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Gram-scale synthesis of pinusolide and evaluation of its antileukemic potential

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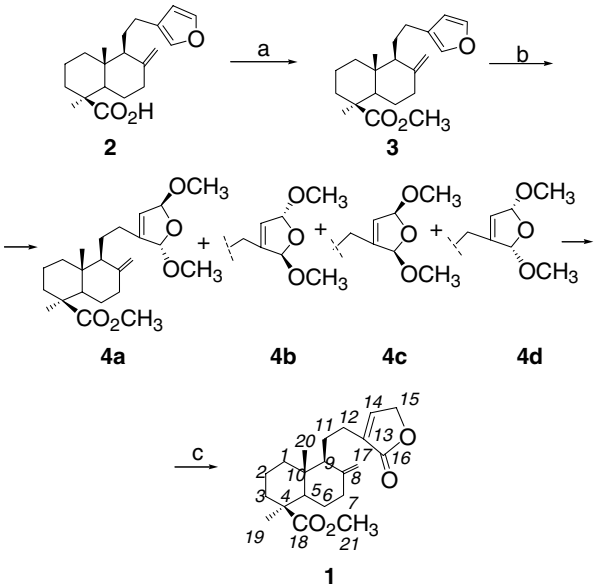
Abstract—Pinusolide (**1**), a known platelet-activating factor (PAF) receptor binding antagonist, was synthesized from lambertianic acid (**2**), a labdane-type diterpene readily accessible in multigram quantities from the Siberian pine tree. It was shown that **1** not only decreases the proliferation activity of tumor cells at relatively low concentrations but specifically induces apoptosis at 100 μM via the mitochondrial pathway in the Burkitt lymphoma cell line BJAB. Also, using primary lymphoblasts and leukemic cells from children with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), a significant DNA fragmentation in pinusolide-treated cells could be detected in an ex vivo apoptosis assay.

Pinusolide (**1**) (Scheme 1), a labdane-type diterpene lactone occurring as a secondary metabolite in several plants such as *Biota orientalis*,[1,2] *Pinus sibirica*, and *Pinus koraiensis*,[3] is known to be a potent and specific platelet-activating factor (PAF) receptor binding antagonist.[4,5] In addition, in vitro antiplasmodial and erythrocyte membrane-modifying effects of **1**[2] and potent activities also of semi-synthetic analogs[6] reflect the general biological potential of this class of compound. Therefore, the search for a convenient access to **1** and the further evaluation of its biological profile represent interesting challenges.

Herein, we describe a convenient method for the gram-scale (partial) synthesis of **1** from the diterpene lambertianic acid (**2**), a main component (7.7%) of the pine oleoresin of *P. sibirica* J. Mayr from which it can be readily isolated by extraction and chromatography.[7–10] Moreover, we disclose experimental results showing that



Scheme 1. Synthesis of compound **1**. Reagents and conditions: (a) CH_2N_2–Et_2O, rt, 2 h, 96%; (b) $PhSO_2NHCl$ (2.1 equiv), MeOH, 0–5 °C, 30 min, 90%, or NBS (2.1 equiv), MeOH, 0 °C, 10 min (86%) isomeric mixture; (c) 20% HCl, dioxane, 30 min, 77%.

Keywords: Natural products; Synthesis; Labdane diterpenes; Apoptosis; Leukemia.
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pinusolide (**1**) exhibits significant apoptosis inducing properties both in vitro (using BJAB tumor cells) and ex vivo (using primary lymphoblasts and leukemia cells of children with ALL or AML).

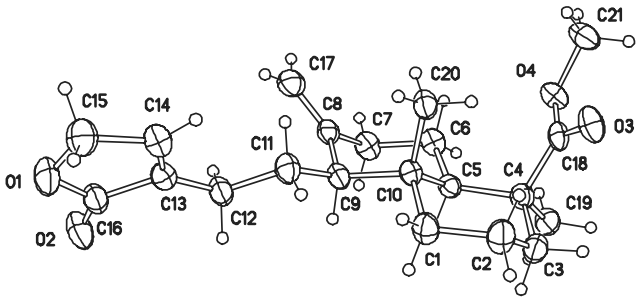

Figure 1. Single-crystal X-ray structure of pinusolide (**1**).

