A Novel Class of Complex Flavonoids from the Frond Exudate of *Pityrogramma trifoliata*

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Fronds of the neotropical fern *Pityrogramma trifoliata* produce a farinose exudate which consists mainly of a dihydrochalcone. The structure of some minor constituents has been elucidated by spectroscopic methods. They are shown to be representatives of a novel class of complex flavonoids, composed of a flavonoid and a neoflavonoid moiety. In other terms, they are 5,7-dihydroxy-8-cinnamoyl-4-phenyl-dihydrocoumarins. The occurence of these and closely related compounds in this fern's exudate supports its inclusion in the genus *Pityrogramma*.

Introduction

Pityrogramma trifoliata (L.) Tryon is a tropical American fern of wide geographical distribution (Mexico to temperate South America and the Carribean Islands) and ample amplitudinal range, being found from sea level up to 2000 m elevation. It grows preferably in marshy areas with low herbaceous vegetation such as road ditches, river banks and open fields, fully exposed to sunlight. Like most other sun ferns, it is resistant to long periods of desiccation. While other species suffer from greater herbivore pressure during such a period of stress, it shows no signs of being subject to predatory activity (as is also the case of other *Pityrogramma* species). *P. trifoliata* thrives in lixiviated lateritic soils and in sandy alluvial deposits, and is commonly associated with *Nephrolepis*, *Equisetum*, and *Selaginella*, as well as with other members of the genus, such as *P. tartarea*, *P. ferruginea*, *P. calomelanos*. With the latter two species it crosses to produce hybrids which, like *P. trifoliata*, form clonal populations by means of stolons.

P. trifoliata with its usually 1- to 4-foliate pinnae (*cf.* [1]) is rather different in its frond morphology from the other species. Therefore it is treated by some authors as *Trismeria trifoliata* (L.) Diels. The plants are usually large – fronds measuring up to 2 m in length – and vary in general habit according to the presence or absence of fertile fronds. Produced between August and December, fertile fronds are longer than the sterile fronds and have the upper pinnae somewhat contracted and covered on the dorsal surface by a farinose indument, thus producing a slight case of leaf dimorphism.

In the scope of investigations on flavonoid exudates in gymnogrammoid ferns (see [2]), the farina of this species has been studied for its chemical composition. As has been shown already elsewhere [3], the major constituent in most samples seen is 2′,6′-diOH,4′-OMe dihydrochalcone, sometimes accompanied by varying amounts of 2′,6′-diOH, 4′,4-diOMe dihydrochalcone. These flavonoids usually form white farinas [2]. The indument of *P. trifoliata*, however, often appears slightly yellowish. This tint is due to the presence of minor constituents, provisionally called **T-1**, **T-2**, and **T-3**. From bulk material of population collections we have succeeded in isolating **T-1** and **T-2** in sufficient amounts for

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structural analysis by spectroscopic methods. In the present paper we report on the structural identification of these compounds, which are representatives of a novel class of flavonoids.

Materials and Methods

Farinose pinnae of fertile fronds of *Pityrogramma trifoliata* were collected in their natural habitat in Costa Rica in 1977 and thoroughly air-dried. Vouchers are kept at the Museo National de Costa Rica at San José (CR 6783 – 6785 and 6792). The dry material was rinsed with acetone to dissolve the farinose exudate and the solution was taken to dryness under reduced pressure. The yellow solid material was treated with boiling benzene which dissolved most of it. From the concentrated solution thus obtained a good deal of the dihydrochalcone crystallized on cooling. The remaining solution was chromatographed over several columns with polyamide (Polyamid SC-6, Macherey & Nagel, Düren) by elution with toluene and increasing quantities of methyl-ethyl-ketone and methanol. Compound **T-1** was obtained in several fractions together with traces of other flavonoids. These fractions were combined and taken to dryness. **T-1** could be purified by crystallization from boiling ethanol to form deep yellow needles, m. p. 207 – 208 °C.

T-2 was present mostly in the material that was not soluble in benzene, and also in some fractions of the previous procedure. These samples were combined and dried onto silica gel (Kieselgel N, Macherey & Nagel). This was placed on top of a column packed with silica gel and eluted as described above. Those fractions containing **T-2** were combined and dried again, then redissolved in methanol and further purified by passing over Sephadex LH-20 (Pharmacia). The material thus obtained was dissolved in boiling acetone and toluene was added. After evaporation of the acetone and cooling to room temperature, **T-2** precipitated as an orange-yellow crystalline powder, m. p. 172 °C.

