Solving the Confusion of Gnaphaliin Structure: Gnaphaliin A and Gnaphaliin B Identified as Active Principles of *Gnaphalium liebmannii* with Tracheal Smooth Muscle Relaxant Properties†

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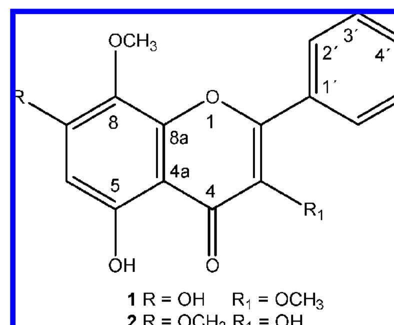
Inflorescences of *Gnaphalium liebmannii*, commonly known as "Gordolobo", is the most important remedy in Mexican traditional medicine to treat respiratory diseases, including asthma. By a bioguided fractionation of the *n*-hexane extract of this plant, following the relaxant effect on guinea pig tracheal smooth muscle, the flavones 5,7-dihydroxy-3,8-dimethoxyflavone (**1**) and 3,5-dihydroxy-7,8-dimethoxyflavone (**2**) were identified as the active relaxant compounds. Compounds **1** and **2** showed more potent relaxant properties than aminophylline in this model. Both **1** and **2** have been described as gnaphaliin in the past; here EIMS data, NMR experiments for both compounds, and X-ray diffraction analysis for **1** provided structural information to suggest that **1** and **2** should be named gnaphaliins A and B, respectively.

Diseases such as asthma and COPD (chronic obstructive pulmonary disease) involve bronchoconstriction of airways. The treatment of these diseases is based on the elevation or maintaining of cAMP levels[1] by the use of β_2-agonists such as salbutamol[2] or by the use of phosphodiesterase (PDE) inhibitor drugs such as aminophylline.[3] Other treatment consists in the antagonism of the muscarinic receptor by drugs such as ipratropium.[4] On the other hand, several flavonoids[5,6] and medicinal plants have shown the ability to produce relaxation of airway smooth muscle by phosphodiesterase inhibition.[7] *Gnaphalium liebmannii* Sch. Bp ex Klatt (family Asteraceae), commonly known as "gordolobo", is used in Mexican traditional medicine for the treatment of several respiratory diseases such as asthma, cough, bronchitis, and bronchial infections.[8−10] Previously we reported the relaxant properties on guinea pig trachea smooth muscle of the *n*-hexane extract obtained from inflorescences of this plant, suggesting that the mechanism of action of this extract was related to the production or conservation of cAMP levels.[11] Here, we are reporting the bioguided study of the *n*-hexane extract of *G. liebmannii* with the aim to identify the relaxant active principles using the model of guinea pig isolated trachea.

