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Complex Flavonoids of *Pityrogramma trifoliata*: Absolute Configuration, Antifungal Effects, and Localization in the Plant

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Abstract

Chemical investigation of the fern *Pityrogramma trifoliata* (L.) R. M. Tryon (Pteridaceae) yielded a new complex flavonoid, here named trifoliatol, and 4 known complex flavonoids in addition to known chalcones and flavanones. The absolute configuration of the complex flavonoids is proposed, based on circular dichroism (ECD), crystal x-ray diffraction, and $[\alpha]_D$ measurements. White and yellow farinas, as well as 10 flavonoids were tested for antifungal activity against a panel of clinically important fungi, including yeast and dermatophytes. Both farinas and 2 of the flavonoids were notably active against *Trichophyton rubrum* and *T. mentagrophytes* (MICs and MFCs between 7.8 and 31.2 µg/mL). The localization of flavonoids in the plant trichomes was accomplished by use of scanning electron microscopy and histochemical methods.

Keywords

Pityrogramma trifoliata, complex flavonoids, absolute configuration, antifungals, flavonoids histochemical localization

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Introduction

Pityrogramma (Pteridaceae) is a fern genus distributed in humid regions of North and South America. Some species exhibit farinose exudates "*farina*" on the underside frond surface. White and yellow exudates are mainly constituted by mixtures of simple and complex flavonoids.[1]

P trifoliata (L.) R. M. Tryon is a tropical American fern[2] distributed from Mexico to South America from sea level up to 2100 masl. It grows in roadside ditches, river banks and open fields, fully exposed to sunlight. The plant resists long periods of desiccation and shows no sign of predatory activity.[3] Previously, in Argentina, this species had been taxonomically described under the genus *Trismeria*,[4] widely spread in subtropical regions of the country.

P. trifoliata produces both gold and white farina, and the color is related to the chemical structure of the farina's flavonoids.[5] Ethnomedical uses for eye infections have been reported in Peru for this species.[6]

Fungal infections in humans are increasing considerably, particularly those involving skin and mucosal surfaces, with dermatophytes and species of *Candida* being the most frequent pathogens.[7] Although there are several medicines for the treatment of mycoses,[8] side effects have been detected during treatments and quick development of resistance occurs due, in part, to intensive prophylactic use.[9] There is an urgent need for new antifungal agents with natural metabolites being promising alternatives.[10] Species of *Pityrogramma* are used for different ethnomedical purposes, particularly as topical antifungals and leishmanicides.[11]

As part of our ongoing natural product investigation of Argentine ferns,[12] the isolation and structural characterization of 1 new and 4 known complex flavonoids, together with other common flavonoids from the white and yellow exudates of *P trifoliata,* are described herein. The absolute configuration of the complex flavonoids is proposed, based on circular dichroism (ECD), crystal x-ray diffraction, and $[\alpha]_D$ measurements. The localization of flavonoids in the plant trichomes was accomplished by use of scanning electron microscopy and histochemical methods. The antifungal activity was

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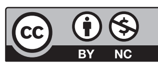

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determined of the white and yellow farina, as well as that of 10 flavonoids from P. trifoliata.

Results and Discussion

Structure Elucidation

Fresh fronds containing white and yellow farinas were rinsed separately with EtOAc and the farinas were completely dissolved. After solvent removal under reduced pressure, both white and yellow residues were separately processed by chromatographic methods (a residue portion was reserved for antifungal activity assays). Silica gel chromatography, followed by HPLC on normal and reverse phase of the yellow farina yielded the new complex flavonoid, here named trifoliatol (**1**), the known complex flavonoids **2** and **3**,[3] the chalcone **4**,[13] the 3 dihydrochalcones **5**, **6**,[14] and **7**,[15] and the flavanones **8** and **9**,[16] which were exhaustibly purified by HPLC and finally identified by spectroscopic techniques. The white farina yielded together with the dihydrochalcones **5**, **6**, and **7** the known complex flavonoids **10** and **11**.[17]

