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Chromenes of polyketide origin from *Peperomia villipetiola*

Karina J. Malquichagua Salazar [a], Guillermo E. Delgado Paredes [b], Luis Ripalda Lluncor [b], Maria Claudia Max Young [c], Massuo Jorge Kato [a,*]

[a] *Instituto de Química, Universidade de São Paulo, CP 26077, 05508-900 São Paulo, SP, Brazil*
[b] *Facultad de Biología, Universidad Pedro Ruiz Gallo, Lambayeque, Peru*
[c] *Seção de Fisiologia e Bioquímica de Plantas, Instituto de Botânica, CP 4005, 01051 São Paulo, SP, Brazil*

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Abstract

An extract of leaves and stems of *Peperomia villipetiola* has been found to contain myristicin (3-methoxy-4,5-methylenedioxy-allylbenzene) and seven chromenes, whose structures are methyl 5-hydroxy-7-methyl-2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**1**), methyl 5-methoxy-7-methyl-2,2-dimethyl-2*H*-1-chromene-8-carboxylate (**2**), methyl 7-hydroxy-5-methyl-2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**3**), methyl 7-methoxy-5-methyl-2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**4**), 5-methanol-7-hydroxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid (**5**), 5-methanol-7-methoxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid (**6**), and methyl 5-acetoxymethanol-7-hydroxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylate (**7**). A biosynthetic rationale for **1**–**7** suggests that orsellinic acid may be a common intermediate. The anti-fungal activities of the chromenes were measured bioautographically against *Cladosporium cladosporioides* and *Cladosporium sphaerospermum*: compounds **6** and **7** were found to be the most active.

Keywords: *Peperomia villipetiola*; Piperaceae; Structural elucidation; Anti-fungal activity; Chromenes; Orsellinic acid

1. Introduction

The chemistry of the Piperaceae family is based on the occurrence of phenylpropanoids (Orjala et al., 1993), lignan/neolignans (Monache and Compagnone, 1996; Parmar et al., 1997; Benevides et al., 1999), pyrones (Singh, 1992), aliphatic and aromatic amides (Alécio et al., 1998; Silva et al., 2002), alkaloids (Dodson et al., 2000), polyketides (Cheng et al., 2003) and chromenes (Moreira et al., 1998; Baldoqui et al., 1999; Lago et al., 2004). Most of the studies reported to date have concerned species of the genus *Piper*. Only a few species of *Peperomia* have been subjected to systematic chemical investigation even though many members of this genus are to be found worldwide, particularly in collections of ornamentals.

Of the secondary metabolites that have been isolated from *Peperomia* species, the most noteworthy are the seco-compounds, e.g., the secolignans from *Peperomia glabella* (Monache and Compagnone, 1996) and *Peperomia dindigulensis* (Govindachari et al., 1998), and the cyclobutane compound from *Peperomia pellucida* which seems to be produced by dimerisation of styryl phenol (a seco-phenylpropanoid) (Bayma et al., 2000). Together with these, a number of acylphloroglucinol or phenolic compounds with long aliphatic chains have been isolated from *Peperomia clusiifolia* (Seeram et al., 1998), *Peperomia vulcanica* (Mbah et al., 2002), *Peperomia obtusifolia* (Tanaka et al., 1998), *Peperomia galiodes* (Mahiou et al., 1996) and *Peperomia proctorii* (Seeram et al., 2000).

* Corresponding author. Tel.: +55 11 3091 3813; fax: +55 11 3815 5579.
E-mail address: majokato@iq.usp.br (M.J. Kato).

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In the present work the isolation, structural elucidation and determination of the anti-fungal activities of seven new chromenes from *Peperomia villipetiola* are reported. The bioautographic assay used has been successfully employed in our laboratories over a number of years for screening anti-fungal compounds in Piperaceae species (Alécio et al., 1998; Baldoqui et al., 1999; Navickiene et al., 2000; Silva et al., 2002; Lago et al., 2004).

