **4**, and allobetulone (**8**). To increase the number of compounds for biological testing, we also included several known heterocycles (**17**, **26**, and **27**).

2. Material and methods

2.1. Chemicals

Betulin (**1**), betulinic acid (**2**), and dihydrobetulonic acid (**4**) were obtained from company Betulinines (www.betulinines.com). All other chemicals and solvents were obtained from Sigma–Aldrich.

2.2. Cell lines

The CCRF-CEM (CEM), A549, K562, BJ and MRC-5 cell lines were purchased from the American Tissue Culture Collection (ATCC). Resistant clones, CEM daunorubicine resistant (CEM-DNR) and K562 paclitaxel resistant (K562-TAX), were prepared by increasing concentration of cytotoxic compounds and then characterized.[42] Isogenic colorectal cancer cells bearing wild type (HCT116) or deleted p53 gene (HCT116p53$^{-/-}$) were obtained from Horizon Discovery Ltd (www.horizondiscovery.com). The cells were maintained in nunc/corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium according to ATCC recommendations (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

Table 2
Cytotoxic activity of selected compounds **4**–**6**, **12**, **18**, **19** and **29** against seven tumor and two normal fibroblast cell lines

Compound	IC_{50}[a] (μmol/L)								
	CEM	CEM-DNR	A549	K562	K562-TAX	HCT116	HCT116p53$^{-/-}$	BJ	MRC-5
04	3.7	9.4	5.9	2.5	4.0	4.1	6.1	22.6	11.7
05	1.4	11.4	11.9	1.6	1.7	4.3	4.1	17.8	14.6
06	1.0	7.0	8.4	0.9	1.1	2.4	2.6	13.3	8.0
12	3.5	29.6	17.6	8.4	12.7	12.1	13.7	55.5	29.9
18	2.8	10.4	5.2	2.6	3.0	4.5	3.3	35.8	14.1
19	2.6	8.2	3.6	3.5	2.7	3.9	2.8	22.4	13.4
29	3.5	11.2	7.0	4.8	6.9	5.1	4.3	24.9	15.7

[a]The lowest concentration that kills 50% of tumor cells. The standard deviation in cytotoxicity assays is typically up to 10% of the average value.

2.3. Cytotoxic MTT 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 μL) into 96-well microtiter plates. Inoculates were allowed to pre-incubate for a period of 24 h at 37 °C and 5% CO_2 for stabilization. Four-fold dilutions of the intended test concentration were added in 20 μL aliquots at time zero to the microtiter plate wells. All tested compounds were dissolved in 10% DMSO and the experiments were performed in quadruplicate. The cells were incubated with the tested compounds for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed by using the MTT test. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and incubated for additional 1–4 h. After this incubation period, the produced formazan was dissolved by the addition of 10% aq SDS (100 μL/well, pH = 5.5), and the cells were incubated at 37 °C overnight. The optical density (OD) was measured at 540 nm with a Labsystem iEMS Reader MF. Tumor cell survival (TCS) was calculated by using the following equation: TCS = ($OD_{\text{drug-exposed well}}$/mean $OD_{\text{control wells}}$) × 100%. The IC_{50} value, the drug concentration that is lethal to 50% of the tumor cells, was calculated from the appropriate dose-response curves.

3. Results and discussion

3.1. Chemistry

Allobetulin (**7**), allobetulon (**8**), and their derivatives were prepared by the acidic rearrangement of betulin (**1**) by using montmorillonite K10 in chloroform and subsequent oxidation with sodium dichromate.[14,40] One equivalent of Br_2 was used to introduce bromine at position 2 to give bromo derivatives **5** and **9**, or 2 equiv of Br_2 were used to obtain dibromo compounds **6** and **10** (Scheme 1). All of these procedures were described earlier.[11,14,32,34–37]

A modified procedure[38] was used to synthesize aldehydoketones **11**–**13**. 3-Oxo derivatives **3**, **4**, or **8** were treated with ethyl formate in a presence of a large excess of NaH (10 equiv) in anhydrous dioxane at reflux. These aldehydoketones exist predominantly in their enol form. A similar procedure was used for the preparation of β-keto esters **14**–**16**, which also predominantly exist in their enol form.

The 5-membered heterocycles were synthesized by condensation reactions. The major issue with all of these reactions was the limited solubility of the starting compounds in most of the solvents that are usually used for similar reactions, and no reactivity in the solvents in which the starting materials were soluble. For this reason, unusual solvents, such as morpholine or a mixture of EtOH and pyridine, were used frequently.

Pyrrazoles **17**–**19** and pyrazolones **23**–**25** were prepared by the reaction of β-dicarbonylic compounds **11**–**16** with anhydrous hydrazine hydrate (Scheme 2). The optimum conditions for this reaction was 2–3 h in dioxane at reflux, after which most of the products crystallized from the reaction mixture upon cooling. The use of EtOH resulted in a much longer time to achieve the full conversion of the reactant into product. In contrast, the reactions of compounds **11**–**13** with phenylhydrazine only proceeded in glacial acetic acid and not in any other solvent (dioxane, DMF, diglyme, pyridine, pyridine/EtOH, morpholine, EtOH). This is probably because of a lower reactivity of phenylhydrazine in these solvents. No reaction occurred between β-keto esters **14**–**16** and phenylhydrazine at reflux in any of the solvents mentioned. The preparation of both isoxazoles **26** and **27** by the reaction of aldehydoketone **11** with hydroxylamine hydrochloride in a mixture of pyridine and EtOH at reflux proceeded as described in the literature,[32,33], whereas an inseparable mixture was obtained from the same reaction of aldehydoketones **12** and **13**. This problem is probably a result of the free C-28 carboxyl group being attached to position 17 in both of these compounds. This moiety may undergo various side reactions. The reaction of both 2-bromo ketones **5** and **9** with thiourea gave the best yield of the corresponding aminothiazoles **28** and **29** in morpholine at reflux, whereas larger amounts of the side-product diosphenol **33** were obtained when other solvents were used. Amines **28** and **29** were further modified into the bromo derivatives **30** and **31** by converting them into the corresponding diazonium salts (Scheme 2). We attempted to prepare various heterocycles by reactions of bromo ketone **9** with urea, guanidine, or acetamide, however these reactions were not successful. Running these reactions in morpholine at reflux resulted in the morpholino derivative **32**, which easily hydrolyzed to diosphenol **33**. The reaction of **9** with urea in pyridine, DMF, *N*-methylpyrrolidone, or DMSO at reflux yielded only diosphenol **33** and urea. No reaction occurred in diglyme, EtOH, AcOH, THF, toluene, dioxane, or xylene at reflux. The structure of the **32** (Fig. 1) was proved by X-ray crystallography.[41]