Trifoliatol (**1**) is a white amorphous solid. Its molecular formula $C_{24}H_{18}O_6$ was deduced from a molecular ion peak observed at *m/z* 402.1102 (calcd. 402.1103) in its HR-EI-MS. The IR spectrum showed absorptions at 3528, 1780, and 1631 cm^{-1} due to a nonassociated OH group, a δ-lactone and, a pyrone ring, respectively.[18] The ^{1}H-NMR spectrum of compound **1** in acetone-d_6 (Table 1) showed 2 double doublets, one at δ(H) 5.61 and the other at δ(H) 4.69. They were assigned to CH protons vicinal to CH_2 groups in hexacyclic rings. The signal at δ(H) 5.61 (1H, dd, $J = 12.5$, 3) was coupled to the methylene protons at δ(H) 3.34 (1H, dd, $J = 17$, 12.5) and 2.88 (1H, dd, $J = 17$, 3), which were assigned to the protons on C-2 and C-3 of a flavanone nucleus, while the other methyne proton at δ(H) 4.69 (1H, dd, $J = 7$, 2) was in a $PhCHCH_2CO$ unit (neoflavonoid part of the complex flavonoid) with the methylene protons at δ(H) 3.30 (1H, dd, $J =$ 16, 7) and 3.01 (1H, dd, $J = 16$, 2). The aforementioned evidence and the presence of aromatic signals in the ^{1}H and ^{13}C-NMR spectra indicated that **1** could be a complex flavonoid. A *p*-disubstituted phenolic ring was deduced by the 2 doublets at δ(H) 6.95 (2H, d, $J = 8$) and 7.45 (2H, d, $J = 8$) assigned to the B ring (Figure 1) of the flavanone part of the molecule. The spectrum also showed 3 signals corresponding to protons of a monosubstituted benzene (D ring of the neoflavonoid part) at δ(H) 7.23 (2H, brd, $J = 7$) assigned to H-2″, H-6″, δ(H) 7.28 (1H, t, $J = 7$) assigned to H-4″, and δ(H) 7.34 (2H, brd, $J = 7$) assigned to protons 3″, 5″ (Figure 1). The ion at *m/z* 325 in the mass spectrum $[M - 77]^+$ also indicates that a non-substituted benzene ring is present in the molecule. A singlet signal at δ(H) 6.29 was identified as the only proton (H-8) from the A ring (Figure 1). This was corroborated by comparison with literature data of similar compounds.[3] The complex flavonoid nature of **1** was also deduced by the ^{13}C-NMR spectrum in acetone-d_6 (Table 1), which could be fully assigned by crosspeaks of the HSQC bidimensional spectrum. The location of the 2 carbonyl groups was confirmed by the HMBC correlations (Figure 2) observed between protons at C-2 and C-3 and the ketone carbon at δ(C) 199.8 (C-4) and H-7″, H-8″ and the ester carbon at δ(C) 167.8 (C-9″). HMBC correlations between carbons 2 and 3 and the protons H-2′, H-6′ evidenced the location of the B ring, while HMBC correlations between H-7″ and C-5, as well as with the ring carbons C-1″, C-2″, and C-6″, allowed us to locate the D ring at C-7″. HMBC correlations between H-8 and carbons 6, 7, 9, and 10 (Figure 2) confirmed the substitution pattern of the A ring as described. Based on the mentioned spectroscopic data, the structure of **1** could be deduced as 7″,8″-dihydro-7-hydroxy-9,10-(4′-hydroxyphenyl-2,3-dihydro-1-benzopyran-4-one)-7″-phenylbenzopyran-9″-one.

Table 1. ^{1}H-, ^{13}C-, and HMBC NMR Data for Compound **1** in Acetone-d_6 (δ in ppm, J in Hz).

Position	δ (H)	**1** δ (C)	HMBC (H→C)
2	5.61 (*dd*, $J = 12.5$, 3, Hα)	81.3	2′, 6′
3	2.88 (*dd*, $J = 17$, 3, Hα)	44.4	4, 10, 1′
	3.34 (*dd*, $J = 17$, 12.5, Hβ)		2, 4, 1′
4		199.8	
5		160.9[a]	
6		107.5	
7		164.0	
8	6.29 (*s*)	98.0	6, 7, 9, 10
9		160.9[a]	
10		106.8	
1′		131.2	
2′/6′	7.45 (*d*, $J = 8$)	130.2	2, 4′
3′/5′	6.95 (*d*, $J = 8$)	117.2	
4′		159.8	
1′′		143.5	
2′′/6′′	7.23 (*t*, $J = 7.5$)[a]	130.7	1′′, 7′′
3′′/5′′	7.34 (*t*, $J = 7$)[a]	128.5	4′′
4′′	7.28 (*t*, $J = 7.5$)	129.0	3′′, 5′′
7′′	4.69 (*dd*, $J = 7$, 2, Hα)	35.7	5, 1′′, 2′′, 6′′, 9′′
8′′	3.01 (*dd*, $J = 16$, 2, Hα)	38.3	6, 1′′, 7′′, 9′′
	3.30 (*dd*, $J = 16$, 7 Hβ)		6, 1′′, 7′′, 9′′
9′′		167.8	

^{1}H- and ^{13}C-NMR spectra were recorded at 500 and 125 MHz, respectively.
[a]overlapped.