2. Results and discussion

The CH_2Cl_2:MeOH (2:1) extract of the aerial parts of *P. villipetiola* was fractionated by CC and TLC to yield myristicin (3-methoxy-4,5-methylenedioxy-allylbenzene) and compounds **1**–**7**. The basic skeleton of **1**–**7** was determined to be of the chromene type by virtue of a common set of ^{1}H NMR spectra (Table 1) that displayed an AB system at δ 5.52–5.69 (*d*, 10Hz) and 6.13–6.69 (*d*, 10Hz), and intense singlets at δ 1.39–1.46 (6H) characteristic of the 2,2-dimethyl-2*H*-1-chromene moiety. This structure was further confirmed by inspection of the UV, IR and ^{13}C NMR spectra (see Section 3 and Table 2). MS data, together with elemental analysis and NMR spectra, were employed to determine the molecular formula of each compound. Additionally, a fragment $[M-15]^+$, associated with the loss of a methyl radical and observed in all MS, provided complementary evidence of the identity of the chromene moiety (Diaz et al., 1987). A common feature in the NMR spectra of all compounds was a unique singlet for the aromatic hydrogen (δ 6.19–6.46, *s*, 1H) in the ^{1}H NMR spectra with a corresponding aromatic methine carbon (δ 98.1–111.9) in the ^{13}C NMR spectra. The three remaining substituents on the aromatic ring were determined to be hydroxyl for **1**, **3**, **5** and **7**, methoxyl for **2**, **4** and **6**, methyl for **1**–**4**, carboxyl for **5**–**6**, methyl carboxylate for **1**–**4** and **7**, methanol for **5** and **6**, and acetoxy-methanol for **7**. Assignments of the substituents in each case were achieved by interpretation of spectrometric data including IR, ^{1}H NMR and, especially, HMBC.

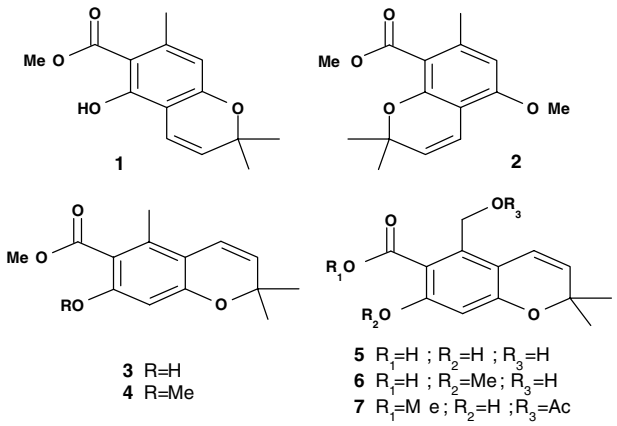
Table 1
^{1}H NMR spectroscopic data (300 MHz, $CDCl_3$) for chromenes **1**–**7** isolated from *Peperomia villipetiola*

Hydrogen	**1**	**2**	**3**	**4**	**5**	**6**	**7**
3	5.52 (1H, *d*, 10)	5.53 (1H, *d*, 10.0)	5.60 (1H, *d*, 10.0)	5.55 (1H, *d*, 10.0)	5.61 (1H, *d*, 10.0)	5.59 (1H, *d*, 10)	5.69 (1H, *d*, 10.0)
4	6.69 (1H, *d*, 10)	6.59 (1H, *d*, 10.0)	6.56 (1H, *d*, 10.0)	6.44 (1H, *d*, 10.0)	6.13 (1H, *d*, 10.0)	6.15 (1H, *d*, 10)	6.53 (1H, *d*, 10.0)
6		6.23 (1H, *s*)					
8	6.19 (1H, *s*)		6.29 (1H, *s*)	6.28 (1H, *s*)	6.33 (1H, *s*)	6.35 (1H, *s*)	6.46 (1H, *s*)
9	2.46 (3H, *s*)	2.29 (3H, *s*)	2.48 (3H, *s*)	2.20 (3H, *s*)	5.23 (2H, *s*)	5.15 (2H, *s*)	5.40 (2H, *s*)
10, 11	1.43 (6H, *s*)	1.39 (6H, *s*)	1.41 (1H, *s*)	1.40 (6H, *s*)	1.45 (6H, *s*)	1.46 (6H, *s*)	1.43 (6H, *s*)
2′	3.92 (3H, *s*)	3.87 (3H, *s*)	3.93 (3H, *s*)	3.89 (3H, *s*)			3.90 (3H, *s*)
1″		3.81 (3H, *s*)		3.77 (3H, *s*)		3.92 (3H, *s*)	
2″							2.07 (3H, *s*)
CH_2OH					7.62 (1H, *s*)		
Ar-OH	11.97 (1H, *s*)		11.39 (1H, *s*)				11.32 (1H, *s*)

Table 2
^{13}C NMR spectroscopic data[a] (75 MHz, $CDCl_3$) for chromenes **1**–**7** isolated from *Peperomia villipetiola*