3.2. Cytotoxicity

All of the compounds prepared in the study were tested for cytotoxicity in highly chemosensitive T-lymphoblastic leukemia CEM cell line in vitro, by using the MTT test[39] (Table 1). In agreement with previous results, all of 18α-oleanane derivatives were found to be inactive. Betulinic (**2**) and betulonic acid (**3**) were only moderately active (IC_{50} = 30 and 20 μmol/L, respectively), whereas dihydrobetulonic (**4**), 2-bromo dihydrobetulonic (**5**), 2,2-dibromo dihydrobetulonic acid (**6**) and aldehydo-ketone **12**, as well as pyrazoles **18**, **19**, and thiazole **29** were an order of magnitude more active (IC_{50} = 4–1 μmol/L) on CEM cell line. Two more compounds, diosphenol **33** and pyrazolone **24**, were also moderately active on these cells (Table 1).

Therefore, we chose five most active compounds **6**, **12**, **18**, **19**, **29** together with two starting compounds **5**, **6** for extended testing in the panel of seven cancer cell lines including multidrug resistant and two non-tumor cell lines MRC-5 and BJ fibroblasts (Table 2). The extended testing surprisingly shows that the most active

Table 3
Crystalographic data of compound **32**

Empirical formula	$C_{34}H_{53}NO_3$, $CHCl_3$
Formula mass	643.14
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	$P2_12_12_1$ (No. 19)
Unit cell dimensions	a = 9.6402(2) Å; α = 90°
	b = 11.4835(3) Å; β = 90°
	c = 30.2137(8) Å; γ = 90°
Volume	3344.75(14) $Å^3$
Z	4
Density	1.277 g cm^{-3}
Absorption coefficient	0.309 mm^{-1}
$F(000)$	1384
Crystal size	0.50 × 0.20 × 0.18 mm
Θ Range for data collection	1.90–27.47°
Index ranges	$-12 \le h \le 12$, $-8 \le k \le 14$,$-38 \le l \le 39$
Total reflections collected	7474
Independent reflections	6266 [R(int) = 0.0483]
Completeness to Θ	Θ = 27.47°; 97%
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	7474/0/386
Goodness-of-fit on F	1.023
Final R indices [$I>2\sigma(I)$]	R_1 = 0.0483, wR_2 = 0.1061
R indices (all data)	$R1$ = 0.0626, wR_2 = 0.1149
Largest difference peak/hole	0.405/−0.407 e $Å^{-3}$

compounds are bromo acids **5**, **6** or aldehydoketone **12**, which reached favorable in vitro therapeutic index >10 (ratio of cytotoxicity in cancer versus non-cancer cell lines). While pyrazoles **18**, **19** or thiazole **29** showed potency comparable to or slightly worse than dihydrobetulonic acid (**4**) (Table 2).

Interestingly, the most active compounds were among the starting materials, and generally, all heterocyclic modifications to the A-ring decreased the cytotoxicity. The majority of the heterocycles were almost insoluble in any solvent, which might have played an important role in the cytotoxicity testing. Solubility would also be significant problem for the in vivo tests.

3.3. X-Ray structure of morpholino derivative 32

Single crystals of **32** were grown from mixture of chloroform/methanol. A colorless crystal was measured at Bruker Nonius KappaCCD diffractometer by monochromatized Mo K_α radiation (l = 0.71073 Å) at 150(2) K. The structure was refined by full-matrix least squares based on F2 (SHELXL97). CCDC-809288[41] (Table 3).

4. Conclusions

Starting from betulin (**1**) and betulinic acid (**2**), we prepared a set of lupane, lup-20(29)-ene, and 18α-oleanane derivatives (**3**–**16**) that were further converted into their heterocyclic analogues. These analogues have a 5-membered heterocycle connected to their A-ring in position 2 and 3. In spite of many problems (especially with solubility), we obtained a set of 15 heterocycles. All of synthesised compounds were tested for cytotoxicity against the sensitive acute lymphoblastic leukemia cell line (CEM). Five most active compounds **6**, **12**, **18**, **19**, **29** and starting compounds **4**, **5** were selected and tested against seven cancer cell lines derived from various solid and hematological tumors including lung (A549), colon (HCT116p53WT) and myeloid leukemia (K562) cancer cell lines in vitro; Importantly, the most active compounds are bromo dihydrobetulonic acid (**5**), dibromo dihydrobetulonic acid (**6**) and aldehydoketone **12** with preferential toxicity to leukemia cancer cells and not to normal human fibroblasts (BJ or MRC-5), demonstrating promising therapeutic index (>10) under in vitro conditions. These compounds have also high cytotoxic activity against multidrug resistant P-glycoprotein positive (K562-TAX) and p53 inactivated (HCT116p53$^{-/-}$) cancer cell lines, suggesting therapeutic potential against these therapeutically problematic tumors. However, the compounds failed to exhibit significant cytotoxic activity in lung resistant protein positive cells (CEM-DNR) suggesting their limited use in these cancers.[14] Poor solubility of the heterocyclic derivatives in water-based media and other solvents might be the main reason for their lower activity in comparison with dihydrobetulonic acid (**4**).