In order to determine the absolute configuration of the 2 chiral centers of compound **1**, the amorphous solid was induced to crystallize in various solvents. During work up, hydrolysis followed by esterification of **1** occurred to yield the isomer **12** (Figure 3) which was identified as the known flavonoid calomelanol H, previously isolated from P. calomelanos. Further spectroscopic analysis of **12** showed results coincident with those previously reported.[19] The colorless crystals of the isomer **12** obtained from CH_2Cl_2 were suitable for x-ray crystallographic analysis. Crystals of **12** are orthorhombic belonging to the space group $P2_12_12_1$ and, as shown in the ORTEP drawing in Figure 4, compound **12** possesses a complex flavonoid

Figure 1. Structures of compounds 1-11.

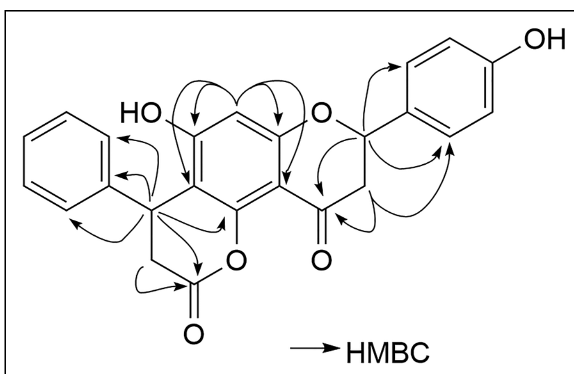

Figure 2. The key HMBC (arrows) data of **1.**

skeleton C_6-C_3-C_6-C_3-C_6 with 2 asymmetric centers, as depicted. As the chiral carbons of **1** were not involved in the isomerization reaction, the absolute configuration, *S* at C-7″ and *R* at C-2 can be proposed for our new compound **1,** in coincidence with the obtained X ray crystallographic analysis of **12**.

Regarding the absolute configuration results, although the value of the Flack parameter *x* is −0.2(5), based on the Hooft *y* of 0.0(4) and the P2(true) value of 0.931, it is considered that the statistics validate the proposed structure.[20, 21]

The circular dichroism (CD) spectrum of compound **2** exhibited diagnostic negative and positive Cotton effects at 335 and 234 nm, respectively ($\Delta\epsilon_{335}$ −4.64 and $\Delta\epsilon_{234}$ + 1.67). The absolute configuration of **2** (possesses 1 chiral carbon) at C-4 was established as *R* by comparing the experimental CD spectrum with the simulated electronic CD (ECD) spectra of both enantiomers (Figure 5).[22] The simulated spectrum for the 4*R* enantiomer showed positive bands at 230 and 270 nm, and negative bands at 250 and 348 nm (Figure 5). As compound **3** is an analog of **2** from the same plant species, we can unambiguously suggest the same absolute configuration at C-4 for the natural compound **3**, as depicted.

The absolute configuration of the only chiral carbon of compound **10** could not be determined by ECD measurements followed by theoretical calculations. Therefore, we decided to obtain the absolute stereochemistry of C-4 in compound **10**

