Workup-Dependent Formation of 5-Lipoxygenase Inhibitory Boswellic Acid Analogues

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Received February 11, 2000

Pentacyclic triterpenes from the 11-*keto*-boswellic acid series were identified as the active principal ingredients of *Boswellia* resin, inhibiting the key enzyme of leukotriene biosynthesis, 5-lipoxygenase (5-LO). Of the genuine boswellic acids hitherto characterized, 3-*O*-acetyl-11-*keto*-β-boswellic acid, AKBA (**1**), proved to be the most potent inhibitor of 5-LO. In the course of purification of further boswellic acid derivatives from *Boswellia* resin, we observed the degradation of the natural compound 3-*O*-acetyl-11-hydroxy-β-boswellic acid (**2**) to the thermodynamically more stable product 3-*O*-acetyl-9,11-dehydro-β-boswellic acid (**4**). The metastable intermediate of this conversion, under moderate conditions of workup in methanolic solutions, was identified as 3-*O*-acetyl-11-methoxy-β-boswellic acid (**3**). The novel artifactual boswellic acid derivatives inhibited 5-LO product formation in intact cells with different characteristics: **4** almost totally abolished 5-LO activity, with an IC_{50} of 0.75 μM, whereas **3** and 9,11-dehydro-β-boswellic acid (**5**), the deacetylated analogue of **4**, were incomplete inhibitors. The data suggest that the conditions chosen for the workup of *Boswellia* extracts could significantly influence the potency of their biological actions and their potential therapeutic effectiveness.

Frankincense, the gum resin of *Boswellia* species, is used for the treatment of inflammatory diseases in traditional folk medicine. Ethanolic extracts from *B. serrata* resin were shown to inhibit leukotriene (LT) biosynthesis in intact neutrophils in vitro.[1] Boswellic acids from the resin were identified as the active principles with relevant biological actions.[2,3] For example, the pentacyclic triterpene, 3-*O*-acetyl-11-*keto*-β-boswellic acid AKBA (**4**), attenuates LT biosynthesis with an IC_{50} of about 2 μM by inhibiting 5-lipoxygenase (5-LO), the key enzyme of LT biosynthesis. AKBA acts by a unique mechanism, in which it binds to 5-LO in a calcium-dependent and reversible manner and acts as a nonredox-type, noncompetitive inhibitor.[4,5] Pilot trials using *B. serrata* preparations containing AKBA suggested promising effects, which might be promoted, in part, by inhibition of LT biosynthesis; for example, reduction of peritumoral brain edema in patients with malignant glioblastomas, along with a decreased urinary LTE_4 excretion,[6,7] and improvement of symptoms of colitis[8] and bronchial asthma.[9]

Boswellia gum resins are complex mixtures, and crude organic extracts thereof contain a mixture of natural mono-, sesqui-, di-, and triterpenes.[10−14] The normally occurring penta- and tetracyclic triterpenes consist of structurally related members, which, depending on the nature of functional groups, can exert an inhibitory, partial inhibitory, noninhibitory, or even a potentiating influence upon the LT biosynthesis.[4,15,16] In addition, partial-inhibitory and noninhibitory terpenes antagonize AKBA inhibition of 5-LO by competitive binding to 5-LO.[4,5,15] Thus, the overall effect of *Boswellia* extracts on 5-LO activity is the result of complex actions of structurally related triterpenes.[17] Because of their relevance to therapeutic application, we have assayed the presence and the biological effects of other triterpenes in *Boswellia* resins. Here, we report the presence of 3-*O*-acetyl-11-hydroxy-β-boswellic acid (**2**) as an unstable natural ingredient in *Boswellia* resins from African and Indian origin that decomposes to a stable diene product. Because this artifactually formed novel pentacyclic diene exerts potent 5-LO inhibitory activity and shows strong UV absorption at 280 nm, the processing of resins could affect both the clinical efficacy and the optical methods used for the standardization of drugs.

Results and Discussion

In crude ethanolic extracts from *Boswellia* resins, we consistently observed the presence of component **2**, which appears as a trailing shoulder on the signal for AKBA (**1**) in reverse-phase (RP) HPLC monitored at 210 nm (Figure 1). Attempts to isolate and purify this unknown compound by rechromatography led to the disappearance of the shoulder, with concomitant appearance of two new compounds. As illustrated in Figure 2, the naturally occurring compound **2** yielded two decomposition products (**3** and **4**), which were eluted from the RP-HPLC column with increased elution times: compound **3** was more hydrophobic than **2**, and compound **4** was more hydrophobic than **3**. These observations suggested the presence of an unstable natural product in original resins, which was chemically modified in the course of workup.