5. Experimental

5.1. General experimental procedures

Melting points were determined with a Kofler block and are uncorrected. Optical rotations were measured in $CHCl_3$ solutions (unless otherwise stated) on an Autopol III (Rudolph Research, Flanders, NJ) polarimeter. Infrared spectra were recorded in $CHCl_3$ solution or DRIFTS (KBr) on a NICOLET Impact 400D. Wave numbers are given in cm^{-1}. NMR spectra were recorded on a Varian Unity INOVA 400 instrument (^{1}H NMR spectra at 399.95 MHz) in $CDCl_3$ solutions (unless otherwise stated), with $SiMe_4$ as an internal standard. EI-MS were recorded on an INCOS 50 (Finigan MAT) spectrometer at 70 eV and an ion source temperature of 150 °C. The samples were introduced through a direct exposure probe at a heating rate of 10 mA/s. Direct infusion APCI–MS experiments were performed on an LCQ Fleet ion-trap mass spectrometer; the system was controlled by a personal computer with Xcalibur software (all by Thermo Fisher Scientific, San Jose, CA, USA). The APCI vaporizer and heated capillary temperatures were set to 400 and 250 °C, respectively; the corona discharge current was 3.0 μA. Nitrogen served as both the sheath and auxiliary gas at a flow rate of 40 and 14 arbitrary units, respectively. The MS spectra of the positively charged ions were recorded from 180 to 700 *m*/*z*. The sample solution in acetonitrile/chloroform (99:1, flow rate of 5–30 μL/min) was introduced into the APCI source. The concentrations and time of data collections were chosen to obtain optimal spectra. Column chromatography was performed on silica gel 60 (Merck 7734). The HPLC system consisted of a high-pressure pump (Gilson model 361), a Rheodyne injection valve, and a preparative column (25 × 250 mm) filled with silica gel (Biospher 7 μm). The differential refractometer detector (Laboratorni Pristroje, Praha, CZ) was connected to a PC (software Chromulan) and an automatic fraction collector (Gilson model 246). A mixture of EtOAc and hexane was used as the mobile phase, its composition specified in each experiment. TLC was carried out on Kieselgel 60 F254 plates (Merck) with toluene/Et_2O (5:1) as eluent, and the results were visualized by spraying the plate with 10% aqueous H_2SO_4 and heating to 150–200 °C. Work-up refers to pouring the reaction mixture into H_2O, extracting the product with an organic solvent, washing the organic layer successively with H_2O, dilute aqueous HCl, H_2O, saturated aqueous $NaHCO_3$, and H_2O again, and drying the organic layer with $MgSO_4$. This process was followed by filtration and evaporation of the filtrate under reduced pressure. Analytical samples were dried over P_2O_5 under reduced pressure. **1**, **2**, and **4** were obtained from Betulinines (www.betulinines.com). All other chemicals and solvents were obtained from Sigma–Aldrich.

5.2. Betulonic acid (3)

Betulonic acid (**3**) was prepared from betulinic acid (**1**) by oxidation using sodium dichromate[11]

5.3. Bromination of 2-oxo compounds

A solution of bromine (2.3 mL, 45 mmol) and sodium acetate (1 g) in acetic acid (80 mL) was added dropwise to a vigorously stirred solution of the corresponding 3-oxo compound (20 g, 43.9 mmol) in $CHCl_3$ (300 mL) and acetic acid (50 mL). The product was isolated by pouring the reaction mixture into water, filtering the precipitate, and drying. The product was purified by chromatography on silica gel (200 g), elution with toluene/diethyl ether (20:1) gave an epimeric mixture of 2α- and 2β-bromo ketones. The same procedure was used to prepare dibromo derivatives **6** and **10** except 2 equiv of bromine were used.

5.3.1. 2-Bromo dihydrobetulonic acid (5)

Yield: 18 g (77%), white microcrystals; mp 255–257 °C ($CHCl_3$/MeOH) for epimeric mixture of 2α- and 2β-bromo ketones; $[\alpha]_D^{25}$ +14 (*c* 0.17, $CHCl_3$) (lit.[32] mp 125–127 °C for 2α-bromo ketone ($CHCl_3$/MeOH).

5.3.2. 2,2-Dibromo dihydrobetulonic acid (6)

Yield: 22 g (85%), white microcrystals; mp 249–249 °C ($CHCl_3$/MeOH; decomp.); $[\alpha]_D^{25}$ −14 (*c* 0.35, $CHCl_3$); ^{1}H NMR δ 0.77 (d, $J_{(H\text{-}29,\ H\text{-}30)}$ = 6.8 Hz), 0.87 (d, $J_{(H\text{-}30,\ H\text{-}29)}$ = 6.8 Hz), 0.92 (s), 0.97 (s), 0.97 (s), 1.22 (s), 1.54 (s) (21H, 7 × CH_3), 1.90 (1H, dd, J_1 = 12.5 Hz, J_2 = 7.2 Hz), 3.13 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 16.0 Hz, H-1a), 3.65 (1H, d, $J_{(H\text{-}1b,\ H\text{-}1a)}$ = 16.0 Hz, H-1b); EI-MS *m*/*z* 614 [M$^+$] (1), 599 (1), 569 (40), 534 (55), 489 (100), 455 (41), 409 (77); Anal. C 58.53, H 7.62, Br 25.95, Calcd for $C_{30}H_{46}Br_2O_3$, C 58.64, H 7.55, Br 26.01.

5.3.3. 2-Bromo allobetulon (9)

7 was converted into allobetulon **8** as previously described,[14,40] and subsequently brominated as described above. Yield (bromination): 17 g (72%), white microcrystals; mp 226–228 °C ($CHCl_3$/MeOH); $[\alpha]_D^{25}$ +68 (*c* 0.55, $CHCl_3$) (lit.[36,37] mp 216–225 °C; $[\alpha]_D^{25}$ +74.

5.3.4. 2,2-Dibromoallobetulon (10)

Yield: 19 g (71%), white microcrystals; mp 217–220 °C ($CHCl_3$/MeOH); $[\alpha]_D^{25}$ −16 (*c* 0.3, $CHCl_3$) (lit.[36] mp 214–216 °C; $[\alpha]_D^{25}$ −14.