Results and Discussion

The bioassay-guided fractionation of the active *n*-hexane extract of *G. liebmannii* (see Figure 1), using the model of isolated trachea from guinea pig, led to the isolation of an active relaxant mixture [$EC_{50} = 99.34 \pm 27.76$ μg/mL ($r^2 = 0.851$)] eluted with *n*-hexane/EtOAc (7:3). From this fraction a yellow solid positive to ferric chloride test in TLC precipitated. From the supernatant were separated by preparative TLC two components. One, colored red with ceric ammonium sulfate, was identified as β-sitosterol by comparison with an authentic sample. The other component was identified as 5,7-dihydroxy-3,6,8-trimethoxyflavone by comparison with spectroscopy data previously reported.[12] This compound was not tested for activity due to the small amount. The yellow solid contained two components, evidenced by HPLC at 272 nm. This mixture showed the major relaxant activity [$EC_{50} = 90.96 \pm 6.48$ μg/mL ($r^2 = 0.954$)], and it was resolved by a type of flash chromatography column into two flavones, **1** and **2**. Compound **2** was slightly more active ($EC_{50} = 43.10 \pm 2.21$ μg/mL = 134.04 ± 6.41 μM) than **1** ($EC_{50} = 63.05 \pm 12.22$ μg/mL = 195.97 ± 36.07 μM) as a relaxant. Aminophylline [$EC_{50} = 534.50 \pm 27.88$ μM ($r^2 = 0.997$)] was used as reference relaxant drug, and it was less active than **1** and **2** (see Figure 2). The ^{1}H NMR spectra (400 MHz; $CDCl_3$; δ ppm) of **1** and **2** were very similar, suggesting that they were regioisomers (see Figure 3). The EIMS and ^{1}H, ^{13}C, and DEPT NMR spectra for **1** indicated a molecular formula of $C_{17}H_{14}O_6$, corresponding to a flavone. The five protons of the B ring appeared as two multiplets at δ_H 7.55 (3-H, H-3′, H-4′, and H-5′) and δ_H 8.11 (2-H, H-2′, and H-6′) with coupling constants for H-2′−H-3′, 9.6 Hz; H-2′−H-4′, 6.4 Hz, and H-3′−H-4′, 9.6 Hz. An exchangeable singlet was observed at δ_H 12.38, which is generally characteristic for the hydrogen-bonded OH-5 of flavones and flavanones.[13] Two characteristic signals for *O*-methyl groups were observed at δ_H 3.88 (OMe-8) and 4.00 (OMe-3); by HSQC and HMBC experiments, C-8 and C-3 were assigned to the signals at δ_C 126.7 and 139.7, respectively. The NOESY experiment disclosed the correlations of a signal at δ_H 6.43 with OH-5 and with the *O*-methyl group at δ_H 3.88 (OMe-8); the signal at δ_H 6.43 is characteristic for H-6 of a 5,7,8-trisubstituted A ring. In the HMBC experiment (see Figure 3) correlations were observed between H-6, C-8 (δ_C 126.7), C-5 (δ_C157.6), C-7 (δ_C 155.1), and the C-4a quaternary carbon at δ_C 105.8. These correlations allowed the placement of an *O*-methyl group (δ_H 3.88) at C-8. The proton of the second hydroxy group was not observed in the ^{1}H NMR spectra; therefore this hydroxy group was located at C-7 (δ_C 155.1). The *O*-methyl protons at δ_H 4.00 showed correlation in the HMBC spectra only with the carbon at δ_C 139.7, therefore locating this *O*-methyl group at C-3. These results suggested that one of the substances responsible for the relaxing activity of *G. liebmannii* is 5,7-dihydroxy-3,8-dimethoxyflavone (**1**). The structure was confirmed by X-ray diffraction (see Figure 4). The molecule crystallizes in the space group $P2_1/n$ with four independent molecules per asymmetric unit. This compound presents intramolecular O−H··· O═C and O−H···O−CH$_3$ hydrogen bonds and intermolecular interactions through groups C═O and OH.



