A Meroterpenoid NF-κB Inhibitor and Drimane Sesquiterpenoids from Asafetida

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Investigation of an acetone extract from asafetida afforded two drimane sesquiterpene dienones (fetidones A and B, **1a**,**b**) and several known sesquiterpene coumarin ethers, one of which (8-acetoxy-5-hydroxyumbelliprenin, **2a**) showed potent and specific NF-κB-inhibiting properties. This, coupled to a negligible cytotoxicity, qualifies **2a** as a new anti-inflammatory chemotype, and its occurrence in asafetida might rationalize the use of this gum resin to alleviate and prevent colon inflammatory disturbances.

Asafetida is a plant-derived culinary spice characterized by a burning taste and a persistent, offensive odor reminiscent of rotten meat, human sweat, and garlic.[1] It is a prominent ingredient of the Indian cuisine, especially among Jains, who cannot eat animal food and vegetables that contain buds, such as onion and garlic.[2] Under the name of *silphion persicum*, asafetida was also popular in ancient Rome, where it was used as a cheap replacement for *silphion cyrenaicum*, a very expensive spice whose culinary merits eventually led to the extinction of its plant source.[3] The popularity of asafetida as a spice continued throughout the Middle Ages, but it disappeared from European cookbooks during the Renaissance.[1,2] Asafetida was also used in medicine for a host of indications, mostly based on the assumption that its vile smell could act as a deterrent to germs and somewhat exert a sedative activity, counteracting hysteria crises as well as alcohol and opium craving.[1,4] None of these properties have been substantiated by modern studies, and asafetida is nowadays considered an obsolete drug. Like other strong spices, asafetida has carminative and eupeptic properties and is commonly added to legumes and beans to help in their digestion.[5]

The botanical origin of asafetida is unclear, and the commercial gum resin comes from a variety of foul-smelling *Ferula* species (Umbelliferae) endemic to Eastern Iran and Western Afghanistan [*F. assa-foetida* L., *F. narthex* Boiss., *F. foetida* (Bunge) Regel, *F. alliacea* Boiss.].[6] The sensory properties of asafetida are due to a host of sulfides and polysufides,[7] while sesquiterpene coumarin ethers are the major constituents of the nonvolatile fraction.[8] A large number of meroterpenoids of this type have been characterized from asafetida, sometimes in very large amounts,[1a] but, apart from the chemopreventive activity reported for farnesylferol C[9] and the antibacterial properties of galbanic acid,[10] their biological profile is still largely unknown. As part of an investigation on chemesthetic spices,[11] we have profiled phytochemically a commercial sample of asafetida, isolating two new drimane sesquiterpenoids and discovering a powerful NF-κB inhibitory activity for 8-acetoxy-5-hydroxyumbelliprenin (**2a**), the major constituent of the gum resin.[8]