5.4. General procedure for the preparation of aldehydoketones 11–13

A modified procedure from the literature[36] was used. Each of the 3-oxo derivatives **3**, **4**, and **8** (20 g, 45 mmol) was dissolved in dry dioxane (400 mL) and excess NaH (22 g) was added slowly to the solution, under argon. The mixture was heated slowly to boiling and then ethyl formate (6 mL) was slowly added over 3 h. The mixture was cooled to room temperature and any remaining NaH was quenched with 30 mL of EtOH in dioxane (100 mL). The crude product was precipitated out of the solution by adding 10% HCl (1 L) and was then extracted into chloroform. The product was purified by chromatography on silica gel (200 g) in toluene (for compound **11)** or by using gradient from toluene to 10% diethyl ether in toluene (for acids **12** and **13**) and was subsequently crystallized from $CHCl_3$/MeOH to give the pure aldehyde.

5.4.1. 2-Hydroxymethylene-allobetulone 11

Yield: 71%; mp 242–244 °C ($CHCl_3$/MeOH); $[\alpha]_D^{25}$ +84 (*c* 0.40, pyridine) (lit.[38] mp 253–257 °C (benzene/EtOH); $[\alpha]_D^{25}$ +76).

5.4.2. 2-Hydroxymethylene-betulonic acid 12

Yield: 70%; mp 235–238 °C ($CHCl_3$/MeOH); $[\alpha]_D^{25}$ +41 (*c* 0.30, $CHCl_3$); ^{1}H NMR contains singlet peak of methylene at 8.60 ppm as described in lit.[6]

5.4.3. 2-Hydroxymethylene-dihydrobetulonic acid 13

Yield: 72%; mp 284–287 °C($CHCl_3$/MeOH); $[\alpha]_D^{25}$ +12 (*c* 0.42, $CHCl_3$); ^{1}H NMR contains singlet peak of methylene at 8.60 ppm as described in lit.[6]

5.5. General procedure for a preparation of β-keto esters 14–16

Each of the 3-oxo derivatives **3**, **4**, and **8** (20 g, 45 mmol) was dissolved in dioxane (400 mL) and excess NaH (30 g) was slowly added to the solution, under argon. The mixture was slowly heated up to its boiling point and then diethyl carbonate (25 mL) was slowly added over 3 h. The work-up procedure was same as for aldehydes **11**–**13**.

β-Keto ester ***14*** was purified by chromatography on silica gel (200 g) with gradient elution from toluene to toluene/diethyl ether (9:1). Yield: 67% mp 137–140 °C (EtOH); $[\alpha]_D^{25}$ +75 (*c* 0.37, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3666, 1736 cm^{-1}; ^{1}H NMR δ 0.80, 0.86, 0.93, 0.94, 1.01, 1.08, 1.17 (21H, all s, 7 × CH_3), 1.30 (3H, t, $J_{(CH3,\ CH2)}$ = 7.2 Hz, CH_3–Et), 2.43 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.4 Hz, H-1a), 3.45 (1H, d, *J* = 7.9 Hz, H-28a), 3.55 (1H, s, H-19), 3.79 (1H, dd, J_1 = 7.8 Hz, J_2 = 1.3 Hz, H-28b), 4.20 (2H, m, CH_2–Et), 12.59 (1H, s, 3-O**H**); EI-MS *m*/*z* 512 [M^+] (17), 439 (22), 424 (100), 409 (35), 393 (11), 381 (52), 355 (53), 342 (21), 189 (57); Anal. C 77.17, H 10.28, Calcd for $C_{33}H_{52}O_4$, C 77.30, H 10.22.

β-Keto ester ***15*** was purified by chromatography on silica gel (200 g) with gradient elution from toluene to toluene/diethyl ether (9:1).Yield: 52%; mp 234–236 °C (EtOH); $[\alpha]_D^{25}$ +38 (*c* 0.34, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3516, 1736, 1694, 1646, 1612 cm^{-1}; ^{1}H NMR δ 0.83, 0.97, 0.99, 1.06, 1.16 (15H, all s, 5 × CH_3), 1.29 (3H, t, *J* = 7.2 Hz, CH_3–Et), 1.70 (3H, m, H-30), 1.94–2.07 (2H), 2.18–2.34 (2H), 2.39 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.4 Hz, H-1a), 3.04 (1H, td, $J_{(H\text{-}19\beta,\ H\text{-}18\alpha)}$ = 10.4 Hz, $J_{(H\text{-}19\beta,\ H\text{-}21\alpha)}$ = 10.4 Hz, $J_{(H\text{-}19\beta,\ H\text{-}21\beta)}$ = 4.7 Hz, H-19β), 4.18 (2H, m, C**H**$_2$–Et), 4.61 (1H, m, H-29 *pro E*), 4.75 (1H, bd, *J* = 1.9 Hz, H-29 *pro Z*), 12.59 (1H, s, 3-O**H**); EI-MS *m*/*z* 526 [M^+] (100), 511 (9), 480 (39), 465 (14), 452 (10), 434 (13), 419 (7), 391 (42), 317 (56), 189 (27); Anal. C 75.08, H 9.66, Calcd for $C_{33}H_{50}O_5$, C 75.25, H 9.57.

β-Keto ester ***16*** was purified by chromatography on silica gel (200 g) with gradient elution from hexane to hexane/ethyl acetate (9:1).Yield: 46%; mp. 231–234 °C (EtOH); $[\alpha]_D^{25}$ +12 (*c* 0.45, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3517, 1733, 1693, 1647, 1612 cm^{-1}; ^{1}H NMR δ 0.76 (d, $J_{(H\text{-}29,\ H\text{-}30)}$ = 6.8 Hz), 0.83 (s), 0.86 (d, $J_{(H\text{-}30,\ H\text{-}29)}$ = 6.8 Hz), 0.97 (s), 0.97 (s) (15H, 5 × CH_3), 1.30 (3H, t, *J* = 7.2 Hz, CH_3–Et), 2.19–2.31 (2H), 2.41 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.6 Hz, H-1a), 4.19 (2H, m, C**H**$_2$–Et), 11.00 (1H, br s, 28-COOH), 12.58 (1H, s, 3-O**H**); EI-MS *m*/*z* 528 [M^+] (100), 513 (7), 482 (29), 467 (12), 454 (13), 358 (44); Anal. C 75.11, H 9.84, Calcd for $C_{33}H_{52}O_5$, C 74.96, H 9.91.