Compound **2** and artifacts **3** and **4** were measured with on-line RP-HPLC−ESIMS coupling: **2** shows *m*/*z* 515 (M + 1), **3** has *m*/*z* 529 (M + 1) and **4** shows *m*/*z* 497 (M + 1). If isolation were performed using moderately acidified aqueous MeOH-solutions, **3** and **4** could be obtained. Refluxing of fractions containing **2** under more drastic conditions (e.g., extended reaction times and/or increased TFA contents) led to the disappearance of **2** with sole formation of **4**, suggesting that **3** is the metastable intermediate product from **2** and the precursor of **4**. In contrast to **2** and **3**, the thermodynamically stable compound **4** shows UV absorption at 280 nm, indicating a structure with a conjugated double-bond system.

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10.1021/np000069k CCC: $19.00
Published on Web 07/11/2000

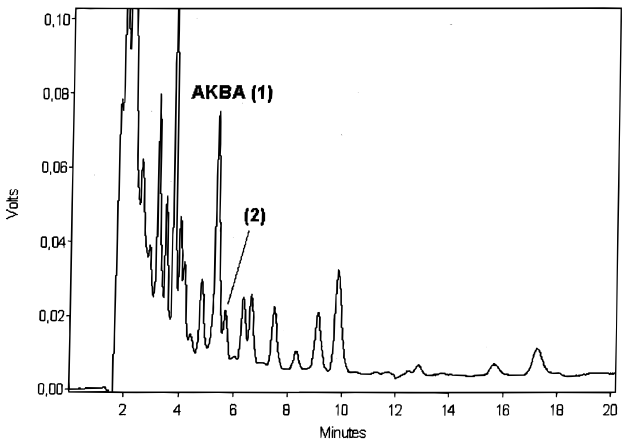


Figure 1. Elution profile of an ethanolic extract from *B. carteri* resin. AKBA (**1**) and shoulder component (**2**) were detected in reversed-phase HPLC monitored at 210 nm, as described in the Experimental Section.

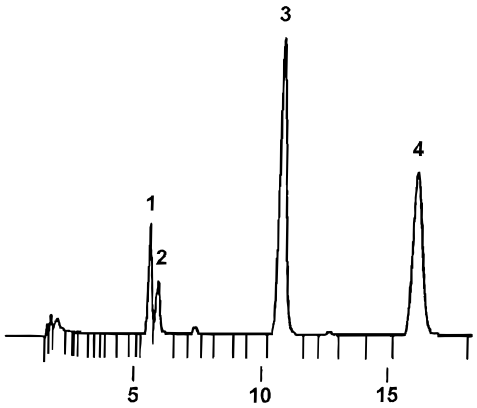


Figure 2. Elution profile of AKBA (**1**), unstable component **2**, and the artifactual products **3** and **4** formed in the course of purification. The compounds were separated with reversed-phase HPLC and detected at 210 nm, as described in the Experimental Section.

Compounds **3** and **4** were prepared by refluxing a crude extract fraction containing **2** under modified conditions (for details see Experimental section). The metastable intermediate **3** was purified by RP-HPLC using an acid-free eluent and identified by RP-HPLC−ESIMS, IR, ^{1}H NMR, EIMS, and HREIMS as 3-*O*-acetyl-11-methoxy-β-boswellic acid (**3**). The thermodynamically stable product was identified as 3-*O*-acetyl-9,11-dehydro-β-boswellic acid (**4**).

An analogous decomposition was also observed in our attempt to isolate a substance eluting as a trailing shoulder on the peak for KBA (11-*keto*-β-boswellic acid). The resulting stable product, which gave *m*/*z* 455 (M + 1, RP-HPLC−ESIMS) and is strongly UV absorbing, was identified as 9,11-dehydro-β-boswellic acid (**5**). Compound **5** was previously detected by others as an impurity in some samples of β-boswellic acid and was tentatively characterized on the basis of its optical properties and named "γ-boswellic acid".[18,19] In analogy to the decomposition of **2**, we suggest that the unstable natural product that elutes as a shoulder on the KBA peak is 11-hydroxy-β-boswellic acid, which is converted in aqueous MeOH solvents to the stable diene **5** via the metastable intermediate 11-methoxy-β-boswellic acid.