Figure 3. Hydrolysis followed by esterification of **1.**

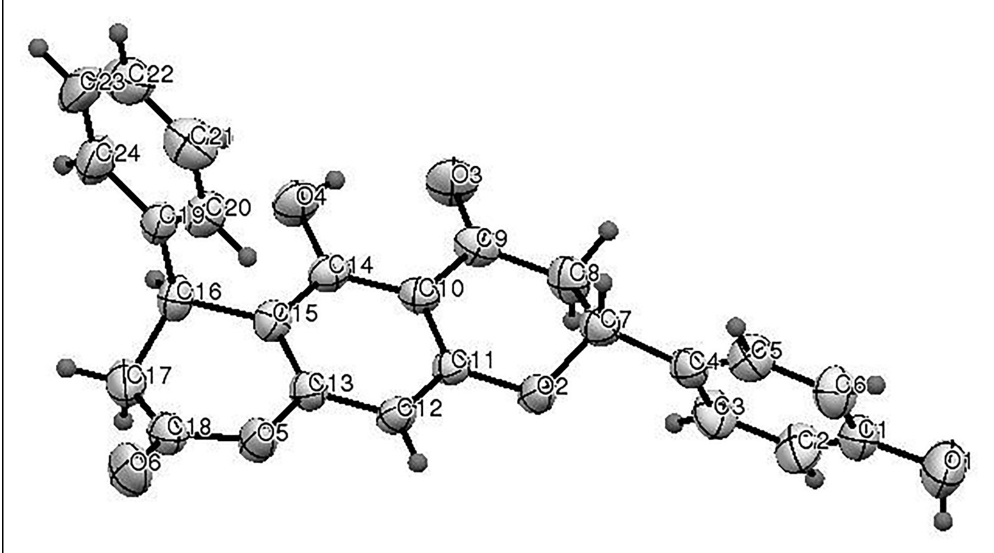

Figure 4. ORTEP diagram for compound **12**.

by comparison with that obtained for compound **2.** Compound **2** was submitted to hydrogenation of the double bond C-7″–C-8″ with Pd/C under a hydrogen atmosphere to give a dihydrogenated derivative identical to our natural compound **10**, as corroborated by comparison of the spectroscopic data of the dihydrogenated derivative of **2** with those of compound **10**. In addition, the same $[\alpha]_D$ values were obtained for the semisynthetic compound and for **10**, indicating that the same *R* absolute configuration at C-4 can be assigned to compound **10**. As could be expected, compound **11**, biosynthesized by the same plant biogenetic pathway, might share the same *R* absolute configuration at C-4.

Antifungal Activity

The yellow and white farina, as well as compounds **2-11** were tested in the range from 250 to 7.8 μg/mL against clinically important fungi by the microbroth dilution method according to the CLSI guidelines.[23, 24] Compound **1** was not evaluated for its antifungal activity because it underwent chemical transformation to compound **12** (Figure 3) during the process of recrystallization when exposed to various solvents. As shown in Table 2, the 2 farinas, as well as compounds **8** and **10**, were notably active against both strains of *Trichophyton* (MICs and MFCs between 7.8 and 31.2 μg/mL) while compounds **2-4** displayed moderate activities (MICs and MFCs between 31.2 and 125 μg/mL). Interestingly, methoxylation of the B ring in flavanone **9** plays an inactivating role, when compared to flavanone **8**, one of the most active compounds among the tested flavonoids. The remaining compounds were barely active or inactive against these dermatophytes. Compounds **4**, **8**, and **10** were moderately active against *M. gypseum* with MICs = 62.5 μg/mL and MFCs = 125 μg/mL while the rest of the compounds were shown to be inactive against this fungus indicating that in the chalcone **4** the double bond α, β-conjugated to carbonyl and phenyl is important for the activity in relation to the corresponding dihydrochalcone **6** (inactive). Both yellow and white farina displayed an important activity against *C. neoformans* (MIC = MFC = 15.8 μg/mL and MIC = MFC = 31.2 μg/mL, respectively). However, the isolated compounds were not active against this yeast, indicating a possible synergistic effect in the farinas. Regarding *C. albicans*, neither the farinas nor the pure compounds were active. Although MIC values for reference drugs amphotericin B, terbinafine, and ketoconazole against the 5 strains are lower than those obtained for our samples, the activity of the farinas and that of compounds **8** and **10** with MIC and MFC values lower than 20 μg/mL is relevant when compared with other natural