5.6. General procedure for the preparation of pyrazoles 17–19

Hydrazine hydrate, 100% (1 mL, 20 mmol) was added dropwise to a solution of each aldehyde **11**–**13** (2.1 mmol) in refluxing dioxane (20 mL) and the solution was heated for another 2 h. White crystals precipitated from the reaction mixture upon cooling to room temperature. The dioxane was evaporated and the crystalline product was washed with $CHCl_3$ (3 mL, twice) to remove unreacted aldehyde.

5.6.1. Pyrazole 17

Yield: 85%; mp. 342–343 °C (dioxane); $[\alpha]_D^{25}$ +60 (*c* 0.22, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3467, 3282vb cm^{-1}; ^{1}H NMR δ 0.81, 0.82, 0.95, 1.03, 1.19, 1.29 (21H, all s, 7 × CH_3), 2.00 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 14.6 Hz, H-1a), 2.68 (1H, d, $J_{(H\text{-}1b,\ H\text{-}1a)}$ = 14.8 Hz, H-1b), 3.46 (1H, d, *J* = 7.8 Hz, H-28a), 3.55 (1H, s, H-19α), 3.79 (1H, d, *J* = 7.8 Hz, H-28b), 4.69 (1H, br s, NH), 7.23 (1H, s, H-31); EI-MS *m*/*z* 464 [M^+] (100), 450 (19), 433 (8), 393 (12). Physical constants were same as reported in the lit.[27]

5.6.2. Pyrazole 18

Yield: 85%; mp 219–220 °C (dioxane); $[\alpha]_D^{25}$ +30 (*c* 0.35, $CHCl_3$); IR (KBr) ν_{max} 3209b, 1680 cm^{-1}; ^{1}H NMR δ 0.79, 105, 1.02, 1.03, 1.17, 1.28 (15H, all s, 5 × CH_3), 1.71 (3H, s, 3 × H-30), 1.90–2.04 (2H), 1.98 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 14.5 Hz, H-1a), 2.27 (1H,dt, J_1 = 12.6 Hz, J_2 = J_3 = 3.16 Hz), 2.34 (1H, td, J_1 = J_2 = 12.6 Hz, J_3 = 3.5 Hz, 2.63 (1H, d, $J_{(H\text{-}1b,\ H\text{-}1a)}$ = 14.9 Hz, H-1b), 3.05 (1H, td, $J_{(H\text{-}19\beta,\ H\text{-}18\alpha)}$ = 10.9 Hz, $J_{(H\text{-}19\beta,\ H\text{-}21\alpha)}$ = 10.9 Hz, $J_{(H\text{-}19\beta,\ H\text{-}21\beta)}$ = 4.7 Hz, H-19β), 4.61 (1H, m, H-29 pro-E), 4.74 (1H, bd, *J* = 2.0 Hz, H-29 pro Z), 7.17 (1H, s, H-31); EI-MS *m*/*z* 478 [M^+] (100), 463 (38), 450 (2), 434 (13), 419 (1), 396 (19), 269 (32); Anal. C 77.63, H 9.71, N 5.73 Calcd for $C_{31}H_{46}N_2O_2$, C 77.78, H 9.69, N 5.85.

5.6.3. Pyrazole 19

Yield: 91%; mp 237–239 °C (dioxane); $[\alpha]_D^{25}$ +9 (*c* 0.35, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3692, 3466, 1691 cm^{-1}; ^{1}H NMR δ 0.77 (3H, d, *J* = 6.8 Hz), 0.79 (3H, s), 0.87 (3H, d, *J* = 6.8 Hz), 0.99 (3H, s), 1.00 (3H, s), 1.19 (3H, s), 1.30 (3H, s, 7 × CH_3), 2.18–2.39 (3H), 2.65 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 14.8 Hz, H-1a), 7.25 (1H, s, H-31); MS EI-MS *m*/*z* 480 [M^+] (41), 465 (12), 452 (3), 436 (100), 419 (16), 409 (2), 267 (38); Anal. C 77.57, H 10.02, N 5.78, Calcd for $C_{31}H_{48}N_2O_2$, C 77.45, H 10.06, N 5.83.

5.7. General procedure for the preparation of phenylpyrazoles 20–22

Phenylhydrazine (1 mL, 20 mmol) was added dropwise to a solution of each aldehyde **11**–**13** (2.1 mmol) in acetic acid (20 mL) at reflux, under argon. The solution was heated for another

2 h and then poured into water. The product was extracted into ethyl acetate, washed with aqueous solution of $NaHCO_3$, and dried with $MgSO_4$. The ethyl acetate was removed under reduced pressure and the crude product was purified by chromatography on silica gel (150 g).

Phenylpyrazole ***20*** was eluted with a gradient from toluene to toluene/diethyl ether (10:1) and crystallized from MeOH. Yield: 56%; mp 269–271 °C (MeOH); $[\alpha]_D^{25}$ +73 (*c* 0.32, $CHCl_3$); IR ($CHCl_3$) ν_{max} 1598, 1500, 1384 cm^{-1}; ^1H NMR δ 0.81, 0.88, 0.94, 0.95, 1.01, 1.03, 1.05 (21H, all s, 7 × CH_3), 2.09 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.0 Hz, H-1a), 2.73 (1H, d, $J_{(H\text{-}1b,\ H\text{-}1a)}$ = 15.0 Hz, H-1b), 3.46 (1H, d, *J* = 8.0 Hz, H-28a), 3.57 (1H, s, H-19α), 3.80 (1H, d, *J* = 7.6 Hz, H-28b), 7.37–7.48 (5H, m, 5 × H-Ph), 7.40 (1H, s, H-31); MS EI-MS *m*/*z* 540 [M^+] (100), 525 (43), 509 (6), 469 (12); Anal. C 81.83, H 9.52, N 5.21, Calcd for $C_{37}H_{52}N_2O$, C 82.17, H 9.69, N 5.18.