The artifacts **3**, **4**, and **5** showed concentration-dependent inhibition of calcium- and ionophore-stimulated 5-LO product formation from endogenous substrate in intact polymorphonuclear leukocytes (PMN) (Figure 3 and Table 1). Compounds **3** and **5**, with incomplete inhibition of the 5-LO activity, were partial inhibitors. The stable product

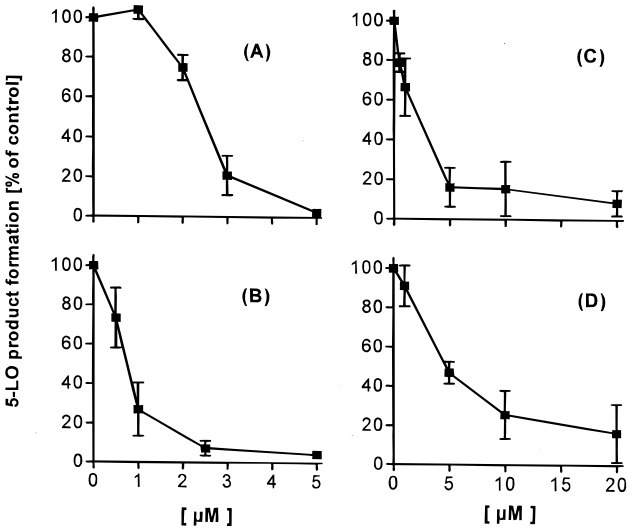
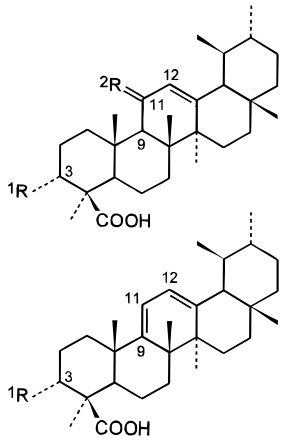


Figure 3. Inhibition of 5-LO product formation in intact PMN by AKBA and artificial boswellic acids. Concentration dependent effects of AKBA (compound **1** in **A**), 3-*O*-acetyl-9,11-dehydro-β-boswellic acid (compound **3** in **B**), 3-*O*-acetyl-11-methoxy-β-boswellic acid (compound **2** in **C**) and 9,11-dehydro-β-boswellic acid (compound **5** in **D**) on product synthesis from endogenous substrate in calcium/ionophore stimulated intact PMN were measured, as described in the Experimental Section. 5-LO product formation [i.e., LTB_4, 6-*trans*-LTB_4, 6-*trans*-12-*epi*-LTB_4, and 5(*S*)-HETE] are presented as percent of products in controls (means ± SD; $n = 3-8$).

Table 1. Structures and Structure-Dependent 5-LO Inhibitory Actions of Boswellic Acid Derivatives in Intact PMN



	^{1}R	^{2}R	IC_{50} (μM)
AKBA (**1**)	AcO	O	2.7 (1.5)[a]
acetyl-11-OH-BA (**2**)	AcO	OH/H	not tested
acetyl-11-MeO−BA (**3**)	AcO	MeO/H	partial inhibition
KBA	OH	O	3[a]
β-BA	OH	2H	partial inhibition[a]
acetyl-β-BA	AcO	2H	partial inhibition[a]

	^{1}R	IC_{50} (μM)
acetyl-9,11-dehydro-BA (**4**)	AcO	0.75
9,11-dehydro-BA (**5**)	OH	partial inhibition

[a] Data from refs 2, 4, and 17.

4 almost totally abolished 5-LO activity at 5 μM (IC_{50} = 0.75 μM). The artifacts **4** and **5** were also inhibitory to 5-LO activity in a cell-free assay system in the presence of exogenous substrate (IC_{50} ca. 5 μM), demonstrating that both **4** and **5**, like other inhibitory boswellic acids,[4,15] modulate 5-LO activity but do not impair release of the arachidonate substrate by phospholipase A_2 (Figure 4).