An acetone extract from asafetida was fractionated by gravity column chromatography on silica gel, and the fractions obtained were further purified by passing through neutral alumina and/or crystallization. Four major sesquiterpene coumarin ethers were obtained, along with various minor constituents of meroterpenoid and sesquiterpenoid structure (see Experimental Section). Among the latter, **1a** and **1b** are new. The HRMS and ^{13}C NMR spectra of **1a** and **1b** suggested that these two compounds are a ketone−alcohol pair [molecular formula $C_{15}H_{20}O_2$ (HRMS) for **1a** and $C_{15}H_{22}O_2$ (HRMS) for **1b**; replacement of the ketone carbonyl at δ 214.1 in the ^{13}C NMR spectrum of **1a** with an oxymethine at δ 78.8 in **1b**]. The ^1H NMR spectrum of **1a** (Table 1) was rather simple and showed three olefin resonances [an exomethylene (δ 5.40, s; 5.27 d, $J = 1.6$ Hz) and one vinyl methine (δ 5.90, s)], one allylic methyl (δ 2.01, d, $J = 1.2$ Hz), three quaternary methyls (δ 1.44, s; 1.40, s; 1.32, s), an ABMX ethylidene system (δ 2.91, ddd, $J = 14.7, 14.7, 5.6$ Hz; 2.40, ddd, $J = 14.7, 4.2, 3.2$ Hz; 2.18, ddd, $J = 13.1, 5.6, 3.2$ Hz; 2.10, ddd, $J = 14.7, 13.1, 4.2$ Hz), and an aliphatic methine singlet (δ 2.58). Apart from the resonances corresponding to these carbons, the ^{13}C NMR spectrum showed five additional singlets, sorted out by chemical shift consideration into two ketone carbonyls (δ 214.1 and 197.8), two olefinic carbons (δ 150.3 and 154.2), and one aliphatic quaternary carbon (δ 47.0). The molecular formula of **1a** indicated six degrees of unsaturation, only four of which were accounted for by double bonds, and therefore this compound had to be bicyclic. On the basis of the HMBC considerations, the NMR resonances could be combined into the dienone drimane structure **1a**. The relative stereostructure was determined by the correlations observed in the NOESY spectrum. Thus, strong correlations between axial H-1, H-5, and the 14-methyl group showed that these protons are all located on the same side of the molecule, while correlations between the 13- and 15-methyl groups and axial H-2 indicated that these groups are located on the opposite side of the decalin system. Since all drimanes of known absolute configuration obtained from umbelliferous plants belong to the 15α-methyl, 5β-H series,[8] the same absolute configuration was also assumed for **1a**. Compound **1b** differed from **1a** only in the reduction of the 3-keto group to a secondary alcohol, whose relative configuration was assessed by NOE experiments (NOE correlations of H-3 with H-5 and the 13-methyl). Drimane sesquiterpenoids occur in *Ferula* species as umbelliferyl conjugates, and it is therefore likely that **1a** and **1b** are formed by elimination of umbelliferone from precursors of the Δ^7 or Δ^8 drimenyl type, as shown in Scheme 1. Indeed, the presence of the 6-keto group makes the 11-umbelliferyl moiety susceptible

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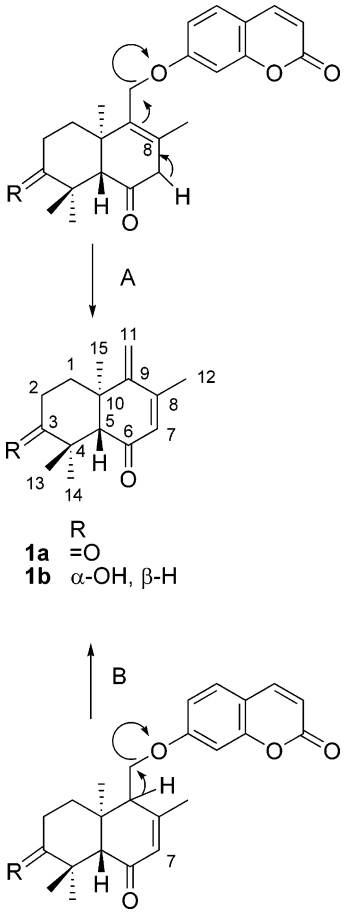
Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data for Fetidones A (**1a**) and B (**1b**)[a]