Carbon	**1**	**2**	**3**	**4**	**5**	**6**	**7**
2	77.3 (C)	76.4 (C)	76.2 (C)	76.0 (C)	77.9 (C)	78.1 (C)	76.6 (C)
3	127.4 (CH)	128.6 (CH)	128.8 (CH)	128.3 (CH)	129.6 (CH)	129.1 (CH)	130.5 (CH)
4	116.4 (CH)	116.5 (CH)	119.1 (CH)	118.8 (CH)	116.4 (CH)	116.4 (CH)	118.1 (CH)
4a	107.3 (C)	108.6 (C)	113.9 (C)	113.1 (C)	108.5 (C)	107.6 (C)	115.4 (C)
5	159.8 (C)	155.7 (C)	137.4 (C)	132.3 (C)	142.8(C)	145.7 (C)	132.2 (C)
6	105.1 (C)	104.8 (CH)	106.4 (C)	117.1 (C)	103.8(C)	106.0 (C)	106.2 (C)
7	142.7 (C)	137.3 (C)	164.0 (C)	157.2 (C)	157.4 (C)	159.6 (C)	163.9 (C)
8	111.9 (CH)	116.0 (C)	102.9 (CH)	98.1 (CH)	103.7 (CH)	100.9 (CH)	105.6 (CH)
8a	157.5 (C)	151.6 (C)	158.9 (C)	155.2 (C)	160.9 (C)	160.0 (C)	158.9 (C)
9	24.4 (CH_3)	20.3 (CH_3)	17.6 (CH_3)	15.4 (CH_3)	69.2 (CH_2)	67.2 (CH_2)	60.2 (CH_2)
10, 11	28.3 (CH_3)	27.57 (CH_3)	28.01 (CH_3)	27.7 (CH_3)	28.26 (CH_3)	28.3 (CH_3)	28.0 (CH_3)
1′	172.4 (C)	168.44 (C)	172.16 (C)	169.0 (C)	172.3 (C)	168.8 (C)	171.0 (C)
2′	51.8 (C)	51.74 (CH_3)	51.9 (CH_3)	52.0 (CH_3)			52.3 (CH_3)
1″		55.6 (CH_3)		55.8 (CH_3)		56.1 (CH_3)	170.6 (C)
2″							20.7 (CH_3)

[a] Multiplicities (CH_n; n = 0–3) given for ^{13}C NMR/Dept 135° spectrum associated with HETCOR data (300 and 75 MHz, $CDCl_3$).



The EIMS spectrum of **1** showed a molecular ion peak $[M]^+$ at *m/z* 248 corresponding to the molecular formula $C_{14}H_{16}O_4$, which also matched the H and C atom counts in the respective NMR spectrum. The IR showed bands assignable to a hydroxyl group (3447 cm^{-1}, broad band) chelated to a conjugated carbonyl ester at 1644 cm^{-1}: the phenolic hydrogen was accordingly observed in the low field region of the ^1H NMR spectrum at δ 11.97 (1H, *brs*). The aromatic ring of **1** should thus possess an hydroxyl *ortho* to a carboxymethyl ester group. The dispositions of the other substituents on the aromatic ring were determined from the HMBC spectrum. The aromatic hydrogen, which correlated with C4a, C6, C8a and the methyl carbon (C9), was located at C8. The aromatic methyl group (C9) was located at C7 based on observed correlations with C8, C7 and C6. The signal of H4 was correlated with that of C5, at which position was located the hydroxyl group that in turn was correlated with carbon signals of C4a, C5 and C6. Hence chromene **1** was determined to be methyl 5-hydroxy-7-methyl-2,2-dimethyl-2*H*-1-chromene-6-carboxylate.

The molecular formula of **2** was established to be $C_{15}H_{18}O_4$ based on elemental analysis and EIMS data ($[M]^+$ at *m/z* 262). The IR spectrum showed bands corresponding to a conjugated ester group at 1729 cm^{-1}, but no hydroxyl stretching bands were observed. The substituents of the penta-substituted aromatic ring included one aromatic hydrogen at δ 6.23 (*s*, 1H), a methyl at δ 2.29 (*s*, 3H), a carboxymethyl at δ 3.87 (*s*, 3H) and a methoxyl group at δ 3.81 (3H). Important HMBC correlations were observed between H4 and C5 and C8a, and between H6 and C5, C4a, C9 and C8. Finally, the correlations between methyl hydrogens (H9) and C6 and C8 confirmed the location of all of the substituents in the aromatic ring. Chromene **2** was thus deduced to be methyl 5-methoxy-7-methyl-2,2-dimethyl-2*H*-1-chromene-8-carboxylate.