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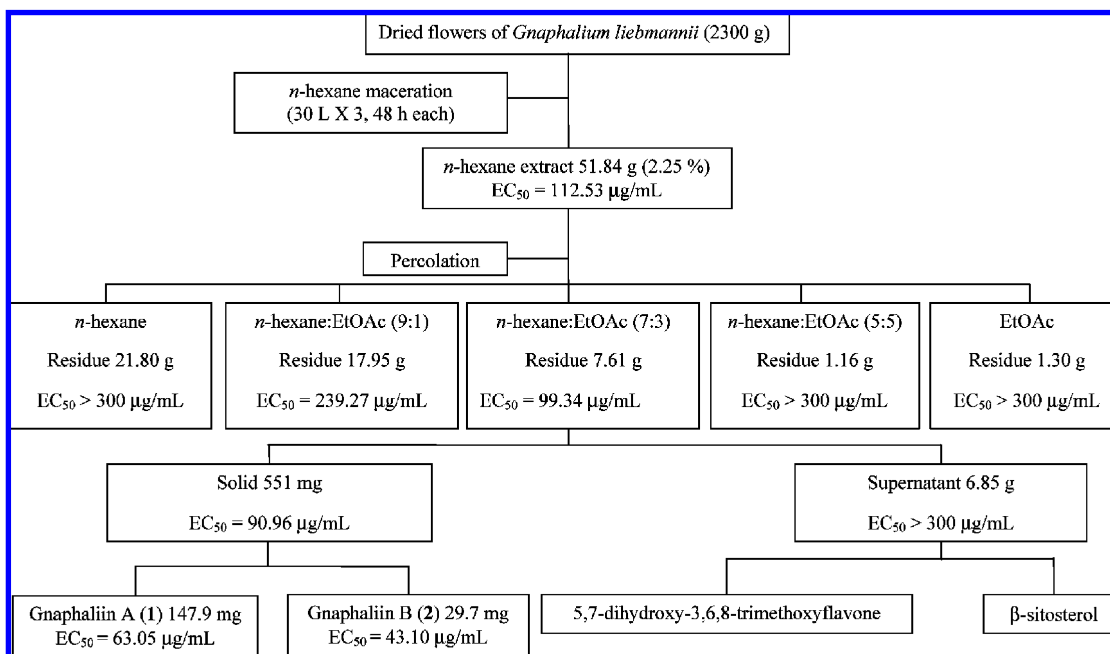

Figure 1. Bioassay-guided fractionation of the *n*-hexane extract of *Gnaphalium liebmannii*.

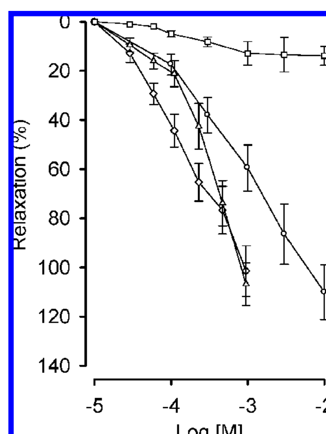

Figure 2. Dose−response curves plotting % of relaxation on carbachol (3 μM)-precontracted guinea pig trachea. **1** (Δ), **2** (◇), aminophylline (○), and vehicle (□; Tween 80, 0.0005% in water). Each point represents the mean ± SEM, $n = 6$.

Compound **2** was identified as 3,5-dihydroxy-7,8-dimethoxyflavone, whose major difference from **1** was observed in the HMBC experiment (see Figure 3), where the correlations between H-6 (δ_H 6.57) and C-7 (δ_C 139.5), C-8 (δ_C 130.3) were observed. NMR data for **1** and **2** are collated in Table 1. The EIMS fragmentation patterns for **1** and **2** were also different. The molecular ion *m*/*z* 314 was also the base peak for **2**, whereas for **1** it showed a relative abundance of 68%; the peak at *m*/*z* 299 $[M - CH_3]^+$ was the base peak for **1**, whereas for **2** it showed a relative abundance of 48%. These results suggest that the molecular ion is more stable when *O*-methyl groups are at C-7 and C-8 rather than at C-3 and C-8.

Both **1**[14−17] and **2**[13,18,19] have been known indistinctly as gnaphaliin. However, it is clear that there is confusion related with the correct designation of these compounds. To resolve this confusion, we propose that **1** should be named gnaphaliin A and **2** gnaphaliin B.

In conclusion, compounds **1** and **2** were identified as active relaxant principles of *G. liebmannii* on guinea pig tracheal muscle. This is the first time that **1** and **2** were isolated from the same natural source. These flavones could be potentially used in the treatment of diseases related to airway smooth muscle contraction.