	1a		**1b**	
position	δ_H	δ_C	δ_H	δ_C
1α	2.10 (ddd; 4.2, 13.1, 14.7)	36.6 (t)	1.81 (m)	35.8 (t)
1β	2.18 (ddd; 3.2, 5.6, 13.1)		1.89 (ddd; 3.3, 3.3, 12.7)	
2α	2.40 (ddd; 3.2, 4.2, 14.7)	34.0 (t)	1.79 (m)	26.8 (t)
2β	2.91 (ddd; 5.6, 14.7, 14.7)		1.73 (m)	
3		214.1 (d)	3.21 (dd; 4.4, 11.0)	78.8 (s)
4		47.0 (s)		38.1 (s)
5	2.58 (s)	60.6 (d)	2.27 (s)	60.3 (d)
6		197.8 (s)		199.1 (s)
7	5.90 (s)	128.2 (d)	5.83 (s)	128.2 (d)
8		150.3 (s)		149.7 (s)
9		154.2 (s)		155.5 (s)
10		42.3 (s)		42.6 (s)
11a	5.40 (s)	113.0 (t)	5.32 (s)	112.3 (t)
11b	5.27 (d; 1.6)		5.25 (d; 1.6)	
12	2.01 (d; 1.2)	20.3 (q)	2.01	20.2 (q)
13	1.32 (s)	24.8 (q)	1.28 (s)	28.2 (q)
14	1.44 (s)	21.9 (q)	1.18 (s)	15.0 (q)
15	1.40 (s)	22.5 (q)	1.14 (s)	23.3 (q)

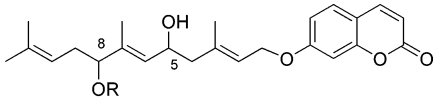
[a] Spectra were recorded in $CDCl_3$, with solvent signal of $CDCl_3$ (7.26 ppm for ^{1}H and 77.0 for ^{13}C) as reference, coupling constants (*J*) in Hz, and ^{13}C NMR multiplicities from HMQC experiments.

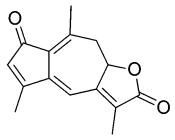
Scheme 1. Possible Biogenetic Derivation of the Dienones **1a**,**b** from a Drimanyl Coumarinyl Ether of the Δ^8 (A) or Δ^6 (B) Type



to loss via β-elimination or a vinylogous version of it, as outlined in Scheme 1.

One of the medicinal indications for asafetida is the treatment of colon disturbances, especially in children.[1a] NF-κB inhibitors are under clinical investigation for irritable bowel syndrome, [12] and many terpenoids containing electrophilic double bonds show NF-kB inhibitory activity.[13] Since an electrophilic behavior is expected for vinylogous exomethylene ketones such as **1a** and **1b**, these compounds, as well as a few major constituents of the gum resin, were assayed for their capacity to interfere with the activation of NF-κB.





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To study the effects of the isolated compounds on the activation of the NF-κB pathway, we used the cloned 5.1 cell line that contains the luciferase gene driven by the HIV-1-LTR promoter, which is responsive to TNFα through the NF-κB pathway.[14] The HIV-1 promoter contains two NF-κB binding sites that are absolutely required for TNFα-induced transactivation.[15] The cells were pre-incubated with increasing doses of the compounds for 30 min, then stimulated with TNFα for 6 h, and finally lysed, and the reporter luciferase activity was measured. An almost 15-fold increase in luciferase activity over the nonstimulated control cells was noted upon stimulation with TNFα. While dienones **1a** and **1b** showed negligible activity on the TNFα-mediated activation of NF-kB in the 5.1 cell line,[16] the sesquiterpene coumarin ether **2a**[8] showed specific NF-κB inhibitory activity with an IC_{50} of 15.09 μM, coupled to a lack of cytotoxicity. Interestingly, compound **2b**, the deacetyl derivative of **2a**,[8] was less active (IC_{50} 37.51 μM), while the unusual guaianolide **3** (taraxacin)[17] was even less active (IC_{50} 57.57 μM). The effects of compound **2a** on cellular toxicity were evaluated by propidium iodide staining and flow cytometry, with no cytotoxic activity found in either 5.1 cells or in other cell lines such as A549 and HeLa (data not shown). To further confirm that inhibition of HIV-1-LTR was mediated through the NF-κB sites located in this promoter, the lung carcinoma cell line A549 was transfected with either the KBF-Luc or the AP-1-Luc plasmids, and 24 h later the cells were preincubated with increasing concentrations of **2a** for 30 min and then stimulated with TNFα for 6 h. The KBF-Luc and the AP-1-Luc plasmids contain the