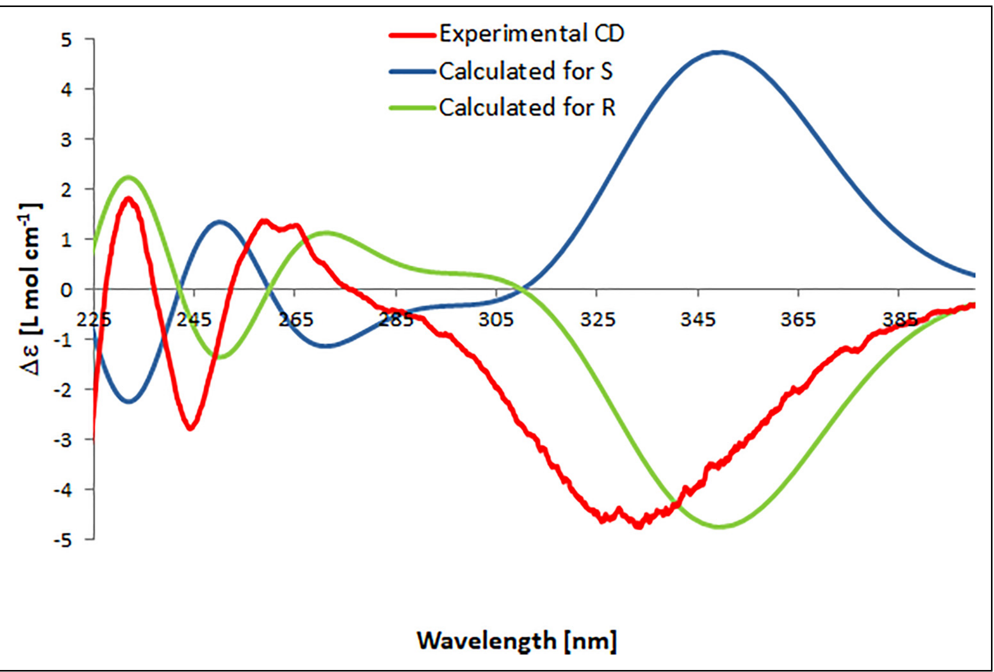

Figure 5. Experimental ECD spectrum of **2** (red) and calculated spectra of both hypothetical enantiomers (green and blue).

compounds cited in the literature. Plant extracts or isolated compounds with MICs lower than 20 μg/mL are considered to be promising when used in combination with synthetic compounds in antifungal therapies.[8–10]

Histochemical Localization of Flavonoids in the Fern

Farinose exudates of ferns of the big family Pteridaceae are considered to play an important role in plant protection.[25, 26] In *P. trifoliata* fronds, farina (Figure 6) is secreted by glandular trichomes located in the abaxial surface, associated with the development of sporangia (Figure 7A). This is the reason why only fertile pinnae have farina, as previously reported for *Onychium siliculosum* (Desv.) C. Chr. (Pteridaceae).[25]

P trifoliata shows 3 types of trichomes, 1 eglandular (100-500 μm) (Figure 7B), a glandular trichome with a single cell basal short stalk and a spherical secreting head (60-70 μm) (Figure 7C), and a glandular trichome with a long 2-celled stalk and oval head (150-240 μm) (Figure 7D). The gland surface is smooth in both types of glandular trichomes, due to the detachment of the cuticle from the gland cell wall. It forms a small subcuticular chamber where secretory substances accumulate before their release.[26]

Histochemical tests were useful to localize in situ the flavonoids present in trichome secretions. Results show (Figure 8) that in plants with yellow farina both glandular trichome types contain flavonoids. The fluorochromes for flavonoid detection, such as the Benedict reagent (Figure 8A) and aluminum trichloride (Figure 8B) induced an intense green and light yellow fluorescence in the whole secreting head of glandular trichomes. Staining with vanillin/HCl, cherry-red vanillin-flavonoid condensates were formed immediately in both the head and neck cells (Figure 8C). In plants with white farina only, long glandular trichomes secrete flavonoids. With Benedict reagent, these trichomes showed intense yellow fluorescence in the head and neck cells (Figure 8D), while with aluminum trichloride they gave florescence mainly in the head cell (Figure 8E). Red drops were observed in head and neck cells and in the stalk when vanillin/HCl was applied (Figure 8F). Similar results were reported for *Notholaena sulphurea*.[26]

Conclusions

One new and 10 known flavonoids have been isolated from an Argentinian collection of *P. trifoliata*. The absolute stereochemistry for all quiral centers of the isolated complex flavonoids is proposed. Antifungal activities of white and yellow farinas, and those of 10 flavonoids isolated from them, were determined against a panel of dermatophytes and yeasts. Farinas and some flavonoids displayed antifungal effects with MIC and MFC values lower than 20 μg/mL. The activity is relevant when compared with other natural compounds cited in the literature. Histochemical studies demonstrated that flavonoids accumulate in glandular trichomes located in the abaxial surface of fronds and play a defensive role in the plant. Complex flavonoids are solid compounds which have been isolated from many other species of ferns, as shown in the literature cited, though they have not been investigated by x-ray crystallographic diffraction methods to confirm their structures and stereochemistry. Determination of ECD spectra has not

Table 2. Antifungal Activities of *Pityrogramma trifoliata* Farinas and Isolated Compounds Against a Panel of Human Opportunistic and Pathogenic Fungi.