Experimental Section

General Experimental Procedures. All chromatographic solvents were redistilled. Silica gel used for partition column chromatography was obtained from Merck (Darmstadt, Germany). For qualitative analysis an HPLC system with a photodiode array detector (Waters Corp., Milford, MA) was used. Detection was at 272 nm in H_2O with H_3PO_4 (0.1%)/MeOH/CH_3CN (50:30:20), using an Inertsil ODS-3 (150 × 4.6 mm i.d., 5 μm) column prefilled with C_{18} silica (GL Science Inc., Tokio, Japan). Melting points were determined on an Electrothermal digital IA9100 apparatus. EIMS spectra (ionization energy of 70 eV) were obtained on a Hewlett-Packard model 5890 spectrometer. Infrared (IR) spectra were registered on a Perkin-Elmer spectrometer model 599, and NMR spectra were obtained using a Varian model Unity INOVA spectrometer at 75 MHz for ^{13}C NMR and 300 or 400 MHz for ^{1}H NMR.

Plant Material and Extraction. *G. liebmannii* aerial parts were collected in San Pablo Ixayoc, Texcoco Estado de México, México, during February 2003. The specimens were identified by Abigail Aguilar, botanist from the Herbarium of the Instituto Mexicano del Seguro Social. A reference sample was deposited in this herbarium with the voucher number 14766. The plant material (2.3 kg) was dried at room temperature, ground, and extracted with *n*-hexane (3 × 13 L) by maceration at room temperature for 3 days. After evaporation of the solvent under vacuum, a green semisolid was obtained (51.84 g).

Bioguided Fractionation. The *n*-hexane extract of *G. liebmannii* flowers (50 g) was absorbed on the same amount of silica gel (Kieselgel 60; 0.063−0.200 mm) and separated by percolation in a silica gel column (6.5 cm × 21 cm, 500 g) by the consecutive addition of 4 L of *n*-hexane, *n*-hexane/EtOAc (9:1, 7:3, or 1:1), or EtOAc. Each of these fractions was tested in the guinea pig isolated trachea model (9.37−300 μg/mL), and the EC_{50} values were calculated (see Figure 1). The fraction eluted with *n*-hexane/EtOAc (7:3) was the most active fraction (7.61 g). This fraction was resuspended in *n*-hexane/EtOAc (7:3), which led to the precipitation of a yellow amorphous solid, which was separated by simple filtration, obtaining 551 mg of product (EC_{50} = 90.96 μg/mL). The supernatant was filtered, and after evaporation of the solvent, the residue (6.85 g) was tested but was inactive. A 150 mg sample of this residue was separated by preparative TLC, eluting with a mixture of *n*-hexane/EtOAc (7:3) to give two pure compounds. One of these was identified as β-sitosterol by comparison with an authentic sample, and the other as 5,7-dihydroxy-3,6,8-trimethoxyflavone by comparison of their spectroscopic and spectrometric data with reported data.[10] The active yellow solid (419 mg) was subjected to a subsequent separation on a silica gel column (1.2 cm × 60 cm, 28 g) eluted with *n*-hexane and *n*-hexane/EtOAc (9:1). From this column flavones **1** (147.9 mg)

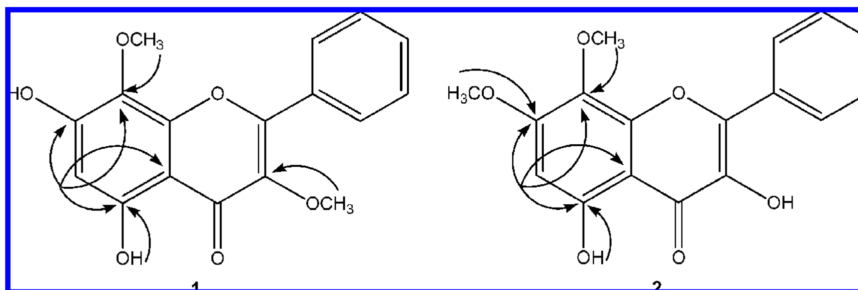

Figure 3. HMBC correlations of **1** and **2**.