The $[M]^+$ peak (at *m/z* 248 from the EIMS spectrum) and the elemental analysis (corresponding to $C_{14}H_{16}O_4$) of **3** were the same as for **1**. The IR spectrum showed bands typical of a hydroxyl group (3442 cm^{-1}, broad band) chelated to a conjugated carbonyl group (1655 cm^{-1}). The ^1H NMR (Table 1) ^{13}C NMR and DEPT 135° (Table 2) spectroscopic data suggested that **3** was an isomer of **1** with the only difference, inferred from the HMBC spectrum (Table 3), being the reversed positions of the hydroxyl and the methyl groups. Thus it was concluded that chromene **3** was methyl 7-hydroxy-5-methyl-2,2-dimethyl-2*H*-1-chromene-6-carboxylate.

The molecular formula of **4** was established to be $C_{15}H_{18}O_4$ based on elemental analysis and the EIMS spectrum ($[M]^+$ at *m/z* 262). All spectrometric data indicated that **4** was either an isomer of **2** or a methyl derivative of **3**. Consideration of the HMBC data (Table 3) led to the placement of the aromatic hydrogen at C8, the methoxyl group at C7, the methyl ester function at C6, and the methyl group at C5. Chromene **4** was thus determined to be methyl 7-methoxy-5-methyl-2,2-dimethyl-2*H*-1-chromene-6-carboxylate.

Compound **5** had the molecular formula $C_{13}H_{14}O_5$ as indicated by elemental analysis and EIMS data ($[M]^+$ at *m/z* 250). Chelated hydroxyl groups gave rise to a set of IR absorption bands in the range 3600–2500 cm^{-1}, with the associated carbonyl band being observed at 1724 cm^{-1}. Two singlets in the ^1H NMR spectrum at δ 6.33 (1H) and 5.23 (2H) were assigned to the aromatic hydrogen and to the methylene group, respectively. The two hydrogen singlet was associated with the methylene carbon (δ 69.2; DEPT 135 and HETCOR) and assigned to C9. The HMBC correlations between the signal of H9 and the carbon signals of C5, C4a, C6 and C1′ (Table 3), and between the signal of the aromatic hydrogen and those of C4a, C8a, C7 and C6, led to the determination of chromene **5** as 7-hydroxy-5-methanol-2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid.

The molecular formula of **6** was established to be $C_{13}H_{14}O_5$ based on elemental analysis, EIMS data ($[M - H_2O]^+$ at *m/z* 246) and the H and C atom counts in the respective NMR spectra. The IR spectrum showed a broad band at 3444 cm^{-1} associated with an hydroxyl group, and an absorption at 1757 cm^{-1} typical of a carboxylic acid. As for **5**, a hydroxy methyl group (δ_H 5.15, 2H; δ_C 67.2) was present in **6**, the hydrogens of which were correlated with carbons signals of C4a, C5, and C6. The aromatic hydrogen was placed at C8 based on its correlations with C4a, C8a, C7 and C6, whilst the methoxyl group (δ_H 3.92; δ_C 56.11) was correlated with the signals of carbons C7, C6, and C-8 and was hence located at C7. Chromene **6** was thus deduced to be 5-methanol-7-methoxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylic acid, i.e., a methyl ether derivative of **5**.

The EIMS spectrum of **7** showed $[M]^+$ at *m/z* 360 corresponding to the molecular formula $C_{14}H_{16}O_4$, which was confirmed by elemental analysis. The IR spectrum showed a broad band at 3457 cm^{-1} typical

Table 3
HMBC data (500 and 125 MHz, δ ppm, $CDCl_3$) for chromenes **1–7** isolated from *Peperomia villipetiola*

Position (H → C)	1	2	3	4	5	6	7
3	C2, C4a, C10/11	C2, C4a, C10/11	C2, C4a, C10/11	C2, C4a, C10/11	C2, C4a, C10/11	C2, C4a, C10/11	C2, C4a, C10/11
4	C2, C5, C8a	C2, C5, C8a	C2, C4a, C5, C8a	C2, C4a, C5, C8a	C2, C4a, C5, C8a	C2, C4a, C5, C8a	C2, C4a, C5, C8a
6		C4a, C5, C8, C9					
8	C4a, C6, C8a, C9		C4a, C6, C7, C8a	C4a, C6, C7, C8a	C4a, C6, C7, C8a, C1′	C4a, C6, C7, C8a, C1′	C4a, C6, C7, C8a
9	C6, C7, C8	C6, C7, C8	C4a, C5, C6	C4a, C5, C6	C4a, C5, C6, C1′	C4a, C5, C6, C1′	C4a, C5, C6, C1′
10, 11	C2, C3	C2, C3	C2, C3	C2, C3	C2, C3	C2, C3	C2, C3
2′	C1′	C1′	C1′	C1′			C1′
1″		C5		C7		C7	
2″							C1″
Ar-O*H*	C4a, C5, C6		C6, C7, C8				C6, C7, C8