Phenylpyrazole ***21*** was eluted with a gradient from toluene to toluene/diethyl ether (3:1) and crystallized from $CHCl_3$. Yield: 66%; mp 258–260 °C ($CHCl_3$); $[\alpha]_D^{25}$ +25 (*c* 0.41, pyridine); IR ($CHCl_3$) ν_{max} 1734, 1694 cm^{-1}; ^1H NMR (in $CDCl_3/CD_3OD$) δ 0.86, 0.99, 1.00, 1.01, 1.03 (15H, all s, 5 × CH_3), 1.72 (3H, s, H-30), 2.05 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 14.7 Hz, H-1a), 2.68 (1H, d, $J_{(H\text{-}1b,\ H\text{-}1a)}$ = 14.9 Hz, H-1b), 3.04 (1H, td, $J_{(H\text{-}19\beta,\ H\text{-}18\alpha)}$ = 10.4 Hz, $J_{(H\text{-}19\beta,\ H\text{-}\ 21\alpha)}$ = 10.4 Hz, $J_{(H\text{-}19\beta,\ H\text{-}21\beta)}$ = 4.7 Hz, H-19β), 4.63 (1H, m, H-29 pro-E), 4.75 (1H, bd, *J* = 2.4 Hz, H-29 pro Z), 7.37 (1H, s, H-31), 7.34–7.40 (2H, m, Ph), 7.42–7.50 (3H, m, Ph); MS EI-MS *m*/*z* 554 [M^+] (100), 539 (47), 510 (16), 495 (3), 485 (1), 467 (2), 441 (5), 198 (31), Anal. C 80.43, H 9.31, N 5.16, Calcd for $C_{37}H_{50}N_2O_2$, C 80.10, H 9.08, N 5.05.

Phenylpyrazole ***22*** was eluted with a gradient from toluene to toluene/diethyl ether (3:1) and crystallized from $CHCl_3$. Yield: 70%; mp 298–300 °C ($CHCl_3$); $[\alpha]_D^{25}$ 0 (*c* 0.28, pyridine); IR (KBr) ν_{max} 2200–3450 vb, 1696 cm^{-1}; ^1H NMR δ 0.78 (3H, d, *J* = 6.8 Hz), 0.87 (3H, s), 0.88 (3H, d, *J* = 6.8 Hz), 0.99 (3H, s), 1.00 (6H, s), 1.04 (3H, s, 7 × CH_3), 1.74 (1H), 1.85 (2H), 2.06 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 14.8 Hz, H-1a), 2.20–2.40 (3H), 2.70 (1H, d, $J_{(H\text{-}1b,\ H\text{-}1a)}$ = 14.8 Hz, H-1b), 7.38 (1H, s, H-31), 7.35–7.40 (2H, m, Ph), 7.43–7.50 (3H, m, Ph); MS EI-MS *m*/*z* 556 [M^+] (100), 541 (67), 512 (7), 497 (3), 469 (4), 198 (52); Anal. C 79.74, H 9.16, N 5.03, Calcd for $C_{37}H_{52}N_2O_2$, C 79.81, H 9.41, N 5.03.

5.8. General procedure for the preparation of pyrazolones 23–25 from β-keto esters 14–16

Hydrazine hydrate, 100% (1 mL, 20 mmol) was added dropwise to a solution of each β-keto ester **14–16** (2.0 mmol) in dioxane (20 mL) at reflux, under argon. The solution was heated for another 3 h and then poured into 8% aqueous HCl. The product was extracted into ethyl acetate, washed with an aqueous solution of $NaHCO_3$, and dried with $MgSO_4$. The ethyl acetate was removed under reduced pressure and the crude product was purified by chromatography on silica gel (50 g).

Pyrazolone ***23*** was eluted with toluene/isopropanol (10:1) and crystallized from $CHCl_3$/MeOH. Yield: 59%; mp over 300 °C ($CHCl_3$/MeOH); $[\alpha]_D^{25}$ +68 (*c* 0.15, pyridine); IR ($CHCl_3$) ν_{max} 3487, 3159, 1615 vb cm^{-1}; ^1H NMR δ 0.76, 0.81, 0.92, 0.93, 0.99 (15H, all s, 5 × CH_3), 2.5 (1H, bd, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 12.7 Hz, H-1a), 3.45 (1H, d, *J* = 7.5 Hz, H-28a), 3.55 (1H, s, H-19α), 3.79 (1H, d, *J* = 7.5 Hz, H-28b), 8–9 (1H, br s, NH); MS EI-MS *m*/*z* 480 [M^+] (100), 465 (9), 449, (8), 409 (13), 138 (92); Anal. C 77.26, H 9.94, N 5.71, Calcd for $C_{31}H_{48}N_2O_2$, C 77.45, H 10.06, N 5.83.

Pyrazolone ***24*** was eluted with $CHCl_3$/MeOH (10:1) and crystallized from $CHCl_3$/MeOH. Yield: 64%; mp 241–243 °C ($CHCl_3$/MeOH); $[\alpha]_D^{25}$ +15 (*c* 0.31, pyridine); IR ($CHCl_3$) ν_{max} 1734, 1694 cm^{-1}; ^1H NMR δ 1.00, 1.01, 1.16, 1.26, 1.70 (15H, all s, 5 × CH_3), 1.96 (3H), 2.29 (3H), 3.00 (1H, m, H-19β), 4.61 (1H, m, H-29 pro-E), 4.74 (1H, bd, H-29 pro Z); EI-MS *m*/*z* 494 [M^+] (41), 479 (37), 467 (19), 438 (62), 410 (43), 239 (100); Anal. C 79.31, H 9.25, N 5.79, Calcd for $C_{31}H_{46}N_2O_3$, C 75.26, H 9.37, N 5.66.