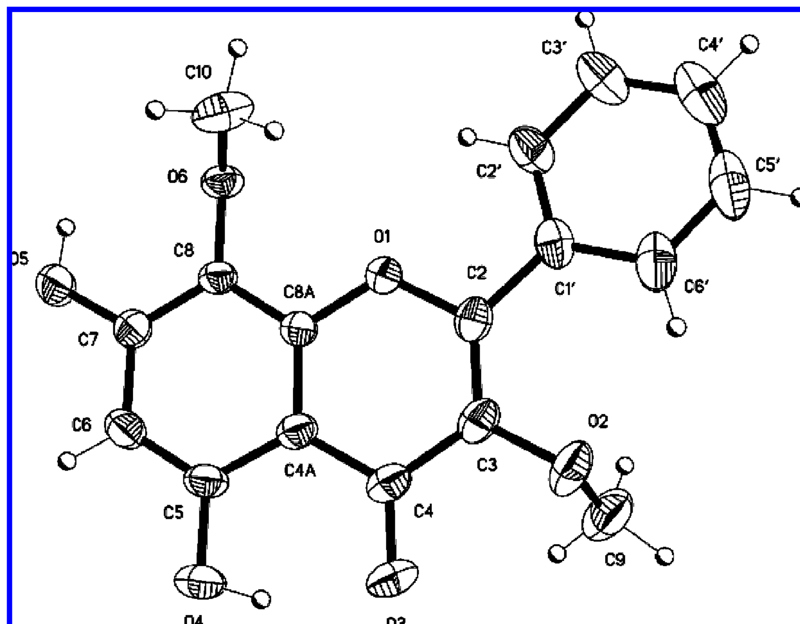

Figure 4. ORTEP diagram of **1**.

Table 1. ^{13}C and ^{1}H NMR Data of Compounds **1** and **2** in $CDCl_3$

	1[a]			**2**[b]		
position	δ_C (ppm)	δ_H (ppm)	multiplicity (J in Hz)	δ_C (ppm)	δ_H (ppm)	multiplicity (J in Hz)
2	155.5					
3	139.7					
4	179.1					
4a	105.8					
5	157.6			155.2		
6	98.5	6.43 s		93.1	6.57 s	
7	155.1			139.5		
8	126.7			130.3		
8a	148.0			152.4		
1′	130.5			130.7		
2′	128.2	8.11 ddd	(9.6, 6.4, 1, 2)	128.4	8.07 ddd	(8.1, 6.9, 1.2)
3′	128.7	7.55 ddd	(9.6, 6.4, 1.2)	128.6	7.52 ddd	(8.1, 6.9, 1.2)
4′	131.0	7.55 dd	(9.6, 6.4)	130.9	7.52 dd	(8.1, 6.9)
5′	128.7	7.55		128.6	7.52	
6′	128.2	8.11		128.4	8.07	
OH-5		12.38 s			12.86 s	
OMe	60.4 (3)	4.00 s		60.3 (7)	4.05 s	
	61.9 (8)	3.88 s		60.8 (8)	3.86 s	

[a] Recorded at 400 MHz. [b] Recorded at 300 MHz. All data were assigned on the basis of the observed 2D NMR correlations.

and **2** (29.7 mg) were isolated and tested in the guinea pig isolated trachea model (9.37−300 μg/mL). Both compounds were active as relaxants, **2** being more active than **1**.

Gnaphaliin A (1): yellow crystals (*n*-hexane/EtOAc); mp 175−176 °C; UV λ_{max} (CH_3CN) 273.7 nm; TLC R_f 0.33 [*n*-hexane/EtOAc (7:3)]; IR (KBr) ν_{max} 3282 (OH), 1654 (C=O) cm^{-1}; ^{1}H and ^{13}C NMR (see Table 1); EIMS m/z (% relative intensity) 314 [M$^+$] (68), 299 [M$^+$ − CH_3] (100), 271 (16), 256 (5), 157 (4), 139 (8), 118 (6), 105 (8), 77 (7).