In conclusion, the data presented demonstrate that artifact formation during the extraction procedure can alter

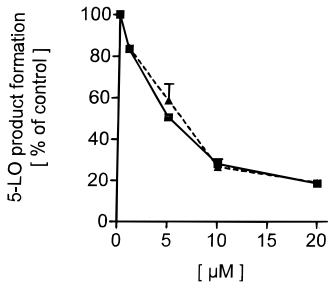


Figure 4. Inhibition by artificial boswellic acids of 5-LO product formation in a cell-free assay system. Concentration-dependent effects of 3-acetoxy-9,11-dehydro-β-boswellic acid (▲) and 9,11-dehydro-β-boswellic acid (■) on the 5-LO product synthesis from exogenous substrate (10 μM arachidonic acid) in PMN homogenates. 5-LO product formation [i.e., the sum of LTB_4, 6-*trans*-LTB_4, 6-*trans*-12-*epi*-LTB_4, 5(*S*)-HpETE, and 5(*S*)-HETE) are presented as percent of products in controls (means ± SD; $n = 3-8$).

the composition of boswellic acids in crude extracts, and thus, modify the overall 5-LO inhibitory potency of such extracts. In addition, the strong UV absorbing properties of these artifacts suggest that optical methods used heretofore for extract and drug standardization should be critically reevaluated.

Experimental Section

General Experimental Procedures. For boswellic acid purification, an isocratic RP-HPLC−UV-detection system using a Hypersil-ODS column, 5 μm, 250 × 8 mm (Knauer, Berlin, Germany) with MeOH−H_2O (90:10) and either with 0.007% (pH 2.8) or without TFA as mobile phase was used. Analytical runs were performed on a prepacked Hypersil-ODS column, 5 μm, 250 × 4 mm with MeOH−H_2O−TFA (90:10:0.007, v/v) eluent at 33 °C and 1.2 mL/min. RP-HPLC−ESIMS experiments were carried out on an API III TAGA 6000E (Perkin-Elmer, Toronto, Canada) with an electrospray ion source and a *m/z* range of 2400 amu. Spectra were recorded in the positive mode. For data acquisition, data processing, and the control of the mass spectrometer, a MacIntosh Quadra 650 was employed. EIMS were recorded on a TSQ 70 (Analytical Systems, Egelsbach, Germany). The ionization energy was 70 eV, and a direct inlet system was used. NMR spectra were recorded in $CHCl_3$-d_1 at room temperature on an AMX 400 (Bruker, Rheinstetten, Germany). UV spectra were recorded in MeOH employing an Ultraspec 2000 (Pharmacia Biotech, Uppsala, Sweden).

Chemicals. Olibanum in granis, representing a mixture of *Boswellia carteri* Birdw. and *Boswellia bhau dajiana* Birdw. (Ch.B. 74830028), was obtained from Caelo (Hilden, Germany). *Boswellia serrata* Roxb. resin batches were gifts from Pharmasan (Freiburg, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany) in the highest available analytical grade.

Isolation of Boswellic-Acid Fraction and Boswellic Acids. For preparation of the boswellic acid fraction, diethyl ether extracts of resins were chromatographed on Si gel (LiChrosorb Si 60, Merck) using a *n*-hexane−EtOAc gradient (9:1 to 1:1, v/v). We obtained single compounds by rechromatography of fractions on RP-HPLC with acidified (0.007% TFA, pH 2.8) or acid-free eluents. To obtain products from **2**, a crude fraction was refluxed with acidic RP-HPLC−eluent for 2 h, and monitored by UV−RP-HPLC (210 and 280 nm) every 30 min. After 30 min, **2** had been converted into a mixture of **3** and the second, more lipophilic compound **4** (Figure 2). By increasing the TFA content and refluxing for an additional 1 h, **3** was completely converted to **4**. Alternatively, **4** was prepared by refluxing the extract fraction in 1 N HCl for 2 h and isolation by rechromatography on RP-HPLC.

3-*O*-Acetyl-11-hydroxy-β-boswellic acid (2): could not be isolated as a pure compound due to its instability; RP-HPLC−ESIMS *m/z* 515 [M + 1]; MS/MS *m/z* 497 (100), 437 (39).