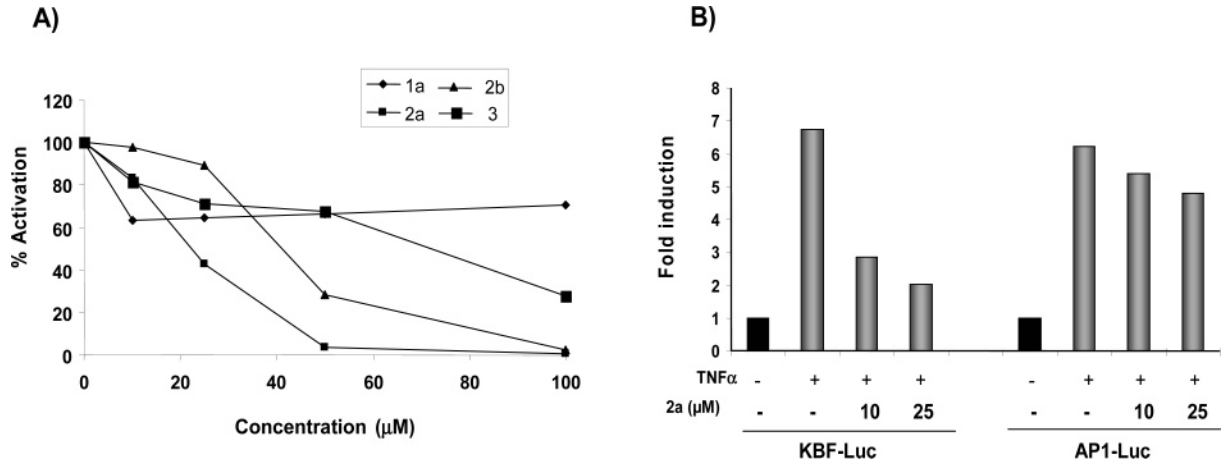

Figure 1. 8-Acetoxy-5-hydroxyumbelliprenin (**2a**) inhibits NF-κB-dependent luciferase gene expression. 5.1 cells (A) or A549 cells transiently transfected with either the KBF-Luc or the AP-1-Luc plasmids (B) were pretreated with different doses of the compounds and treated with TNFα for 6 h, after which luciferase activity was measured. The results show the percentage of transactivation in 5.1 cells (A) or the fold induction over the untreated control cells (B).

luciferase gene driven by multimerized binding sites for the NF-kB and AP-1 transcription factors, and therefore the TNFα-induced luciferase expression reflects the transactivation activity of these transcription factors. The dose-dependent inhibition of NF-κB-dependent luciferase expression in A549 cells was very similar to the inhibition observed in the stable cell line, and, interestingly, compound **2a** showed negligible activity on AP-1 activation, thus highlighting the specificity of the NF-kB pathway inhibition (Figure 1B).

Taken together, the results of this study show that asafetida contains bioactive compounds and suggest that old drugs are worth revisiting as a source of drug leads, since certain "obsolete" medicinal plants might have been dismissed prematurely because of lack of knowledge on their mechanism of activity.[18] We are nowadays well poised to translate anecdotal accounts of use into mechanism-based hypotheses, and the wealth of information on plant uses available from pre-modern medicine is worth investigation as a potential shortcut to the isolation of bioactive compounds.[19]

Experimental Section

General Experimental Procedures. Optical rotations were determined at 22 °C on a Perkin-Elmer 141 polarimeter equipped with a sodium lamp (589 nm) and 10 cm microcell. IR spectra were obtained on a Shimadzu DR 8001 spectrophotometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH} = 145$ Hz and $^nJ_{CH} = 10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra (HRESI) were recorded with a Micromass Q-TOF MICRO instrument. Macherey-Nagel silica gel 60 (70−230 mesh) and neutral alumina (Brockmann activity I) were used for gravity column chromatography.

