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SYNTHESIS AND INHIBITORY ACTIVITY OF 7-GERANOXYCOUMARIN AGAINST *PENICILLIUM* SPECIES IN *CITRUS* FRUIT

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Key Word Index—*Citrus paradisi*; plutaceae; grapefruit; 7-geranoxycoumarin; synethesis; *Penicillium italicum* and *P. digitatum*.

Abstract—A naturally occurring compound with strong antifungal activity was isolated from the flavedo tissue of "Star Ruby" grapefruits (*Citrus paradisi*) and identified as 7-geranoxycoumarin. A high yield strategy for its synthesis was found and its antifungal activity against *Penicillium italicum* and *P. digitatum* assessed by *in vivo* and *in vitro* test.

INTRODUCTION

Much attention has been paid to preformed and induced antifungal compounds of the flavedo tissue of *Citrus* fruits [1–4]. Among induced compounds or phytoalexins, scoparone (6,7-dimethoxycoumarin) and scopoletin (6 hydroxy-7 methoxycoumarin) are known to have inhibitory activity against microorganisms. Preformed compounds include: terpenes, such as citral (3,7-dimethyl-2,6-octadiennal), coumarins, such as limettin (5,7-dimethoxycoumarin) and 5-geranoxy-7-methoxycoumarin, and furanocoumarins, such as isopimpinellin (5,8-dimethoxypsoralen). Some of these compounds were shown to be effective *in vitro* and citral, in particular, is known to have potent antifungal properties [5] and it is widely used in food processing [6]. However, because of its phytotoxicity, it is not likely to be viable for use *in vivo* on fresh fruit and vegetables [5]. The fruit defence system is known to depend upon a number of factors [7], including growth stage, and is known to decline rapidly after harvesting [8]. Moreover, this decline has been associated with a decrease in the antifungal activity of flavedo tissue [9].

Preliminary studies in our laboratory showed the occurrence of an unidentified preformed compound in flavedo tissue of "Star Ruby" grapefruits with high fungicidal activity. Indeed, this compound was found to remain unchanged even after long-term storage (at least 3 months at 8°). In the present investigation, we have isolated and characterized this naturally occurring compound and elucidated its structure. We have also developed a procedure to synthesize the compound in order to compare the properties of the natural and synthetic substance. The inhibitory activity of the synthetic compound against *Penicillium italicum* and *P. digitatum* was also evaluated by *in vivo* and *in vitro* tests.

RESULTS AND DISCUSSION

In a previous investigation, Ben-Yehoshua *et al.* [8] isolated several preformed antifungal compounds from flavedo tissue of pumelo fruit, some of which were reported as coumarin derivatives, including 7-geranoxycoumarin. However, no chemical evidence was reported in this paper on the chemical structures of these naturally occurring compounds. Coumarin derivatives have been previously isolated and characterized from juice of *C. hassaku* [10].

In the present study we isolated a preformed compound from flavedo tissue of "Star Ruby" grapefruits. The identity of this product as 7-geranoxycoumarin was proved by GC-mass spectrometry, GC-FTIR, UV and NMR analyses. In order to have sufficient amounts of compound for antifungal tests and to prove its identification, a method was developed to

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Table 1. ED_{50} of 7-geranoxycoumarin, scoparone and scopoletin against *Penicillium italicum* and *P. digitatum*

Compound	ED_{50} (μg ml^{-1})	
	P. italicum	*P. digitatum*
7-Geranoxycoumarin	43	57
Scoparone	55	67
Scopoletin	36	60

synthesize 7-geranoxycoumarin, for which geranoxyl bromide and 7-hydroxycoumarin were used. The chemical structure of the synthesized compound was identical to that of isolated substance.

Tests in vitro

ED_{50} values for 7-geranoxycoumarin with respect to scoparone and scopoletin are shown in Table 1. Inhibitory activity of tested coumarins against *P. italicum* was consistently higher than *P. digitatum*. 7-geranoxycoumarin had the highest inhibitory activity against *P. digitatum* and an intermediate activity against *P. italicum* in comparison with scoparon and scopoletin. The agar diffusion bioassay (Table 2) showed that inhibitory activity of tested coumarins against *P. italicum* was consistently higher than that against *P. digitatum*. When applied at 50 mg l^{-1}, scoparone was more effective than scopoletin and 7-geranoxycoumarin in inhibiting *P. italicum* growth, whilst a higher activity of scoparone against *P. digitatum* and comparable activities against *P. italicum* were measured at 100 mg l^{-1}. Both pathogens showed germ tube elongation which was inversely correlated ($R^2 = 0.929$, $P \leqslant 0.01$ for *P. italicum* and $R^2 = 0.918$, $P \leqslant 0.01$ for *P. digitatum*) with 7-geranoxycoumarin concentration.