	MIC (μg/mL)/MFC (μg/mL)/				
Sample	*Mg*	*Tr*	*Tm*	*Ca*	*Cn*
2	250/ I	31.2/31.2	31.2/31.2	I	I
3	250/ I	62.5/62.5	62.5/62.5	I	I
4	62.5/125	62.5/125	62.5/125	I	I
5	250/I	125/125	125/125	I	I
6	I	250/250	125/125	I	I
7	250/I	125/250	250/250	I	I
8	62.5/125	7.8/31.2	31.2/31.2	I	I
9	I	I	I	I	I
10	62.5/125	7.8/31.2	7.8/7.8	I	I
11	I	I	I	I	I
YFE	7.8/31.2	7.8/31.2	7.8/31.2	250/250	15.8/15.8
WFE	7.8/15.6	7.8/15.6	7.8/7.8	I	31.2/31.2
Amph. B	0.125	0.075	0.075	0.78	0.25
Terbinafine	0.04	0.01	0.025	1.56	0.39
Ketoconazole	0.05	0.025	0.025	0.5	0.25

Abbreviations: MIC, minimun inhibitory concentration (μg/mL); MFC, minimum fungicidal concentration (μg/mL); *Mg*, *Microsporum gypseum* C 115-2000; *Tr*, *Trichophyton rubrum* C113-2000; *Tm*, *Trichophyton mentagrophytes* ATCC 9972; *Ca*, *Candida albicans* ATCC 10231; *Cn*, *Cryptococcus neoformans* ATCC 32264; ATCC, American Type Culture Collection; C, CEREMIC; Amph B, Amphotericin B; YFE, Yellow Farina Extract; WFE, White Farina Extract; I, inactive (MIC or MFC > 250 μg/mL).

been reported for them either, and, therefore, no absolute configurations have been proposed using this method. Based on the mentioned aspects, we consider that the present investigations open new perspectives in these fields of research.

Experimental

Plant Material

Fronds of *P trifoliata* with white and yellow farina were collected separately in Tucumán, Argentina, in March 2011 (GPS data: 902 masl; 27°08′39″ South; 65°45′10″ West). The species has been identified by Dr Marcela A. Hernández and a voucher specimen (LIL 612885 yellow and 612863 white) was deposited at the Herbarium of the Fundación Miguel Lillo, Tucumán, Argentina.

Extraction and Isolation

Fresh fronds with yellow farina (980 g) were rinsed with EtOAc and the extract was then filtered and evaporated to dryness (6.93 g). Part of the extract (4 g) was subjected to CC (SiO_2; hexane and increasing amounts of EtOAc) to afford 12 fractions. Fr. 2 (107.5 mg) was chromatographed by HPLC (SiO_2; hexane/EtOAc/HAc 88:12:1, 2.0 mL/min) to yield 50.5 mg of **8** (t_R 17 min) and 5.3 mg of **9** (t_R 26 min). HPLC (SiO_2; hexane/EtOAc/HAc 88:12:1, 2.0 mL/min) of Fr. 4 (108.5 mg) gave **8** (55.3 mg, t_R 17 min) and **6** (10 mg, t_R 27 min). Fr. 5 (405.3 mg) and Fr.6 (500 mg) contained the crystalline compound **4** as the major constituent that crystallized from the fractions. After recrystallization (hexane-EtOAc 1:9), 576 mg of **4** were obtained. After removal of the crystalline compound **4** from Fr. 5, the remaining material was separated by HPLC (MeOH/H_2O/HAc 4:1:1, 2.0 mL/min) to give **4** (75.5 mg, t_R 14 min), **6** (11.5 mg, t_R 17 min), and **8** (41.1 mg, t_R 19 min). HPLC (SiO_2; hexane/EtOAc/HAc 85:15:1, 2.0 mL/min) of Fr.6, after removal of **4** (crystalline), gave **5** (6.6 mg, t_R 48 min) and **2** (10 mg, t_R 52 min). HPLC (SiO_2; hexane/EtOAc/HAc 4:1:1, 2.0 mL/min) of Fr. 7 (200 mg) afforded **8** (86.4 mg, t_R 18 min) and **1** (2.9 mg, t_R 38 min). Fr. 8 (124.1 mg) was submitted to HPLC (SiO_2; hexane/EtOAc/HAc 3:2:1, 2.0 mL/min) to give **1** (3.8 mg, t_R 19 min), **3** (11.8 mg, t_R 27 min), and **7** (9.7 mg, t_R 35 min). Fresh fronds of white farina (952 g) were rinsed with EtOAc and the extract was filtered and evaporated to dryness (5.43 g). Part of the extract (4 g) was subjected to CC (SiO_2; hexane and increasing amounts of EtOAc) to afford 8 fractions. A portion of Fr. 5 (200 mg) was chromatographed by HPLC (SiO_2 column; hexane/EtOAc/HAc 85:15:1; flow rate 2.0 mL/min) to yield 147 mg of **6** (t_R 19 min), 5 mg of **10** (t_R 26 min), and a mixture further purified by RP-HPLC (MeOH/H_2O/HAc 4:1:1; flow rate 2.0 mL/ min) to give 11.3 mg of **5** (t_R 24 min) and 5.2 mg of **11** (t_R 35 min). Fr 6 (52.7 mg) was submitted to RP-HPLC (MeOH/H_2O/HAc 7:3:1, 2.0 mL/min) to give **5** (5.4 mg, t_R 35 min) and **6** (17 mg, t_R 39 min). RP- HPLC (MeOH/H_2O/HAc 7:3:1, 2.0 mL/min) of Fr. 7 gave **7** (5.3 mg, t_R 18 min).