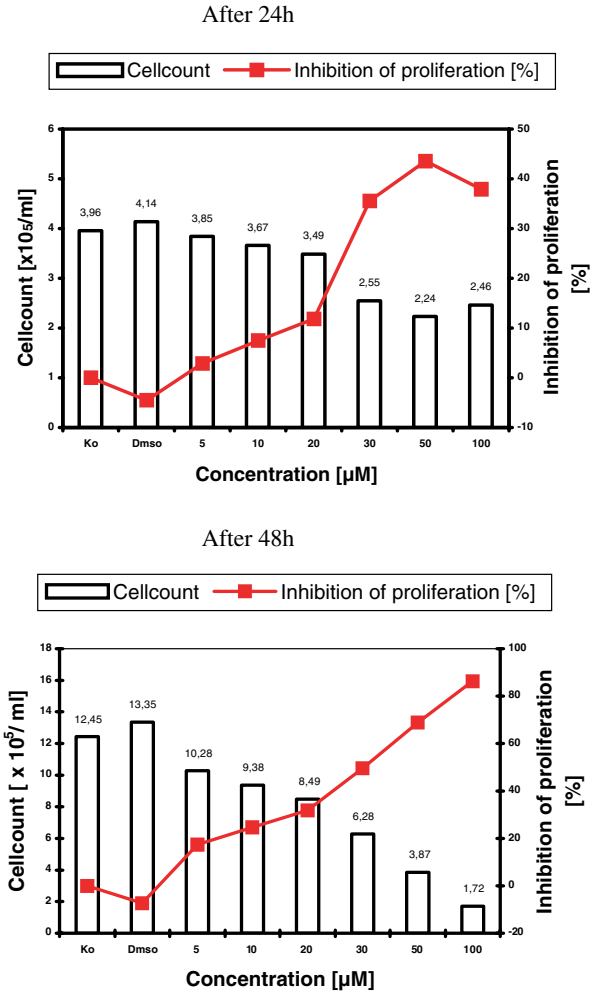

Figure 2. Pinusolide (**1**) inhibits the proliferation of BJAB cells in a concentration-dependent manner. BJAB cells were treated with different concentrations of pinusolide. After incubation for 24 and 48 h, the concentration and viability of the cells were determined by using the CASY® Cell Counter System. The figures show a significant inhibition of the cell proliferation up to 44% after 24 h and up to 86% after 48 h treatment with pinusolide. Data points represent the mean of two determinations from separate cultures with an error less than 3%. The experiments were repeated twice and yielded similar results.

Our synthesis of compound **1** is outlined in Scheme 1. The oxidative methoxylation of methyl lambertianate (**3**), obtained in 96% yield by treatment of **2** with diazomethane, was best achieved with chloramine-B (or NBS) in MeOH to afford a mixture of stereoisomeric 2,5-dimethoxy-dihydrofurane derivatives (**4a**–**d**). The two *cis*- and the two *trans*-isomers were formed at equal amounts, as reflected by the intensity of the ^{1}H NMR signals of the methoxy groups and H-14, H-15, H-16, and confirmed by GC–MS measurements. An analogous result had been obtained in the electrochemical or chemical (Br_2 and MeOH) methoxylation of 2,5-dimethylfuranes.[11] On treatment with acid (HCl), the mixture of compounds **4a**–**d** was smoothly converted into the corresponding 3-substituted (5*H*)-furan-2-one, that is, pinusolide (**1**).[12] The whole sequence can be reliably performed on a gram-scale[13] and thus opens access to substantial amounts of pinusolide (**1**).