Tests in vivo

Active infections of *P. italicum* in "Star Ruby" grapefruits were severely affected by treatments with coumarins which, even at 50 mg l^{-1}, gave exceptional control of the pathogen, i.e., ca 95% inhibition of decay development by scoparone and scopoletin and over 85% by 7-geranoxycoumarin (Fig. 1a). Treatments at 200 mg l^{-1} reduced decay by 50% in comparison with treatments at 50 mg l^{-1}. Scoparone and scopoletin at 500 mg l^{-1} gave no additional advantages with respect to 200 mg l^{-1}, whilst a further improvement was obtained with 7-geranoxycoumarin.

Table 2. Percentage of fungal growth inhibition on PDA by diffusion bioassay following treatment with 7-geranoxycoumarin, scoparone and scopoletin

Compound	Concn (mg l^{-1})	Inhibition (%)	
		P. italicum	*P. digitatum*
7-Geranoxycoumarin	50	92.0	23.0
	100	97.4	59.0
Scoparone	50	100	52.0
	100	100	72.0
Scopoletin	50	94.6	47.0
	100	100	63.0

Treatments on "Miho" satsumas showed that the effectiveness of 7-geranoxycoumarin against *P. digitatum* was much higher than that against *P. italicum* in all samples (Fig. 2b), whilst scoparone and scopoletin at 50 and 200 mg l^{-1} exhibited an opposite trend. Inhibitory activity increased as concentrations increased. All treatment with 200 mg l^{-1} reduced decay by ca 50% with respect to 50 mg l^{-1}. A further 50 % suppression of decay was recorded with 500 mg l^{-1} following scoparone and scopeletin treatments, while negligible differences was recorded with the 7-geranoxycoumarin treatment.

Scoparone and scopoletin treatments induced serious damage in the form of browning and necrosis to the rind, especially in satsumas, and even at 50 mg l^{-1}. In contrast, 7-geranoxycoumarin had no adverse affect to the peel when applied at higher concentrations.

In conclusion, the naturally occurring compound, 7-geranoxycoumarin, showed inhibitory activity against *P. italicum in vitro*, which was comparable to other naturally occurring compounds, like scoparon and scopoletin. Tests *in vivo* showed that only when applied on grapefruit at 500 mg l^{-1} did 7-geranoxycoumarin have antifungal activity against *P. italicum* comparable to scoparone and scopoletin. However, its activity was much higher against *P. digitatum*, the major postharvest pathogen of *Citrus* fruit [11].

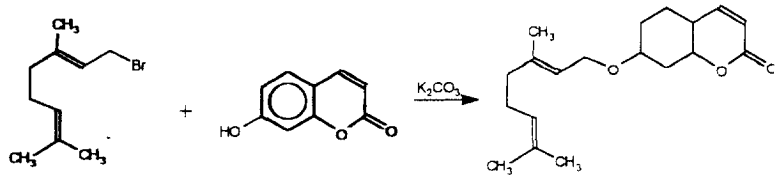
EXPERIMENTAL

Isolation of 7-geranoxycoumarin from grapefruit rind

Late season "Star Ruby" grapefruits (*Citrus paradisi* Macf.) were obtained from 6-year-old trees grown in an experimental grove located in Southern Sardinia, Italy. Flavedo tissue was removed with a vegetable peeler and 20 g aliquots were extracted $\times 2$ for min with CH_2Cl_2 (1:5 w/v) in an Ultraturrax. Combined extracts (100 ml) were taken to dryness by rotary evapn, the ppt. resuspended in MeOH and evapd. The active ingredient ($R_f = 0.7$) was isolated and purified by prep. TLC using toluene–EtoAc (4:1). The structure of 7-geranoxycoumarin was elucidated by GC-MS, GC-FTIR and NMR.

Identification of compound from grapefruit flavedo

Elemental analysis was carried out according to a standard procedure.



1 2 3

Fig. 1. Synthetic pathway for 7-geranoxycoumarin.

GC-MS. A GC equipped with a Durabond fused silica column (30 m × 0.25 mm i.d.) (J&W Scientific) with DB 5 liquid phase (5% phenyl, 95% dimethylpolysiloxane; film thickness 0.25 μm) was used. The sample (2μl) was injected in the on-column mode (100°), the detector was operated at 280° and the oven temp. was programmed as follows: 100° (1 min) raised to 250° (10°/min^{-1}) and then held for 10 min. He was the carrier gas at 1.8 ml/min^{-1} (45kPa). Mass spectrometer operating conditions: electron ionization, 70 eV; ion source 180°; scan mass, range 50–300; scan interval, 2.6 s; solvent delay, 8 min. The mass spectrum showed a [M$^+$] at *m/z* 298. GC-EI spectral fragmentation revealed the presence of a hydroxycoumarin residue (*m/z* 162, 134, 105 and 77) and a geranyl residue (*m/z* 136, 93 and 69).