Structural Characterization of Trifoliatol

Trifoliatol (**1**). White amorphous solid. UV (MeOH): λ_{max} 340, 281. IR: $?_{max}$ 3528 (OH), 1780 (C = O), 1631 (C = O) cm^{-1}. ^1H NMR data (500 MHz, acetone-d_6) and ^{13}C NMR data (125 MHz, acetone-d_6): Table 1. HR-EI-MS: *m*/*z* 402.1102 (calc. for $C_{24}H_{18}O_6$, 402.1103).

Figure 6. *Pityrogramma trifoliata* frond abaxial view (A, B). Aspect of the abaxial frond with farina and sporangia covering the surface, observed in a stereoscopic microscope (C,D).

General Experimental Procedures

Thin layer chromatography: pre-coated silica gel plates (Kiesegel 60 F_{254}; *Merck*); visualized by UV light (254 and 365 nm) and by spraying with Godin reagent followed by heating at 120 °C. Column chromatography (CC): silica gel (230-400 mesh). HPLC: Knauer System, pump S1000 and RI detector S-2400, *Luna* C_8 and C_{18} columns (10 × 250 mm i.d., 5 μm) and Develosil 60-5 column (10 × 250 mm i.d., 5 μm ChemcoPak). IR spectra: Bruker Vektor 22 FTIR spectrometer in an ATR mode. NMR spectra: Bruker Avance 500 spectrometer at 500 MHz (^{1}H) and 125.6 MHz (^{13}C) in acetone-d_6; δ in ppm with the solvent signal as internal reference, J in Hz. High-resolution mass spectra were measured using a Finnigan MAT 95 spectrometer. ECD spectra were registered on a JASCO J-815 spectropolarimeter, using EtOH as solvent.

Single-crystal x-ray Diffraction

Single crystals of **12** were obtained after slow evaporation of the solvent at c.a. 10 °C of a dichloroethane solution. A suitable crystal was then mounted in a loop with radiation of Mo during 20 h. Single-crystal x-ray diffraction data were collected