of a chelated hydroxyl group, a conjugated carbonyl group at 1662 cm^{-1} and, additionally, an ester carbonyl at 1739 cm^{-1}. The presence of an acetate group was clear from the NMR spectra (δ_H 2.07; δ_C 20.7 and 170.6), and its placement at the hydroxyl functionality was evident since the signals of the methylene hydrogens (H9) appeared deshielded at δ 5.40 as expected. The locations of the substituents on the aromatic ring were based on the HMBC spectrum (Table 3), which showed correlations similar to those of chromenes **5** and **6**. Thus chromene **7** was determined to be methyl 5-acetoxymethanol-7-hydroxy-2,2-dimethyl-2*H*-1-chromene-6-carboxylate. The natural occurrence of **7** was confirmed by the HPLC analysis of freshly prepared extracts of *P. villipetiola* obtained using solvents other than MeOH or EtOAC.

The anti-fungal activities of all seven chromenes were evaluated by bioautographic assay against *Cladosporium cladosporioides* and *Cladosporium sphaerospermun* (Table 4). In a preliminary assay, the compounds found to be most active at 100 μg were the chromenes **6** and **7**. Follow-up assays in the range 1–100 μg indicated that **6** was moderately active against *C. sphaerospermun* at 25 μg and strongly active against *C. cladosporioides* at 100 μg. In contrast, **7** was moderately active against both species at 1 μg, and highly active against *C. sphaerospermun* at 5 μg. It thus appears that the introduction of a lipophilic group (as in the methylation or acetylation/methylation of **5** to produce **6** and **7**, respectively) is an important structural requirement for anti-fungal activity. The biological role of the chromenes in *P. villipetiola* is not clear at this time despite their structural relationship with the precocenes (Proksch and Rodriguez, 1983) and their demonstrated anti-fungal activities against *Cladosporium* species.

Chromenes **1–7** isolated from *P. villipetiola* are rare examples of the occurrence of benzoic acid derivatives in members of the Piperaceae. The chromenes in *Piper* and those in *Peperomia* species are probably formed via two distinct biosynthetic routes. *Piper* chromenes possess no aromatic methyl group suggesting an origin from shikimate, whilst the polyketide route is clearly applicable for the chromenes from *Peperomia* species. Since orsellinic acid is commonly found in lichens (Mosbach, 1964; Yamazaki et al., 1965), an hypothesis of horizontal gene transfer, similar to that previously described with respect to mitochondrial *cox1* genes in *Peperomia polybotrya* (Adams et al., 1998), is a tempting possibility. The formation of a pyrone moiety (Fig. 1) might involve the action of a prenyltransferase to link the 3,3-dimethylallyl pyrophosphate as is the case for the precocenes (Vyas and Mulchandani, 1980). A possible lack of specificity could give rise to two distinct pathways in which orsellinic acid would be linked at position 3 or 5 producing chromenes **1/2** or **3–7** after ring closure.

Table 4
Anti-fungal activities against *Cladosporium sphaerospermum* and *C. cladosporioides* of leaves, stems and chromenes **1**–**7** from *Peperomia villipetiola*

Compound/sample	Amount (μg)	*C. sphaerospermum*		*C. cladosporioides*	
		R_f[a]	Anti-fungal activity[b]	R_f[a]	Anti-fungal activity[b]
1	100	0.84	*	0.84	*
2	100	0.83	*	0.83	*
3	100	–	i	–	i
4	100	0.83	*	0.83	*
5	100	0.78	*	0.78	*
6	100	0.76	**	0.76	***
7	100	0.85	***	0.85	***
Leaves	400	0.40/0.48	*/*	0.40/0.48	*/*
		0.61/0.76	***/***	0.61/076	***/***
Stems	400	0.61/0.71	**/**	0.40/0.48	*/*
				0.61/0.71	**/**

[a] R_f values of active components determined on layers eluted with $CHCl_3$:MeOH (9:1).
[b] *, weak; **, moderate; ***, strong; i, compound showed no activity.