Pyrazolone ***25*** was eluted with $CHCl_3$/MeOH (10:1) and crystallized from $CHCl_3$/MeOH. Yield: 64%; mp 218–221 °C ($CHCl_3$/MeOH); $[\alpha]_D^{25}$ +5 (*c* 0.32, pyridine); IR ($CHCl_3$) ν_{max} 1733 cm^{-1}; ^1H NMR δ 0.77 (3H, d, *J* = 6.8 Hz), 0.81 (3H, s), 0.87 (3H, d, *J* = 6.8 Hz), 0.98 (3H, s), 0.99 (3H, s), 1.13 (3H, s), 1.23 (3H, s, 7 × CH_3), 2.5 (1H, bd, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.0 Hz, H-1a), 8–9 (1H, NH); EI-MS *m*/*z* 496 [M^+] (9), 468 (22), 458 (57), 440 (100), 425 (47), 397 (65), 267 (94); Anal. C 74.82, H 9.87, N 5.72, Calcd for $C_{31}H_{48}N_2O_3$, C 74.96, H 9.74, N 5.64.

5.9. Isoxazoles 26 and 27

The procedure used were analogous to those that have been reported in the literature.[27,40] Hydroxylamine hydrochloride (0.5 g, 7.3 mmol) was added to a solution of aldehyde **9** (0.5 g, 1.1 mmol) in a mixture of pyridine/EtOH (1:1, 25 mL) and the mixture was heated to reflux for 7 h. The reaction mixture was then poured into 8% aqueous HCl. The product was extracted with ethyl acetate, washed with aqueous solution of $NaHCO_3$, and dried with $MgSO_4$. The ethyl acetate was removed under reduced pressure and the crude mixture of isoxazoles **26** and **27** was purified by HPLC with 15% ethyl acetate in hexane as the eluent. The HPLC separation was repeated to achieve the full separation of the regioisomers.

5.9.1. Isoxazole 26

Yield: 18%; mp 303–305 °C (hexane/EtOAc); $[\alpha]_D^{25}$ +71 (*c* 0.26, $CHCl_3$); IR ($CHCl_3$) ν_{max} 1610, 1451 cm^{-1}; ^1H NMR δ 0.80, 0.81, 0.94, 1.03, 1.29, 1.38 (21H, all s, 7 × CH_3), 1.92 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 14.9 Hz, H-1a), 2.79 (1H, d, *J* = 15.1, H-1b), 3.46 (1H, d, *J* = 7.8 Hz, H-28a), 3.55 (1H, s, H-19), 3.79 (1H, dd, J_1 = 7.2 Hz, J_2 = 1.1 Hz, H-28b), 8.04 (1H, d, *J* = 1.4 Hz, H-31); EI-MS *m*/*z* 465 [M^+] (95), 450 (22), 437 (100), 422 (24), 394 (87), 384 (23).

5.9.2. Isoxazole 27

Yield: 28%; mp 274–277 °C (hexane/EtOAc); $[\alpha]_D^{25}$ +56 (*c* 0.21, $CHCl_3$); IR ($CHCl_3$) ν_{max} 1641, 1481, 1455 cm^{-1}; ^1H NMR δ 0.81, 0.84, 0.94, 1.03, 1.20, 1.31 (21H, all s, 7 × CH_3), 1.98 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.1 Hz, H-1a), 2.52 (1H, d, *J* = 15.1, H-1b), 3.46 (1H, d, *J* = 7.7 Hz, H-28a), 3.55 (1H, s, H-19), 3.79 (1H, dd, J_1 = 7.8 Hz, J_2 = 1.1 Hz, H-28b), 7.99 (1H, s, H-31); EI-MS *m*/*z* 465 [M^+] (100), 450 (10), 437 (56), 422 (53), 394 (2), 384 (47). The spectra of both isoxazoles were compared with those of identical compounds in the literature.[33]

5.10. Thiazoles 28–31

Thiourea (2 g, 25.6 mmol) was added to a solution of either **5** or **9** (1 g, 1.9 mmol, mixture of α- and β-diastereomers) in morpholine and the mixture was heated at reflux for 6 h, then poured into water. The product was extracted into chloroform, washed with aqueous solution of $NaHCO_3$ and dried over $MgSO_4$. The solvents were removed under reduced pressure and the crude product was purified by chromatography on silica gel (50 g).

Amino thiazole ***28*** was eluted with toluene/diethyl ether (10:1) and crystallized from MeOH. Yield: 63%; mp 313–318 °C (MeOH); $[\alpha]_D^{25}$ +81 (*c* 0.30, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3488, 3391, 1603, 1525 cm^{-1}; ^1H NMR δ 0.81, 0.92, 0.94, 1.03, 1.11, 1.21 (21H, all s, 7 × CH_3), 2.14 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.3 Hz, H-1a), 2.58 (1H, d, *J* = 15.4, H-1b), 3.46 (1H, d, *J* = 7.8 Hz, H-28a), 3.55 (1H, s, H-19), 3.80 (1H, bd, J_1 = 7.3 Hz, H-28b), 4.84 (2H, br s, NH_2); EI-MS *m*/*z* 496 [M^+] (100), 481 (23), 261 (7), 193 (12). FABMS *m*/*z* 497 [M^++H], 481; Anal. C 74.68, H 9.59, N 5.80, S 6.30, Calcd for $C_{31}H_{48}N_2OS$, C 74.95, H 9.74, N 5.64, S 6.45.

Amino thiazole ***29*** was eluted with toluene/diethyl ether (10:1) and crystallized from MeOH. Yield: 57%; mp 310–315 °C (MeOH; decomposition); $[\alpha]_D^{25}$ +22 (*c* 0.37, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3510, 3480, 3385, 1738, 1606 cm^{-1}; ^1H NMR δ 0.77 (3H, d, *J* = 6.8 Hz), 0.86 (3H, d, *J* = 6.8 Hz), 0.89 (3H, s), 0.98 (3H, s), 0.99 (3H, s), 1.10 (3H, s), 1.19 (3H, s, 7 × CH_3), 2.10 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.4 Hz, H-1a), 2.20–2.38 (3H), 2.53 (1H, d, *J* = 15.4, H-1b), 4.07 (2H, br s, NH_2); EI-MS *m*/*z* 512 [M^+] (63), 497 (17), 451 (9), 423 (8), 313 (100); Anal. C 72.55, H 9.37, N 5.46, S 6.33, Calcd for $C_{31}H_{48}N_2O_2S$, C 72.61, H 9.43, N 5.46, S 6.25.