Fractions containing trace amounts of **T-3** were combined and chromatographed over polyamide once more, yielding a still rather unpure product, which was subjected to preparative TLC on silica gel (SIL-GUR 25, Macherey & Nagel; solvent: toluene/acetone/ethanol 9 : 1 : 2, two runs) and finally on polyamide (solvent: toluene/methylethyl-ketone/methanol 60 : 15 : 15). Because of the very small amount obtained, **T-3** could not be crystallized; the material was just sufficient for running UV spectra and MS. An attempt to purify further a minute amount by a passage over Sephadex LH-20 led to complete loss of the sample.

T-1 and **T-2** were reduced at room temperature in ethanol with Pd/C catalysator. Cyclisation was performed by boiling the solution of the substances in aqueous methanol with HCl under reflux for 2 h. Silylation was done by action of BSTFA (+ 1% TMCS) in pyridine.

Results and Discussion

The substances under investigation exhibit the chromatographic properties shown in Table I together with those of 2′,6′-diOH,4′-OMe dihydrochalcone, the major constituent.

Compound **T-1** is the most important of these minor farina components, but **T-3** produces the most remarkable spot on chromatograms because of its intense red colour in UV-light after spraying with "Naturstoffreagenz A". It can thus seem to indicate a reasonable amount of material, whilst in reality only minute amounts are present.

The UV-spectral data of these new compounds as well as those of some known chalcones are presented in Table II. MS and PMR data are listened in Table III.

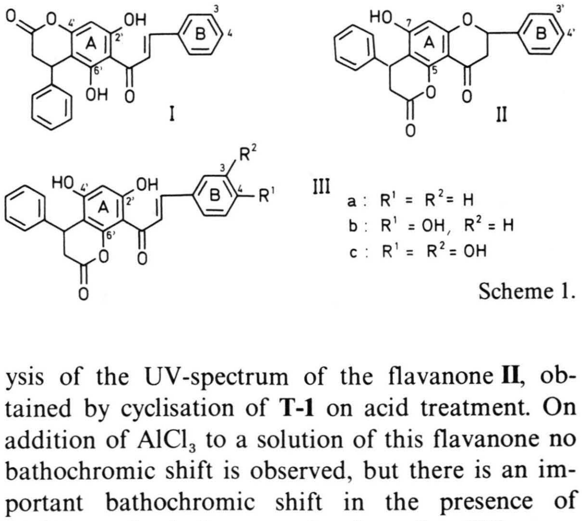
T-1 exhibits the typical UV-spectrum of a chalcone. M^+ at *m/e* 386 and M^+ of its TMSi-derivative at *m/e* 530 indicate the molecular formula

Table I. Chromatographic properties of the **T**-compounds on polyamide-TLC (solvent: toluene/petrol/methylethyl ketone/methanol 60 : 25 : 10 : 5).

Substance	$R_F \times 100$	Colour in UV_{366}	
		before spraying	after 'Nat. A'
T-1	47	dark brownish	dark brown → red
T-2	12	dark brown	orange-yellow
T-3	6	dark	brilliant red
2′,6′-diOH, 4′-OMe-dihydrochalcone	43	faint brown	dull yellow

Table II. UV-spectra (λ_{max} [nm]).

				in MeOH	$+AlCl_3$	$+AlCl_3$ $+HCl$	+NaOAc	+NaOH
new compounds:								
T-1				333	362, (332), (320), (290)	362, (332), (320), (284)	390, (298), 284	390
T-1 cycl.				340, 292	346, 296	348, 295	334, (300)	336
T-1 red.				(324), 286, 232	365, 312, 234	366, 310, 234	336, (260)	
A				(324), 285 236	370, 312 236	368, 310 236	336, 260	
T-2				374, 288	434, (408), 342, 326, 282	426, (400), 340, 324, 280	400, 342, 283	440, 338
T-2 cycl.				(336), 292	(344), 292	(344), 292	334, (286), 260	333
T-3				393, 275	412, 330 277		407, 270	
chalcones:								
2′	4′	6′	4					
OH	OCH_3		OCH_3	366, (306), (296), (260)	424, 380, 332, 318	422, 378, 332, 318	366	
OH	OCH_3	OCH_3		372, (318), 262	412, 336, 322, 272, 254	406, 336, 322, 272, 254	372	358
OH	OH	OCH_3		344, (292)	378, (330), (316), 287	374, (330), (316), 287	380, 306, 270	
OH	OCH_3	OH		340	375, (330), 316	370, (328), 318	324, 290	350, 288
OH	OCH_3	OH	OCH_3	362, (306), (294), (240)	402, (344), (326), (314), (250)	396, (340), (326), (314), (250)	362, (310), (284)	362, 284



Scheme 1.