Gnaphaliin B (2): pale yellow, amorphous solid; UV (CH_3CN) λ_{max} 270.2 nm; TLC R_f 0.4 [*n*-hexane/EtOAc (7:3)]; IR (KBr) ν_{max} 3434 (OH), 1662 (C=O) cm^{-1}; ^{1}H and ^{13}C NMR (see Table 1); EIMS m/z (% relative intensity) 314 [M$^+$] (100), 299 [M$^+$ − CH_3] (48), 271 (34), 253 (8), 228 (8), 157 (4), 143 (3), 118 (6), 105 (12), 77 (12).

Crystal Structure of Gnaphaliin A (1). X-ray data were collected on a Bruker Smart Apex CCD diffractometer with a wavelength of 0.71073 Å. The structure was solved and refined by using full-matrix least-squares on F^2. $C_{17}H_{14}O_6$, M_r = 314.28, data collection was

conducted at 298 K on a monocyclic crystal, $P2_1/n$; $a = 7.820(1)$ Å, $b = 12.225(1)$ Å, $c = 15.690(2)$ Å, $\alpha = 90°$, $\beta = 95.140(2)°$, and $\gamma = 90°$, $V = 1494.0(3)$ Å^3, $Z = 4$; final R indices $[I > 2\sigma(I)]$ R_1 0.0514, $wR_2 = 0.1269$, GOF = 1.039. CCDC-710862 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Drugs, Extracts, and Fractions. Acetylcholine chloride, carbachol chloride, and aminophylline were purchased from Sigma Chemical Co. (St. Louis, MO). These drugs were dissolved in distilled H_2O. The extract and fractions were suspended with 0.05% Tween 80 solution in distilled H_2O. The final concentration of Tween 80 was trace (less than 0.0005%) and did not affect the trachea response. All experiments were compared with one control that contained H_2O and 0.05% Tween 80. The drug solution or suspensions were freshly prepared a few minutes before the experiment.

Guinea Pig Trachea Preparation. Male guinea pigs (300−450 g) bred under conventional conditions and fed with standard diet (Purina pellets) and drinking water were used, following the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999, Especificaciones Técnicas para la Producción, Cuidado y Uso de Animales de Laboratorio) and in compliance with international rules on the care and use of laboratory animals.

The guinea pigs were euthanized by intraperitoneal injection of an overdose of sodium pentobarbital (95 mg/kg). The chest was opened to obtain the trachea, which was transferred to a dish containing warm Krebs solution. After removal of excess connective tissue and fat, the trachea was divided into eight small rings of about 2 mm in length containing two to three cartilaginous segments. Each tracheal ring was hung between two hooks inserted into the lumen and placed in a 10 mL organ bath containing Krebs solution with the following composition (mM): NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2.2H_2O$ 2.5, $NaHCO_3$ 25, and glucose 11.1. This solution was maintained at 37 °C and constantly bubbled with 5% CO_2/95% O_2. Isometric tension was recorded through an eight-channel Biopack System polygraph MP100 via a Grass FT 03E force transducer. The data were digitalized and analyzed using software for data acquisition (Acknowledge 3.9.0). The rings were placed under a resting tension of 1.5 g and allowed to stabilize for 60 min and then washed with fresh Krebs solution at 15 min intervals before starting the experiments. After the stabilization period, the trachea rings were contracted with acetylcholine chloride (3 μM) twice at 30 min intervals and washed after stimulation with fresh Krebs solution. Thirty minutes after the rings were contracted with carbachol chloride (3 μM), cumulative additions of crude extracts or fractions (9.37, 18.75, 37.5, 75, 150, or 300 μg/mL) or reference drug (aminophylline) were made to the bath to yield the required tracheal relaxant effects, which were allowed to reach a steady state at each concentration. The relaxant effects for crude extract, fractions, and substances obtained from the bioguided fractionation study were expressed as EC_{50}.

Data Analysis. The EC_{50} values were calculated by linear regression using only those data of the fraction of the dose−response curve with a good fit and with determination coefficient $r^2 > 0.9$. The values are shown as mean ± SEM of at least six independent experiments.

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