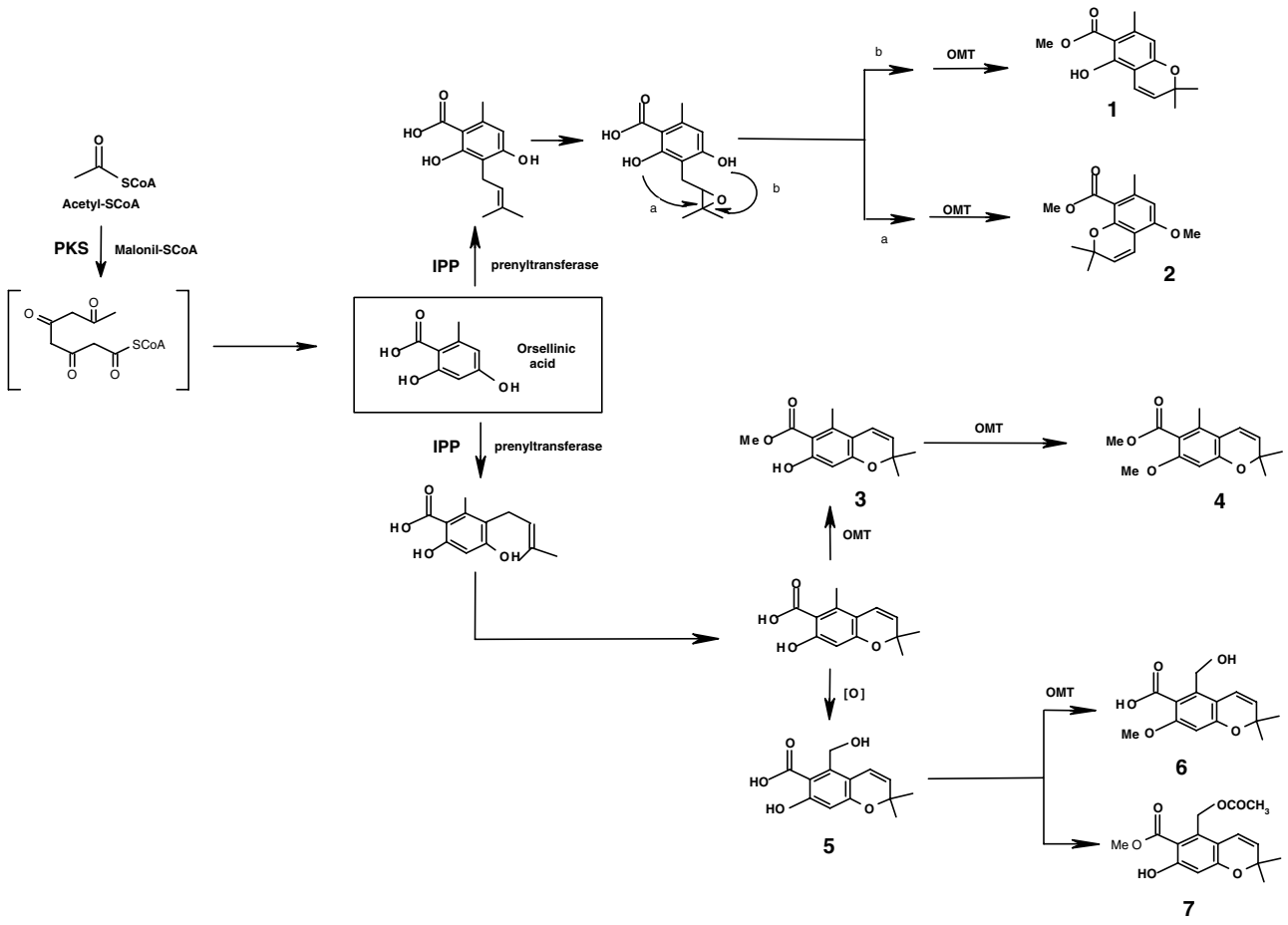


Fig. 1. Possible biogenetic route of chromenes isolated from *Peperomia villipetiola*. PKS: polyketide synthase; IPP: isopentenyl pyrophosphate; OMT: *O*-methyltransferase.