$C_{24}H_{16}O_3(OH)_2$. The PMR spectrum shows an AB system ($J = 16$ Hz), which by chemical shifts and coupling constant is typical of chalcones. Interpretation of this spectrum and uncoupling experiments allow deduction of a fragment $-CO-CH_{2(a)}-\overset{|}{C}H_{(b)}-C_6H_5$. The presence in MS of the fragments m/e 104 ($C_6H_5-CH=CH_2^+$) and m/e 309 (M-77) as well as PMR signals (multiplets at $= 7.67$, 2 H and ca. 7.30 ppm) are in favour of an unsubstituted B-ring. The fragment cited hence must be linked to the A-ring. Since the chemical shift of $CH_{(b)}$ ($\delta = 4.67$ ppm), which clearly is linked with an aromatic ring, is incompatible with linkage via O, two possible structures can be considered. Possibility **I** was discarded after evaluation of the UV-spectra with classical reagents [4]. The important bathochromic shift in the presence of NaOAc is characteristic of a free OH-group at C-4′. This was corroborated by analysis of the UV-spectrum of the flavanone **II**, obtained by cyclisation of **T-1** on acid treatment. On addition of $AlCl_3$ to a solution of this flavanone no bathochromic shift is observed, but there is an important bathochromic shift in the presence of NaOAc, a classical reagent showing a free OH-group at C-7 and a blocked OH (or none) at C-5. Further-

Table III. MS-spectra and PMR-spectra of the new compounds.

	MS *m/e* (rel. int.) 70 eV	PMR (360 MHz, acetone D-6 in ppm/HMDS)
T-1	386 (M^+, 100; calc. 386.115, found 386.116), 385 (M – 1, 30), 358 (M – 28, 10; calc. 358.121, found 358.121), 343 (M – 43, 10), 309 (M – 77, 30), 282 (15), 254 (15), 239 (25), 226 (20), 205 (10), 197 (5), 184 (15), 131 (10), 104 (20)	8.08 1 H d (*J* 16 Hz) 7.75 1 H d (*J* 16 Hz) 7.67 2 H m 7.14 – 7.45 8 H m 6.27 1 H s 4.67 1 H dd (*J* 7.1 and 1.5 Hz) 3.35 1 H dd (*J* 16 Hz and 7.1 Hz) 3.08 1 H dd (*J* 16 and 1.5 Hz)
T-2	402 (M^+, 100; calc. 402.110, found 402.110), 401 (M–1, 30), 359 (M – 43, 5), 309 (M – 93, 10; calc. 309.076, found 309.076), 283 (30; calc. 283.060, found 283.060), 254 (10), 241 (10), 239 (10), 226 (10), 205 (10), 120 (30)	8.01 1 H d (*J* 16 Hz) 7.84 1 H d (*J* 16 Hz) 7.67 2 H d (*J* 8.5 Hz) 7.20 – 7.40 5 H m 6.97 2 H d (*J* 8.5 Hz) 6.36 1 H s 4.76 1 H s 3.34 1 H dd (*J* 16 Hz and 8 Hz) 3.10 1 H dd (*J* 16 Hz and ca. 2 Hz)
T-3	418 (M^+, 23), 417 (M – 1, 5), 367 (13), 323 (9), 309 (M – 109, 5), 294 (8), 283 (12), 268 (9), 256 (7), 238 (9), 210 (8), 196 (11), 183 (16), 156 (10), 140 (12), 136 (14), 109 (11), 99 (21), 83 (26), 41 (100)	

more, the flavanone **II** shows the same $\Delta\lambda_{\max}$ with NaOAc as does **T-1**. The structure thus deduced for **T-1** hence must be that shown by formula **III a**.

Hydrogenation of **T-1** in the presence of Pd/C yields the reduced substance which is identical with compound **A**, that was also isolated recently from the farina of a *Pityrogramma* species. This is obviously the corresponding dihydrochalcone. The structural elucidation of this compound and some analogues are reported elsewhere [5]. The structure of **A** has been corroborated by synthesis of its monomethyl ether. Hence the identity of reduced **T-1** with natural **A** underlines the correctness of the structure elaborated for **T-1**.