Plant Material. Asafetida was purchased from Arjuna Natural Extracts, Ltd, Kerala, India. A voucher specimen (GBA2006-2) is kept at the Novara laboratories. A TLC profile of the commercial sample with its two major constituents (compounds **2a** and **2b**) as reference standards is available as Supporting Information.

Extraction and Isolation. A sample of asafetida (5 g) was dissolved in acetone, mixed with silica gel, and evaporated at 40 °C to a free-floating powder. This was separated by gravity column chromatography on silica gel (100 g, petroleum ether−EtOAc gradient) to gave eight major fractions (A−I), obtained by combining the eluates on the basis of their TLC profile. Fraction A (79 mg) contained umbelliprenin,[8] while the remaining fractions were further purified by column chromatography on alumina using various petroleum ether−EtOAc mixtures to afford methylgalbanate (77 mg)[8] and fetidone A (**1a**, 13 mg) from fraction B, fetidone B (**1b**, 58 mg) from fraction C, umbelliferone (176 mg) and taraxacin[14] (12 mg, **3**) from fraction D, 8-acetoxy-5-hydroxyumbelliprenin[8] (278 mg, **2a**) from fraction E, kataravicinol[8] (217 mg) from fraction F, epifoliferidin[8] (153 mg) from fraction G, and 5,8-dihydroxyumbelliprenin[8] (**2b**, 260 mg) from fraction H.

Fetidone A (1a): colorless oil; $[\alpha]_D^{25}$ +181 (*c* 4.0, CH_2Cl_2); IR (KBr) ν_{max} 1705, 1640, 1610 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 255.1386, calcd for $C_{15}H_{20}O_2$ + Na 255.1385.

Fetidone B (1b): white powder, mp 153 °C; $[\alpha]_D^{25}$ +59 (*c* 5.2, CH_2Cl_2); IR (KBr) ν_{max} 3450, 1707, 1640, 1614 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 257.1533, calcd for $C_{15}H_{20}O_2$ + Na 257.1542.

Cell Lines and Plasmids. The 5.1 clone line is a Jurkat-derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-LTR promoter and was maintained in exponential growth in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 1 mM Hepes, antibiotics (Invitrogen, Barcelona, Spain), and G418 (200 μg/mL).[14] The A549 lung adenocarcinome cell line (ATCC) was maintained in complete DMEM media. The AP-1-luciferase (AP-1-Luc) plasmid was constructed by inserting three copies of an SV40 AP-1 binding site into the *Xho* site of the pGL-2 promoter vector (Promega, Madison, WI). The KBF-Luc contains three copies of the MHC enhancer κB site upstream of the conalbumin promoter, followed by the luciferase gene.[14]

Cytotoxicity Assays. The 5.1 cells were seeded in 96-well plates in complete medium and treated with increasing doses of the selected compounds for the indicated period of time. Samples were then diluted with 300 μL of PBS and incubated 1 min at room temperature in the presence of propidium iodine (10 μg/mL). After incubation, cells were immediately analyzed by flow cytometry.

Transient Transfections and Luciferase assays. A549 cells (10^5/mL) were transiently transfected with the KBF-Luc reporter. The transfections were performed for 24 h using Lipofectamine Plus reagent (Life Technologies, Rockfield, MD), according to the manufacturer's recommendations. After incubation with increasing concentrations of 8-acetoxy-5-hydroxyumbelliprenin (**2a**) for 30 min, transfected cells were stimulated for 6 h with TNFα (2 ng/mL). To determine NF-κB-dependent transcription of the HIV-LTR-Luc, cells were preincubated for 30 min with the compound under investigation, and next stimulated with TNFα (2 ng/mL) for 6 h. Cells were next lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 953 (EG&G Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega), and protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted from each experimental value, the RLU/

μg of protein was calculated, and the specific transactivation was expressed as fold induction over untreated cells. All experiments were repeated at least three times.

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Supporting Information Available: TLC profile of the commercial gum resin employed in this investigation, with its two major constituents (compounds **2a** and **2b**) as standards. This material is available free of charge via the Internet at http://pubs.acs.org.

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