5.11. Bromo thiazoles 30 and 31

Each amino thiazole **28** (200 mg, 0.4 mmol) or **29** (200 mg, 0.4 mmol) was dissolved in chloroform (5 mL) and tetrabutylammonium bromide (300 mg, 1 mmol) and isopentyl nitrite (106 μL, 0.8 mmol) were added to the solution. The reaction mixture was heated to 60 °C for 4 h and then poured into water. The product was extracted with chloroform, washed with aqueous solution of Na_2SO_3 and $Na_2S_2O_3$ and dried with $MgSO_4$. The solvents were removed under reduced pressure and the crude products were purified by chromatography.

Bromo thiazole ***30*** was purified by preparative HPLC on silica gel with hexane/ethyl acetate (95:5) as eluents, and then lyophilized from *t*-BuOH. Yield: 120 mg (53%); mp 271–273 °C (*t*-BuOH); $[\alpha]_D^{25}$ +68 (*c* 0.51, $CHCl_3$); IR ($CHCl_3$) ν_{max} 1603, 1525 cm^{-1}; ^1H NMR δ 0.81, 0.90, 0.94, 1.03, 1.18, 1.28 (21H, all s, 7 × CH_3), 2.21 (1H, bd, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.0 Hz, H-1a), 2.77 (1H, bd, $J_{(H\text{-}1b,\ H\text{-}1a)}$ = 15.0 Hz, H-1b), 3.46 (1H, d, *J* = 6.0 Hz, H-28a), 3.55 (1H, s, H-19α), 3.79 (1H, d, *J* = 6.0 Hz, H-28b); MS APCI$^+$: *m*/*z* 562 [M-^{81}Br+H]$^+$ (100), 560 [M-^{79}Br+H]$^+$ (97); Anal. C 66.22, H 8.41, Br 14.12, N 2.57, S 5.78, Calcd for $C_{31}H_{46}BrNOS$, C 66.41, H 8.27, Br 14.25, N 2.50, S 5.72.

Bromo thiazole ***31*** was purified by chromatography on silica gel (50 g) with chloroform/acetone (100:1), and then lyophilized from *t*-BuOH. Yield: 73 mg (33%); mp 189–191 °C (*t*-BuOH); $[\alpha]_D^{25}$ +30 (*c* 0.48, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3510, 1604, 1521, 1743 cm^{-1}; ^1H NMR δ 0.74 (d, $J_{(H\text{-}29,\ H\text{-}30)}$ = 6.8 Hz), 0.84 (d, $J_{(H\text{-}30,\ H\text{-}29)}$ = 6.8 Hz), 0.85 (s), 0.96 (s), 1.14 (s), 1.23 (s), 1.24 (s) (21H, 7 × CH_3), 2.16 (1H, d, $J_{(H\text{-}1a,\ H\text{-}1b)}$ = 15.0 Hz, H-1a), 2.72 (1H, d, $J_{(H\text{-}1b,\ H\text{-}1a)}$ = 15.0 Hz, H-1b); MS APCI$^+$: *m*/*z* 578 [M-^{81}Br+H]$^+$ (100), 576 [M-^{79}Br+H]$^+$ (96); Anal. C 64.32, H 7.75, Br 14.01, N 2.52, S 5.47, Calcd for $C_{31}H_{46}BrNO_2S$, C 64.57, H 8.04, Br 13.86, N 2.43, S 5.56.

5.12. Reaction of 2-bromoallobetulon 9 with urea, guanidine hydrochloride, formamide, or acetamide under various conditions

General procedure: 5 mmol of urea, guanidine, formamide, or acetamide was added to a solution of **9** (0.5 g, 1.0 mmol) in solvent (10 mL) and the mixture was heated at reflux for 3 h, then worked up. Starting bromo ketone **9** was obtained from the reactions in diglyme, EtOH, AcOH, THF, toluene, dioxane, or xylene. The same compound was obtained when we extended the reaction time to 24 h. A mixture of several products was obtained from the reactions in DMSO, pyridine, *N*-methylpyrolidone, or DMF. We did not separate the mixture, however, we were able to identify the known[31] diosphenol **33** by TLC. We were able to isolate a mixture of two compounds from the reaction of urea in morpholine at reflux, and after separation and crystallization from methanol, **32** (150 mg, 30%) and **33** (100 mg, 23%) were obtained.

5.12.1. Morpholino derivative 32

Mp 210–214 °C (MeOH); $[\alpha]_D$ +172 (*c* 0.28, $CHCl_3$); IR ($CHCl_3$) ν_{max} 1670 cm^{-1}; ^1H NMR δ 0.81, 0.95, 0.97, 0.99, 1.04, 1.06, 1.15 (21H, all s, 7 × CH_3), 2.57 (2H, br s, CH_2 morpholine), 2.89 (2H, br s, CH_2 morpholine), 3.46 (1H, d, *J* = 8.0 Hz, H-28a), 3.56 (1H, s, H-19), 3.78 (1H, d, *J* = 7.6 Hz, H-28b), 3.83 (4H, m, 2 × CH_2 morfoline), 6.01 (1H, br s, H-1); EI-MS *m*/*z* 524 [M^++H] (100), 508 (84), 498 (67), 481 (7), 466 (14), 455 (2); Anal. C 78.12, H 10.02, N 2.51, Calcd for $C_{34}H_{53}NO_3$, C 77.96, H 10.20, N 2.67.

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Supplementary data

Supplementary data (^1H, ^{13}C NMR spectra of new compounds and X-ray crystallographic data for compound **32**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.066. These data include MOL files and InChiKeys of the most important compounds described in this article.

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