GC-FTIR. A GC equipped with a 2000 FT-IR and a fused silica column (30 m × 0.25 mm i.d.) with an SP-2100 liquid phase (polydimethylsiloxane; film thickness 0.25 μm) (Supelco) was employed. The injector and detector were both operated at 300°. The sample (2μl) was injected in the split mode (1:50) and the oven temp. was programmed as follows: 150°, raised to 280 C (5 /min^{-1}). He was the carrier gas at 2 ml/min^{-1}. Scanning conditions were as follows: resolution 8, back ground 8, delay 0, scan by slice, ground Schmidt interferogram gain 25 → 75, range 4000–700 cm^{-1}, heated chamber temperature 200°. The IR spectrum revealed the presence of a lactone group bands at 3500 cm^{-1} (harmonic) and 1764 cm^{-1} (stretching).

NMR. A BRUKER AMX 500 at 500.13 MHz for ^{1}H NMR and at 125.77 MHz was used for ^{13}C NMR analyses. The temp. was fixed at 295 K. Monodimensional (^{1}H, ^{13}C) and two-dimentional (Dept 135, COSY 45, HECTOR) NMR techniques were employed for structural elucidation. Chemical shifts (d) are relative to TMS and locked to CH_2DCOCH_3 (2.04 for ^{1}H and 29.8 for ^{13}C, respectively. The lower field part of monodimensional proton spectrum (δ 6.2 to 7.9) (Table 3) showed a pattern of signals very similar to that of 7-hydroxycoumarin (Aldrich library of NMR spectra, molecule n. H2, 400-3 CAS [93-35-6]) without any phenolic OH signal. The higher field part of the proton spectrum (δ 1.6 to 5.5) showed a pattern of signals similar to that of geraniol (Aldrich library of NMR spectra, molecule n. 16, 333-3, CAS [106-24-1]), but without the OH signal and with the chemical shift of the CH2 at position 10 shifted downfield (δ 4.72) with respect to the spectrum of geraniol (δ 4.15). The presence of all signals after the addition of one drop of deuterated water confirmed the absence of exchangeable hydrogens. These evidence allowed us to propose the proposed structure. The coupling between the various proton was confirmed using the COSY 45 sequence. The monodimentional carbon spectra showed a number of peaks as expected for the proposed structure. The multiplicity for the various

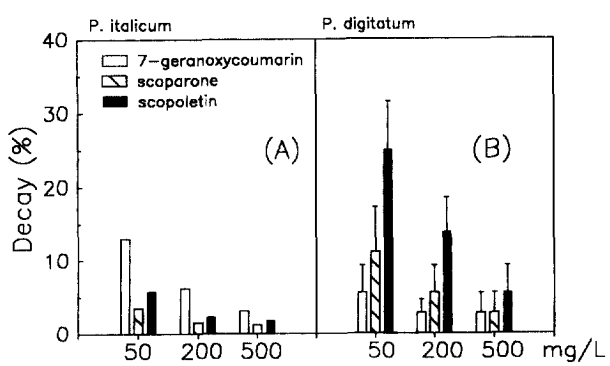


Fig. 2. Influence of scoparone, scopoletin and 7-geranoxycoumarin treatments on the percentage of *P. italicum* infected wounds of "Star Ruby" grapefruits (a) and of *P. digitatum* infected wounds of "Miho" satsumas (b) after storage at 17° for 3 weeks. All data are expressed as percentages of totally infected control. Bars indicate SE.

Table 3. Spectral characteristics of the natural occurring compound from "Star Ruby" grapefruit flavedo and of the synthesized 7-geranoxucoumarin

^{1}H NMR (δ) and ^{13}C NMR (δ):		
C	^{13}C	^{1}H
2	161.12	
3	113.72	6.21 1H, *d*, *J* = 9.5
4	144.71	7.89 1H, *d*, *J* = 9.5
4a	113.58	
5	130.24	7.57 1H, *d*, *J* = 8.6
6	113.92	6.92 1H, *dd*, *J* = 8.6;2.4
7	163.3	
8	102.4	6.88 1H, *d*, *J* = 2.4
8a	157.1	
10	66.42	4.72 2H, *d*, *J* = 6.5
11	120.33	5.49–5.52 1H, *m*
12	142.32	
13	16.9	1.64 3H, *s*
14	40.32	2.09–2.16 4H, *bs*
15	27.17	2.09–2.16 4H, *bs*
16	124.85	5.09–5.12 1H, *m*
17	132.33	
18	17.91	1.6 3H, *s*
19	25.96	1.79 3H, *s*

bs = broad signal; *d* = doublet; *m* = multiplet; *s* = singlet. MS (EI): *m/z* = 298 ([M]$^+$; 1.5%), 162, 136, 105, 69; FT-IR: 960 (γ, > CH *trans*); 1764 (ν), 3500 cm^{-1} (*Hr*, C=O) (γ = bending; ν = stretching; *Hr* = harmonic). UV λ_{max} = 325 nm.

carbons was confirmed using the DEPT 135 sequence. For the assignment of the chemical shifts of the peaks on the carbon spectrum the HECTOR sequence was also used. Therefore, elemental analysis, spectral data and chemical evidence of the compound isolated from the flavedo tissue of "Star Ruby" grapefruits led to the assignment of a structural formula to 7-geranoxycoumarin (Table 3).