3. Experimental

3.1. General

IR spectra were recorded on a Bomen MB-100 spectrometer; ^{1}H NMR (200, 300 and 500 MHz), ^{13}C NMR (50 MHz), DEPT (75 MHz), COSY (75 MHz), HETCOR (75 MHz) and HMBC (125 MHz) spectra were measured in $CDCl_3$ on Bruker AC200E, DPX300 and DRX 500 instruments; EIMS (70 eV) spectra were determined on a Shimadzu QP5050 spectrometer. Silica gel (230–400 mesh) from Merck was used in all CC

separations; TLC layers were of silica gel 60 F_{254} or 60 PF_{254}; all solvents were redistilled prior to use.

3.2. Plant material

Complete specimens of *P. villipetiola* were collected from Chota-Cajamarca (Peru) in March of 2001. Plants were identified by botanist Santos Llatas Quiroz at the Herbarium of the Faculty of Biology, Universidad Pedro Ruiz Gallo (Lambayeque, Peru) and voucher specimens (catalogue numbers 12146 and 12147) are deposited therein.

3.3. Extraction and isolation

Dried and powdered aerial parts (leaves and stems; 5.90 g) of plants were extracted with CH_2Cl_2:MeOH (2:1; 3×50 mL) using a Turrax mixer (1900 rpm; 2 min; room temperature). The combined extracts were concentrated under vacuum and the resulting crude material (0.7 g) fractioned by CC employing gradient elution with hexane:EtOAc from 9.5:0.5 to 0:1. The 29 collected fractions were pooled on the basis of TLC analysis producing F_1–F_8. F_2 (220 mg) was subjected to prep. TLC (hexane:EtOAc; 9:1) to yield **4** (10 mg), **2** (28 mg), **1** (55 mg), **3** (103 mg) and myristicin (4 mg). F_3 (64 mg) was subjected to prep. TLC (hexane:EtOAc; 4:1) to yield **4** (18 mg) and **7** (31 mg). F_6 (17 mg) was subjected to prep. TLC (hexane:EtOAc; 7:3) to yield **6** (12 mg). F_7 (156 mg) and F_8 (81 mg) were subjected to prep. TLC (hexane:EtOAc; 3:2) to yield, respectively, **5** (106 mg), and **5** (21 mg) and **6** (15 mg).

3.4. Methyl 5-hydroxy-7-methyl-2,2-dimethyl-2H-1-chromene-6-carboxylate (*1*)

Amorphous solid. Elemental analysis: found C 67.20, H 6.83, O 25.97%; $C_{14}H_{16}O_4$ requires C 67.74, H 6.45, O 25.81%. IR ν_{max}^{film}: 3447, 3057, 2964, 2934, 2855, 1644, 1617, 1562, 1442 cm^{-1}. EIMS *m/z* (rel. int.): 248 $[M]^+$ (13), 233 $[M-15]^+$ (17), 201(100), 173 (9), 145 (5), 115 (12), 91(8), 77 (7). ^1H NMR (Table 1); ^{13}C NMR (Table 2); HMBC (Table 3).

3.5. Methyl 5-methoxy-7-methyl-2,2-dimethyl-2H-1-chromene-8-carboxylate (*2*)

Oil. Elemental analysis: found C 68.55, H 6.98; O 24.47%; $C_{15}H_{18}O_4$ requires C 68.70, H 6.7, O 24.3%. IR ν_{max}^{film}: 3050, 2975, 2948, 2850, 1729, 1634, 1602, 1577, 1462, 1441 cm^{-1}. EIMS *m/z* (rel. int.): 262 $[M]^+$ (13), 247 $[M-15]^+$ (100), 231 (11), 215 (15), 201 (8), 187 (6), 173 (3), 145 (3), 115 (7), 91(4), 77 (4). ^1H NMR (Table 1); ^{13}C NMR (Table 2); HMBC (Table 3).

3.6. Methyl 7-hydroxy-5-methyl-2,2-dimethyl-2H-1-chromene-6-carboxylate (*3*)

Amorphous solid. Elemental analysis: found C 67.66, H 6.51, O 25.83%; $C_{14}H_{16}O_4$ requires C 67.74, H 6.45, O 25.81%. IR ν_{max}^{film}: 3442, 3038, 2972, 2929, 2871, 1655, 1590, 1467, 1441 cm^{-1}. EIMS *m/z* (rel. int.): 248 $[M]^+$ (19), 233 $[M-15]^+$ (45), 201 (100), 173 (6), 145 (20), 115 (17), 91(12), 77 (9), 69 (10). ^1H NMR (Table 1); ^{13}C NMR, DEPT 135°, HETCOR (Table 2); HMBC (Table 3).