The identity of the synthetic material (**1**) with the natural product was proven by its spectral data and the structure was additionally confirmed by means of a single crystal X-ray diffraction analysis.[14] A perspective view on the structure of **1** is shown in Figure 1. Both six-membered rings adopt a chair conformation with the C20 methyl group and the C18 ester group taking an axial position. The C9–C11–C12–C13 unit (bridge) is almost in the same plane as the planar (±0.005 Å) five-membered ring.

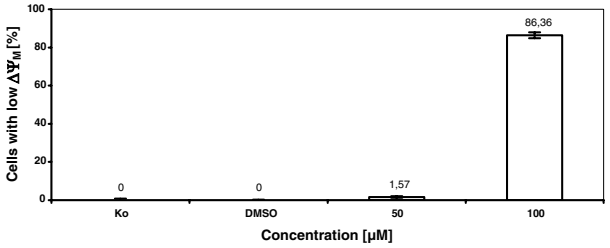

Figure 3. Pinusolide (**1**) induces mitochondrial permeability transition in BJAB cells in a concentration-dependent manner. BJAB cells were treated with different concentrations of **1**. Mitochondrial permeability transition was then measured after 48 h of incubation by staining with JC-1 as described. Values are given as percentage of cells with low $\Delta\psi$ ± SD (n = 3).

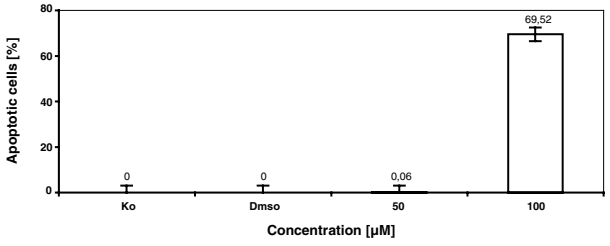

Figure 4. Pinusolide- (**1**) induced apoptosis was measured by DNA fragmentation in BJAB cells. BJAB cells were treated with different concentrations of pinusolide. DNA fragmentation was measured after treatment for 72 h as described. Values are given as percentages of apoptotic cells ± SD (n = 3).

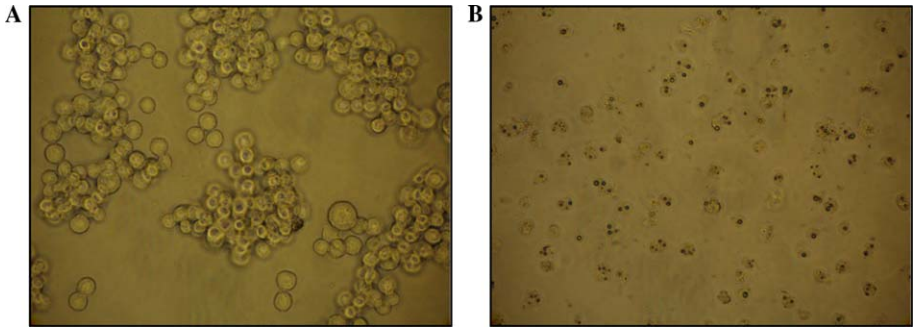

Figure 5. (A) Morphological appearance of untreated BJAB cells. (B) Significant apoptosis induction in BJAB cells after treatment with 100 μM of pinusolide (**1**) for 72 h. Nearly all cells have undergone apoptosis as indicated by shrinking, and fragmenting and the formation of typical 'apoptotic bodies' containing bits of chromatin.

In the crystal, the axial ester group at C4 takes a conformation so that the carbonyl oxygen is eclipsed to the C3–C4 ring bond (the angle C3–C4–C18═O3 equals −4.9°). Whereas the relative configuration of the four chirality centers was unambiguously proven by the X-ray crystal structure, the absolute stereochemistry was assigned based on the known configuration of lambertianic acid.[15] The closest structural analogues of **1** are 14-deoxyandrographolide[16] and the monohydrate of neoandrographolide[17] which, however, have an enantiomorphic skeleton. The conformation of the dihydrofuranylethyl moiety in **1** and the bond lengths are similar to those reported for 14-deoxyandrographolide.[16]

The antileukemic and chemopreventive potential of pinusolide (**1**) was investigated in vitro using the Burkitt lymphoma cell line BJAB. In addition, ex vivo experiments were performed employing primary lymphoblasts and leukemic cells of children with ALL (acute lymphoblastic leukemia) and AML (acute myeloid leukemia), respectively.[18,19]

The results clearly show that compound **1** not only decreases the proliferation activity of tumor cells at relatively low concentrations (Fig. 2), but specifically induces apoptosis at 100 μM (Figs. 4 and 5).