Synthesis of 7-geranoxycoumarin

Synthesis of 7-geranoxycoumarin (**3**) was carried out according to the following strategy (Fig. 1). A 2.3×10^{-3} mol aliquot of geranyl bromide (**1**) was added dropwise to an equimolar amount of 7-hydroxy coumarin (**2**) and K_2CO_3 kept under stirring in 40 ml of Me_2CO. The resulting mixt. was warmed up and refluxed for 10 h. The reaction was monitored by TLC. The solvent was then evapd and the synthesized compound was separated by semi-prep. TLC according to the procedure followed for the extraction of the compound. Yield of the reaction was 85%.

GC-MS, GC-FTIR, UV and MNR analyses of the synthesized compound were identical to those of the isolated substance (Table 3).

Chemicals

$CHCl_2$, THF and MeOH were HPLC grade solvents (Carlo Erba). Standard grade geranyl bromide and 7-hydroxicoumarin (Sigma) were used. Semi-prep. TLC was carried out an silica gel 60 F_{254} (Merck).

Antifungal activity and agar diffusion bioassay

Antifungal activity of 7-geranoxycoumarine, scoparone and scopoletin was tested on *P. italicum* and *P. digitatum* isolates (ICMP 1236, Landcare Research New Zealand Ltd. and local natural infected "Star Ruby" grapefruits, respectively). The pathogen was kept on potato-dextrose agar (PDA) at 20° and its pathogenicity was tested by inoculating citrus fruit every 3 months. Antifungal activity was evaluated from the percentage inhibition of spore germination and germ-tube elongation. The procedure was carried out according to Ref. [12] as modified in Ref. [13]. Median effective doses (ED_{50}) were determined according to Ref. [14]. Agar diffusion bioassay on PDA media was used at 50 and 100 mg l^{-1} of 7-geranoxycoumarin, tested in comparison with those of two compounds with known activity, scoparone (6,7-dimethoxycoumarin, Aldrich cod. 25,488-6) and scopoletin (7-hydroxy-6-methoxycoumarin, Aldrich cod. 24,658-1) [9]. MeOH solns were used to spot the compounds on 13-mm ∅ discs (Whatman). When the solvent had evap, discs were placed in a PDA medium containing *P. italicum* conidia (10^5ml^{-1}) and then incubated at 20° for 5 days. Antifungal activity was measured from the inhibition area around the disc. Assays were repeated twice with 6 dishes for each concn.

Plant material and inoculation

Experiment I. "Star Ruby" grapefruits (*C. paradisi* Maef.) were harvested an the second week of May (late-season) and 400 fruits were selected, surface sterilized by 5 min immersions in a 2% aq. NaOCl soln, rinsed in boiled H_2O and left to dry at ambient temp. Fruits were wounded 1 h before inoculation by six slits (3 × 3 mm) on the stem-end hemisphere. Wounds were inoculated with 20 μl aliquots of a *P. italicum* conidial suspension (10^5 ml^{-1}). Fruits were then kept at 20° for 24 h before treatments. Fruits were subdivided into 10 groups (gps) (40 fruit each), corresponding with the following treatments: (a) 50, 200 and 500 mg l^{-1} scoparone (gps 1–3); (b) 50, 200 and 500 mg l^{-1} scopoletin (gps 4–6); (c) 50, 200 and 500 mg l^{-1} 7-geranoxycoumarin (gps 7–9); (d) deionized H_2O (gp 10).

Experiment II. Mature "Miho" satsumas (*C. unshiu* Marc.) were chosen in this expt because of their sensitivity to *P. digitatum*. Fruit preparation and inoculation procedures were the same as those used for Experiment I. Commercially ripe fruits were harvested

at the end of October 1996, subdivided into 10 groups (40 fruit each) and treated as described in Experiment I. Active ingredients in both exps were dissolved in 2 ml MeOH and taken up in H_2O. All treatments were carried out at 20° by 3 min stem-end hemisphere dipping. Following treatments, fruits were then left to dry for 1 h and stored at 17°, 80–85% relative humidity for 3 weeks. Pathogen spread was monitored weekly by counting the sites with active infections in each fruit and decay was expressed as percentages. During fruit storage, external appearance was also scored in terms of absence or presence of treatment damage on the two fruit hemispheres, one submerged and the other not.

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