T-2 shows M^+ at *m/e* 402 and its TMSi-derivative at *m/e* 618, which leads to the molecular formula $C_{24}H_{15}O_3(OH)_3$. Its UV-spectrum is also characteristic of a chalcone and the PMR spectrum again indicates the fragment $-\mathrm{CO}-\overset{|}{\mathrm{C}}\mathrm{H}_2-\mathrm{CH}-\mathrm{C}_6\mathrm{H}_5$. However, one of the benzene rings in this compound must be *p*-disubstituted ($\delta = 7.97$, 2 H and 7.67 ppm; *J* 8.5 Hz). The presence of a fragmentation ion at *m/e* 309 ($M-C_6H_5-OH$) allows location of the *p*-OH-group at the B-ring (typical fragmentation of chalcones and flavanones). This result is confirmed by the important bathochromic shift in UV observed on addition of NaOEt. $\lambda_{\max\,I} + 66$ nm and increase of the band indicate a free OH-group at C-4′. Again, acid cyclisation of the chalcone **T-2** yields a flavanone, which as we argued for **T-1** demonstrates the presence of a free OH at C-7 of the flavanone, hence at C-4′ of the chalcone. The structure of **T-2** therefore corresponds to formula **III b**.

We are currently isolating a further farina constituent from *P. calomelanos*, which according to its chromatographic behaviour appears to be the dihydrochalcone derivative corresponding to the chalcone derivative **T-2**, thus forming a second pair of products like **T-1/A**.

For **T-3** the M^+ at *m/e* 418 points to the presence of still one more OH-group on the same basic molecule. Its UV-spectrum is also typical of a chalcone. The bathochromic shift of 22 nm on addition of $NaOAc/H_3BO_3$ and the presence of the fragment *m/e* 309 indicate ortho-di-OH substitution at the B-ring. However, because of lack of material no detailed analysis, especially no PMR spectrum, could be done. Hence the structure of this compound can be assigned only tentatively as **III c**.

Our compounds **T-1, T-2,** and **T-3** are composed of a chalcone molecule and of a dihydro-neoflavonoid molecule, linked via a benzene ring belonging to both molecules (ring A of the chalcone). In other terms, they can be called 5,7-dihydroxy-8-cinnamoyl-4-phenyl-dihydrocoumarins (or 5,7-diOH-8-cinnamoyl-4-phenyl-2-H-1-benzopyran-2-ones, *cf.* [5]). Their chromatographic and spectral behaviour, however, is dominated by the chalcone moiety. Therefore in our opinion they should be regarded as a novel class of natural flavonoids.

Three further compounds of this structural type were found as constituents of the farina of other *Pityrogramma* species. They are the dihydrochalcone **A**, the flavone $\mathbf{B}_1$, and the flavonol $\mathbf{B}_2$, corresponding to compound **T-1** in substitution [5]. Compound **A** and its expected homologue (mentioned above as corresponding to **T-2**) shows a colour behaviour typical of some dihydrochalcones and many flavanones: after spraying with "Naturstoffreagenz A" on polyamide thin layer they show light-blue fluorescence on irradiation with longwave UV-light. This observation again leads us to place these new natural compounds into the flavonoids.

The flavonoids **A** and $\mathbf{B}_{1/2}$ are abundant as minor constituents of the farina of *P. calomelanos* (L.) Link. They are also produced by *P. dealbata* (Presl.) Tryon, occasionally by *P. tartarea* (Cav.) Maxon, by most specimens seen of *P. trifoliata,* and by one sample of *P. chrysophylla* (Sw.) Link (*cf.* [3]; therein **A** is listed as **D-1**, and the chromatographically unseparable mixture of $\mathbf{B}_1$ with $\mathbf{B}_2$ is listed as **D-2**). **T-1** is a major farina constituent in the exudate of the endemic ferns of Jamaica, *P. sulphurea* (Sw.) Maxon and *P. williamsii* Proctor.

Because of its different frond morphology *P. trifoliata* had been seggregated by Fée [6] into the monotypic genus *Trismeria.* Tryon in his monograph on the genus *Pityrogramma* [7] transfers it to this genus. Our phytochemical results support this decision, since the novel complex flavonoids found in the farina of *P. trifoliata* (as well as in its hybrid with *P. calomelanos*) are typical for the genus *Pityrogramma.* On the other hand, studies of hybrid behaviour as well as of morphological characters (spore size) present evidence for the separation of *Trismeria* from *Pityrogramma* [8].

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