Apoptotic cell death was measured by a modified cell cycle analysis, which detects DNA fragmentation on the single cell level. Interestingly, pinusolide (**1**) potently induced apoptosis in up to 70% of the cells (Fig. 4).

Furthermore, we could show that pinusolide-induced apoptosis in BJAB cells is mediated by loss of mitochondrial membrane potential (Fig. 3).

In fact, pinusolide (**1**) led to a significant loss of the mitochondrial membrane potential at 100 μM (Fig. 3) indicating that this compound utilizes the mitochondrial apoptosis machinery in the respective death signaling pathway.

We also investigated pinusolide-induced apoptosis in primary lymphoblasts and leukemic cells isolated from bone marrow aspirates of children with ALL and AML. After separation over Ficoll, cells were treated with 100 μM pinusolide (**1**) and incubated for 60 h. Pinusolide significantly induced apoptosis ex vivo. DNA fragmentation in primary lymphoblasts and leukemic cells treated with **1** was detected. We could also demonstrate that compound (**1**) overcomes anthracycline resistance ex vivo in primary lymphoblasts from a high risk ALL patient with a poor clinical chemotherapy response (data not shown).

In conclusion, we have elaborated an efficient method for the gram-scale synthesis and purification of pinusolide (**1**) starting from lambertianic acid (**2**), a natural product readily accessible from the oleoresin of *P. sibirica* J. Mayr.

Most importantly, we found that **1** possesses a significant antileukemic activity originating from apoptosis induction. Therefore, pinusolide (**1**) can be considered as a potential starting point in the search for new antitumoral compounds. Its good availability from a renewable plant source represents a valuable precondition for further derivatization and biological studies.[20]