3.7. Methyl 7-methoxy-5-methyl-2,2-dimethyl-2H-1-chromene-6-carboxylate (*4*)

Oil. Elemental analysis: found C 68.62, H 6.92, O 24.46%; $C_{15}H_{18}O_4$ requires C 68.70, H 6.87, O 24.43%. IR ν_{max}^{film}: 3046, 2970, 2952, 2927, 2849, 1731, 1600, 1576, 1478, 1467, 1436 cm^{-1}. EIMS *m/z* (rel. int.): 262 $[M]^+$ (36), 247 $[M-15]^+$ (100), 231 (22), 215 (25), 201 (8), 187 (6), 173 (5), 159 (7), 145 (8), 115 (17), 91(9), 77 (9). 69 (7). ^1H NMR (Table 1); ^{13}C NMR (Table 2); HMBC (Table 3).

3.8. 5-Methanol-7-hydroxy-2,2-dimethyl-2H-1-chromene-6-carboxylic acid (*5*)

Amorphous solid. Elemental analysis: found C 62.02, H 5.86, O 32.12%; $C_{13}H_{14}O_5$ requires C 62.40, H 5.60, O 32.00%. IR ν_{max}^{film}: 3384, 2980, 2886, 2757, 2629, 1724, 1633, 1600, 1493 cm^{-1}. EIMS *m/z* (rel. int.): 232 $[M-H_2O]^+$ (13), 217 $[M-H_2O-CH_3]^+$ (100), 189 (11), 160 (12), 94 (12), 91 (3), 77 (5), 69 (4) ^1H NMR (Table 1); ^{13}C NMR, DEPT 135°, HETCOR (Table 2); HMBC (Table 3).

3.9. 5-Methanol-7-methoxy-2,2-dimethyl-2H-1-chromene-6-carboxylic acid (*6*)

Amorphous solid. Elemental analysis: found C 63.7, H 6.08, O 30.35%; $C_{14}H_{16}O_5$ requires C 63.64, H 6.06, O 30.30%. IR ν_{max}^{film}: 3444, 3089, 2973, 2928, 2853, 1757, 1624, 1588, 1498, 1436 cm^{-1}. EIMS *m/z* (rel. int.): 246 $[M-H_2O]^+$ (13), 231 $[M-H_2O-CH_3]^+$ (100), 203 (13), 173 (8), 145 (11), 144 (7), 115 (8), 91 (3), 77 (5), 69 (3). ^1H NMR (Table 1); ^{13}C NMR, DEPT 135°, HETCOR (Table 2); HMBC (Table 3).

3.10. Methyl 5-acetoxymethanol-7-hydroxy-2,2-dimethyl-2H-1-chromene-6-carboxylate (*7*)

Amorphous solid. Elemental analysis: found C 62.69, H 5.75, O 31.56%; $C_{16}H_{18}O_6$ requires C 62.75, H 5.88, O 31.37%. IR ν_{max}^{film}: 3457, 3058, 2976, 2956, 2930, 2856,

1739, 1662, 1600, 1586, 1469, 1443 cm^{-1}. EIMS *m/z* (rel. int.): 306 $[M]^+$ (55)], 291$[M-15]^+$ (86), 262 (16), 247 (11), 231 (12), 217 (100), 189 (21), 187 (13), 177 (13), 160 (20), 115 (16), 91 (14), 77 (17), 69 (26). ^1H NMR (Table 1); ^{13}C NMR (Table 2); HMBC (Table 3).

3.11. Determination of anti-fungal activity

Cultures of *C. cladosporioides* (Fresen) de Vries SPC 140 and *C. sphaerospermum* (Perzig) SPC 491 have been maintained at the Instituto de Botânica, São Paulo, SP, Brazil. Anti-fungal assays were carried out according to the procedure described (Homans and Fuchs, 1970). In this assay, 10 μL aliquots of solutions containing either 100 μg of crude extracts or semi-purified fractions, or 50, 10, 5, 1, 0.5 and 0.1 μg of pure compounds, were applied to pre-coated silica gel TLC plates. The layers were developed with hexane:EtOAc (7:3), dried to remove solvent, and sprayed with a spore suspension of *C. cladosporioides* or *C. sphaerospermum* in glucose and salt solution. Plates were incubated for 72 h in a moistened chamber at 25 °C in the dark. Inhibition of fungal growth appeared as clear zones against a dark background from which the minimum amount of compounds **1**–**7** required for inhibition could be determined (Table 4). Nystatin and miconazole were used as positive controls whereas ampicillin and chloramphenicol were used as negative controls.

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