3-*O*-Acetyl-11-methoxy-β-boswellic acid (3): white powder; mp 175−180 °C, UV (MeOH) λ_{max} 206 nm; IR (KBr) ν_{max} 1742, 1080 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.34 (1H, d, J = 3 Hz, H-12), 5.26 (1H, t, J = 3 Hz, H-3), 3.79 (1H, dd, J = 3 Hz, H-11), 3.26 (3H, OCH_3), 2.06 (3H, s, $OCOCH_3$); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 182.05 (s, *C*OOH), 170.41 (s, CH_3COO), 124.07 (s, C-12), 77.02 (s, C-11), 73.2 (s, C-3), 54.6 (s, H_3C-O); EIMS *m/z* 528 [M^+], 496 (100), 436 (15), 392 (43), 255 (81), 218 (68); HREIMS *m/z* [M − 1] 527.3698 (calcd for $C_{33}H_{51}O_5$ 527.3742; error for calc 4.374×10^{-3}); RP-HPLC−ESIMS *m/z* 529 [M + 1].

3-*O*-Acetyl-9,11-dehydro-β-boswellic acid (4): white powder; mp 230 °C, UV (MeOH) λ_{max} 282 nm; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.65 (1H, d, J = 5.83 Hz, H-11) 5.46 (1H, d, J = 5.83 Hz, H-12), 5.3 (1H, t, J = 3 Hz, H-3), 2.1 (3H, s, $OCOCH_3$); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 182.0 (s, *C*OOH), 172.0 (s, CH_3COO), 122.3 (s, C-12) 115.8 (s, C-11), 72.7 (s, C-3); EIMS *m/z* 496 [M^+] (100), 436 (13), 392 (8), 255 (79), 133 (62); RP-HPLC−ESIMS *m/z* 497 [M + 1].

9,11-Dehydro-β-boswellic acid (5): white powder; mp 218 °C, UV (MeOH) λ_{max} 283 nm; ^1H NMR ($CDCl_3$, 400 MHz) 5.64 (1H, d, J = 5.84 Hz, H-11) δ 5.44 (1H, d, J = 5.84 Hz, H-12), 4.07 (1H, t, J = 2.6 Hz, H-3); EIMS *m/z* 454 [M^+] (100), 439 (10), 421 (13), 392 (10), 255 (55), 133 (155); RP-HPLC−ESIMS *m/z* 455 [M + 1].

5-Lipoxygenase (5-LO) Assays. 5-LO product formation from endogenous substrate (5-min incubations at 37 °C) was studied in Ca^{2+} (1.8 mM) and ionophore A23187 (1.9 μM) stimulated intact polymorphonuclear leukocytes (5×10^6 PMN/mL). Upon stimulation, the main 5-LO products in intact PMN were LTB_4 and 5(*S*)-HETE, in addition to minor amounts of two 6-all-*trans*-LTB_4 isomers. Cell purification and buffers, as well as C_{18} solid-phase extraction, RP-HPLC separation, detection (at 280 and 235 nm), and quantification of 5-LO products were as described previously.[4,15] Cell-free 5-LO activity assay was performed with crude homogenates of nitrogen-cavitated PMN in the presence of exogenously added arachidonate substrate (10 μM). The products of the homogenate assay were LTB_4, 6-*trans*-LTB_4, 6-*trans*-12-*epi*-LTB_4, 5(*S*)-HpETE, and 5(*S*)-HETE. Prostaglandin B_2 (PGB_2) (500 pmoles) was used as internal standard for the calculation of extraction efficiency in each sample. The molar extinction coefficients, which were used for the correction of differences in UV absorption, were 28 650 for PGB_2; 39 500 for LTB_4, 44 000 for 6-*trans*-LTB_4, and 6-*trans*-12-*epi*-LTB_4; and 30 500 for 5(*S*)-HETE and 5(*S*)-HpETE. Test compounds dissolved in EtOH were added 5 min before initiation. In all incubations (including controls) the final concentration of EtOH was 0.5%.

Data. Amounts of 5-LO product formation in intact cells [i.e., the sum of LTB_4, 6-*trans*-LTB_4, 6-*trans*-12-epi-LTB_4, 5(*S*)-HETE] are presented as percent of products in controls. In cell-free 5-LO assays, 5(*S*)-HpETE is quantified in addition to former products. Data are shown as means ± SD.

Acknowledgment. This work was supported by the Deutsche Forschungsgemeinschaft Grant Sa. 561/2-3.

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NP000069K