Acknowledgments

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12. For a related method for the conversion of furanes into (5*H*)-furan-2-one derivatives (using NBS and $NaHCO_3$ in $CHCl_3$/EtOH and subsequent acidic hydrolysis), see: Cenal, J. P.; Carreras, C. R.; Tonn, C. E.; Padron, J. I.; Ramirez, M. A.; Diaz, D. D.; Garcia-Tellado, F.; Martin, V. S. *Synlett* **2005**, 1575.
13. *Synthesis of pinusolide (***1***) from* **3**. Step 1: 4.7 g (21 mmol) of the sodium salt of *N*-chloro-benzenesulfonamide ('chloramin-B') was added portionwise (over 10 min) to a stirred solution of 3.3 g (10 mmol) of methyl lambertianate (**3**) in 25 ml of methanol and 1.5 ml of acetic acid at 0–5 °C. After 30 min at 5 °C, the reaction was quenched by addition of 1–2 ml of 5% aqueous Na_2SO_3. The mixture was concentrated by solvent evaporation in vacuo and the residue was partitioned between 50 ml of water and 30 ml of ethyl acetate. The aqueous layer was extracted once again with dichloromethane and the combined organic layers were washed with water and brine. After drying over $MgSO_4$ and filtration, the solvent was evaporated and the residue purified by column chromatography (petrol ether/*t*-BuOMe 1:1) to afford 3.5 g (90%) of a mixture of compounds **4a**–**d** as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.47$ (3H, s, CH_3-20), 0.83–1.29 (3H, m, H-1,3,9), 1.14 (3H, s, CH_3-19), 1.40–2.05 (10H, m, H -1, H-2, H-3, H-5, H-6, H-7, H-11), 2.08–2.25 (2H, m, H-7, H-12), 2.33–2.40 m (1H, H-12), 3.23 s, 3.24 s, 3.26 s, 3.27 s (ratio 1:1:1:1, all s 6H, $2CH_3O$), 3.52 (3H, s, OCH_3–C═O), 4.47 (1H, s, H-17), 4.82 (1H, s, H-17), 5.23 d, 5.27 s, 5.38 d (1H, H-16, for *cis*-isomers, $J = 1.7$ Hz), 5.48, 5.50, 5.59 (2H, all d, $J = 1.7$, 1.6, 1.6 Hz, H-14 and H-15). Calcd. for $C_{23}H_{36}O_5$: 392.22607. Found: 392.22225.
Step 2: to a solution of 1.95 g (5 mmol) of the mixture of compounds **4a**–**d** in 30 ml of dioxane was added 2.5 ml of 20% HCl. The mixture was stirred at rt for 30 min before the reaction was quenched through addition of 15 ml of 3% aqueous K_2CO_3. The product was extracted with dichloromethane and the combined extracts were washed with water and brine. After drying over $MgSO_4$ and filtration, the solvent was evaporated and the residue recrystallized from petrol ether to give 1.18 g (67%) of pinusolide (**1**) as a yellow solid. Mp = 82–84 °C; $[\alpha]_D^{20}$ + 21.5 (*c* 7.1, $CHCl_3$). {Lit.[3]: mp = 83–84 °C; $[\alpha]_D^{20}$ +24 (*c* 2.0, EtOH)}. IR (ATR): 758, 786, 874, 924, 1003, 1023, 1502, 1642, 1727, 1791, 3081 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ [ppm] = 0.39 (3H, s, CH_3-20), 0.91 (1H, dt, H-1, $J = 13.3$, 4.3 Hz), 0.96 (1H, ddd, H-3, $J = 13.2$, 12.8, 4.2 Hz), 1.07 (3H, s, CH_3-19), 1.18 (1H, dd, H-5, $J = 12.6$, 2.9 Hz), 1.40 (1H, m, H-2), 1.47 (1H, m, H-11), 1.53 (1H, dd, H-9, $J = 11.1$, 3.2 Hz), 1.62–1.78 (4H, m, H-1, H-2, H-6, H-11), 1.80 (1H, m, H-7, $J_{gem} = 12.7$ Hz), 1.85 (1H, m, H-6), 1.97 (1H, m, H-12), 2.05 (1H, m, H-3, $J_{gem} = 13.2$ Hz), 2.29 (1H, ddd, H-7, $J = 12.7$, 4.0, 2.6 Hz), 2.33 (1H, m, H-12), 3.50 (3H, s, OCH_3), 4.47 (1H, s, H-17), 4.77 (1H, s, H-17), 4.62 (1H, dd, H15, $J = 18.0$, 1.6 Hz), 6.99 (1H, d, H-14, $J = 1.6$ Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ [ppm] = 12.38 (C-20), 19.72 (C-2), 21.69 (C-11), 24.52 (C-12), 25.99 (C-6), 28.59 (C-19), 38.04 (C-3), 38.49 (C-7) 39.00 (C-1), 40.08 (C-10), 43.98 (C-4), 50.71 (OCH_3), 55.54 (C-9), 56.11 (C-5), 69.48 (C-15), 106.66 (C-17), 134.60 (C-14) 143.23 (C-13), 146.99 (C-8), 173.14 (C-16), 176.61 (C-18). MS (EI, 70 eV): *m*/*z* (%) = 346 $[M]^+$ (4), 314 (8), 286 (48), 271 (22), 217 (14), 189 (19), 161 (27), 121 (100), 109 (27), 81 (36). Calcd for $C_{21}H_{30}O_4$: 346.21439. Found: 346.21489.
14. *X-ray crystallographic analysis of* **1**. A crystal of **1** was chosen [0.80 mm × 0.20 mm × 0.20 mm]. The data were collected on a Bruker P4 diffractometer with Mo K_α radiation. The cell parameters and the orientation matrix for data collection were obtained from least-squares refinement in the range of 25–35° (2Θ). A total of 2442 unique reflections were collected. The structure was solved by direct methods using the SHELXS-97 software. The refining of structural parameters was carried out by the least-squares procedure in the full-matrix anisotropic approximation applying the program SHELXL-97 to $wR_2 = 0.1202$, $S = 1.021$ for all reflections ($R = 0.0485$ for 1768 $F > 4\sigma$). Hydrogen atoms were included in the refinement but restrained to ride on the atom to which they are bonded. The X-ray data were submitted to the Cambridge Crystallographic Data Centre (CCDC 603246), where they can be obtained (www.ccdc.cam.ac.uk/data_request/cif) free of charge.
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18. *Biological investigations: cell culture.* The cells were subcultured every 3–4 days by dilution of the cells to a concentration of 1×10^5/ml. All experiments were performed in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.56 g/l L-glutamine. Twenty four hours before the assay setup, cells were cultured at a concentration of 4×10^5/ml to ascertain standardized growth conditions. For apoptosis induction, the cells were then diluted to a concentration of 1×10^5/ml immediately before addition of **1** (as a solution in DMSO).
Determination of cell concentration and cell viability. Cell viability was determined by CASY®. Cell Counter + Analyzer System of Schaefer System GmbH (Reutlingen, Germany). Settings were specifically defined for the requirements of the used cells. With this system the cell concentration is analyzed simultaneously in three different size ranges: cell debris, dead cells, and viable cells were determined in one measurement. BJAB cells were seeded at a density of 1×10^5cells/ml and treated with different concentrations of the pinustilbene compounds ranging from 5 to 100 μM. After 24 and 48 h of incubation, cells were resuspended properly and 100 μl of each well was diluted in 10 ml CASYton (ready-to-use isotonic saline solution) for an immediate automated count of the cells.*Measurement of the mitochondrial permeability transition.* After incubation with different substance concentrations, BJAB cells were collected by centrifugation at 300*g*, 4 °C for 5 min. Mitochondrial permeability