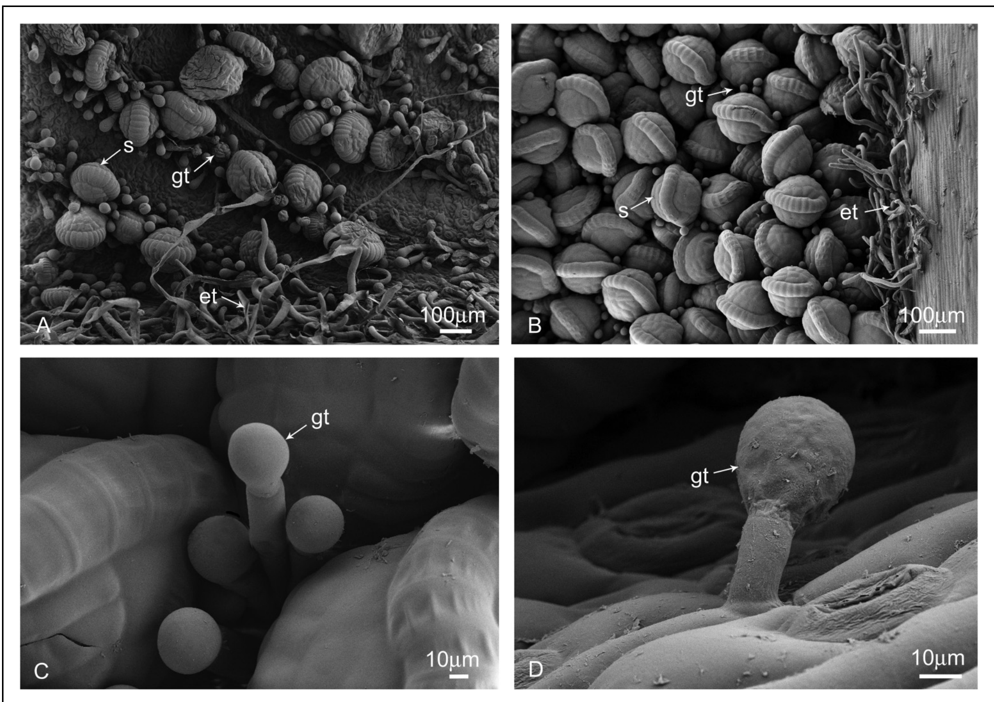

Figure 7. Scanning electron micrographs showing distribution pattern and trichome types in *Pityrogramma trifoliata*. (A): Frond abaxial surface of *P trifoliata* yellow farina and (B): white farina. (C): Glandular trichome with a long 2 celled stalk. (D): Glandular trichome with a single cell basal short stalk. s: Sporangia; et: Eglandular trichomes; gt: Glandular trichomes. Scale bar (A, B) = 100 µm; (C, D) = 10 µm.

at 298 °K using a Gemini E diffractometer (Oxford Diffraction). Data collection strategy and data reduction were performed with standard procedures implemented in CrysAlisPro software.[27] The crystal structure was solved by direct methods, as previously described.[28–30] All H atoms were placed in idealized positions and refined in riding modes such that Uiso(H) = 0.06Ueq(parent). Regarding the absolute configuration results, although the value of the Flack parameter x is −0.2(5), based on the Hooft y of 0.0(4) and the value P2(true) of 0.931, it is considered that these statistics validate the proposed structure. Due to experimental limitations it was impossible to collect the data using Cu radiation. CCDC-1867751 contains the Supplementary crystallographic data for this work.

Pd/C-Catalyzed Chemoselective Hydrogenation

Compound **2** (3.6 mg) was reduced at room temperature in ethanol with Pd/C under a hydrogen atmosphere to yield a reduced substance (3.9 mg) whose spectroscopy and mass spectrometry data are in agreement with those previously reported for compound **10**.

Antifungal Activity Assays

For the antifungal evaluation, strains from the American Type Culture Collection (ATCC) and from Centro de Referencia en Micología, CEREMIC (C), were used: *Candida albicans* ATCC10231, *Cryptococcus neoformans* ATCC32264, *Trichophyton rubrum* C110, *Trichophyton mentagrophytes* ATCC9972, and *Microsporum gypseum* C115. Strains were grown on Sabouraud-Chloramphenicol-Agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-Dextrose-Agar (SDA, Oxoid) and sub-cultured every 15 days to prevent pleomorphic transformations. Inocula of cell or spore suspensions were obtained and quantified following reported procedures (CLSI).[23, 24] Amphotericin B (Sigma), Terbinafine (Novartis), and Ketoconazole (Sigma) were used as positive controls. MICs and MFCs were determined as previously described.[31] MIC represents fungistatic activity of the sample tested and MFC indicates its fungicide capacity.

Scanning Electron Microscopy and Histochemical Assays

Measurements of trichomes were calculated for n = 100 of each type of trichome. Electron microscopy studies were performed as previously described.[26]

For histochemical assays, fresh frond material was free hand sectioned and stained using the following reagents for flavonoids: Benedict reagent, aluminum trichloride, and Vanillin/HCl.[32] Observations were made as previously reported.[26]

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Figure 8. Micrographs of glandular trichomes (A-F). Fluorescence micrographs under UV light (A, B, D, E) showing the response of glandular trichomes of *Pityrogramma trifoliata* fronds with yellow farina (A-C) and with white farina (D-E) to histochemical tests. (A, D) Fluorescence with Benedict reagent for flavonoids. (B, E) Intense green fluorescence of the head cell stained by aluminum trichloride. Light micrographs (C, F) Vainillin/HCl test showing cherry-red staining of flavonoids. **sc**, subcuticular chamber; **nc**, neck cell; **s**, stalk. Scale bar: A-C = 40 µm; D-F = 30 µm.

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Supplemental Material

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