transition was then determined by staining the cells with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanin iodide (JC-1; Molecular Probes, Leiden, The Netherlands) as described. 1×10^5 cells were resuspended in 500 μl phenolred-free RPMI 1640 without supplements and JC-1 was added to give a final concentration of 2.5 μg/ml. The cells were incubated for 30 min at 37 °C and moderate shaking. Control cells were likewise incubated in the absence of JC-1 dye. The cells were harvested by centrifugation at 300*g*, 4 °C for 5 min, washed with ice-cold PBS, and resuspended in 200 ml PBS at 4 °C. Mitochondrial permeability transition was then quantified by flow cytometric determination of cells with decreased fluorescence, that is, with mitochondria displaying a lower membrane potential. Data were collected and analyzed using a FACScan (Becton Dickinson) equipped with the CELLQuest software. Data are given in percentage of cells with low $\Delta\psi$, which reflects the number of cells undergoing mitochondrial apoptosis.

Measurement of DNA fragmentation. Apoptotic cell death was determined by a modified cell cycle analysis which detects DNA fragmentation on the single cell level. For measurement of DNA fragmentation cells were seeded at a density of 1×10^5 cells/ml and treated with different concentrations of pinusolide (**1**) in DMSO ranging from 5 to100 μM. After 72 h of incubation, cells were collected by centrifugation at 300*g* for 5 min, washed with PBS at 4 °C, and fixed in PBS/2% (v/v) formaldehyde on ice for 30 min. After fixation, cells were incubated with ethanol/PBS (2:1, v/v) for 15 min, pelleted, and resuspended in PBS containing 40 μg/ml RNase A. After incubation for 30 min at 37 °C, cells were pelleted again and finally resuspended in PBS containing 50 μg/ml propidium iodide. Nuclear DNA fragmentation was then quantified by flow cytometric determination of hypodiploid DNA. Data were collected and analyzed using a FACScan (Becton Dickinson, Heidelberg, Germany) equipped with the CELLQuest software. Data are given in percentage of hypoploidy (subG1), which reflects the number of apoptotic cells.

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