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Isolation of a New Phenolic Compound, 3, 5-Dihydroxy-2-(Methoxycarbonylmethyl)phenyl 3, 4-Dihydroxybenzoate, from Leaves of *Actinidia chinensis* (Kiwifruit)

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Abstract: Extraction of leaf tissue from the golden-fleshed kiwifruit cultivar *Actinidia chinensis* 'Hort16A' expressing genotype-resistance against the fungus *Botrytis cinerea* revealed the presence of a novel phenolic compound, 3,5-dihydroxy-2-(methoxycarbonyl-methyl)phenyl 3,4-dihydroxybenzoate (1). In addition, the known phenylpropanoids, caffeic acid (2) and quercetin (3) were amongst the principal compounds present in a fungi-toxic phenolics extract from 'Hort16A' leaf tissue. TLC fungi-toxicity bioassays indicated that 100 μg quantities of these pure compounds were not fungi-toxic, but caffeic acid is a central compound in the phenylpropanoid pathway and therefore might function indirectly in host resistance, by acting as a pre-cursor of fungi-toxic secondary metabolites. The other compounds could possibly act in a similar role.

Key words: Kiwifruit, *Actinidia chinesis*, 3,5-Dihydroxy-2-(methoxycarbonylmethyl) phenyl 3,4-dihydroxybenzoate, depside, disease resistance, *Botrytis cinerea*

Introduction

Although many species of *Actinidia* exist in the wild, *A. chinensis* Planch. var. *chinensis* 'Hort16A', which was recently introduced onto the world market, is one of only two kiwifruit cultivars considered commercially important, the other being *A. deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* 'Hayward'. Grey mould, caused by *Botrytis cinerea* is a serious disease of 'Hayward' kiwifruit, but studies have shown that leaves and fruit of Hort16A express greater constitutive resistance to this pathogen than 'Hayward' and that resistance is associated with phenolic compounds (Wurms *et al*., 2003; Wurms, 2004). Given the recent introduction into commercial production of 'Hort16A', there are few detailed studies on the phenolics composition of the 'Hort16A' cultivar, particularly in relation to disease resistance.

The present study was initiated to characterize some of these compounds and determine their fungi-toxicity to facilitate better understanding of resistance mechanisms against *Botrytis cinerea* in 'Hort16A'. Here we present results from a bioassay directed fractionation study of leaf tissue from *Actinidia chinensis* 'Hort16A' and describe the isolation and identification of a novel phenolic compound, 3,5-dihydroxy-2-(methoxycarbonylmethyl)phenyl 3,4-dihydroxybenzoate (1) along with several known phenolic compounds which together constituted the principal components of a fungi-toxic extract.

Materials and Methods

General Experimental Procedures

NMR spectra were determined on a Bruker Avance DRX-400 spectrometer at 400.13 (^{1}H) and at 100.61 MHZ (^{13}C). Homonuclear ^{1}H connectivities were determined by 2D double-quantum filtered

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COSY, long range 2D double-quantum filtered COSY and long range 1D TOCSY experiments. One-bond heteronuclear ^{1}H-^{13}C connectivities were determined by a 2D proton-detected HSQC experiment and two- and three-bond ^{1}H-^{13}C connectivities were determined by a 2D proton-detected HMBC experiment. Chemical shifts are reported relative to DMSO.

LC-MS employed a LCQ Deca ion trap mass spectrometer fitted with an APCI interface (ThermoQuest, Finnigan, San Jose, CA, USA) and coupled to a Surveyor™ HPLC and PDA detector. The analytical column employed was a Prodigy 5 μ ODS(3) 100Å (Phenomenex, Torrance, CA, USA), 150 × 2 mm. A 0.2 μ in-line filter (Alltech, Deerfield, Illinois, USA) was installed before the analytical column and the temperature of the column oven was maintained at 35°C. Compounds were separated isocratically using 36:64 MeOH+0.1% FA (formic acid)-H_2O. Flow rate was 250 μL min^{-1} and the injection volume 10 μL. UV-VIS detection was by absorbance at 200-600 nm. MS data was acquired in the negative mode for most experiments using a data-dependent LC-MS^n method. The APCI vaporizer temperature, capillary temperature, capillary voltage, tube lens offset, sheath gas pressure and auxiliary gas were set at 450, 200°C, -12 and -20 V, 60 and 0 psi, respectively.

High-resolution mass spectra were determined in the FAB mode on a VG70S magnetic sector mass spectrometer.

HPLC was performed using a Shimadzu system (Kyoto, Japan), equipped with a 150×4.6 mm Reversed-phase (RP) column (Prodigy™ 5μ ODS (3), Phenomenex, Torrance, California, USA), with detection at 280 nm and photodiode-array analysis at 200-360 nm.

Standards of known kiwifruit phenolic compounds (Webby, 1990; Webby *et al*., 1994; Dawes and Keene, 1999; Lahlou *et al*., 2001), purchased from Sigma-Aldrich (St. Louis, Missouri, United States) and Apin Chemicals Ltd (Abingdon, England), were analyzed by RP-HPLC and LC-MS and comprised 125 μg mL^{-1} MeOH of chlorogenic acid, hydroxybenzoic acid, caffeic acid, epi-catechin, catechin, ρ-coumaric acid, rutin, quercetin-3-glucoside, quercetin-3-rhamnoside, quercetin, kaempferol and rhamnetin and 100 μg mL^{-1} MeOH of 2α,3β,23-trihydroxy-12,20(30)-ursadien-28-oic acid, otherwise known as actinidic acid (which was a generous gift of Dr Nobuhiro Hirai, Kyoto University, Japan).

A TLC spot bioassay described earlier (Wurms *et al*., 2003) was used to determine anti-fungal activity of crude extracts, standards, purified compounds and chromatographic fractions and thereby to guide compound isolation. In a biological system, a compound is generally considered to be fungi-toxic, if growth inhibition in the TLC-bioassay occurs with <50 μg of pure product (Skipp and Bailey, 1977; Niemann, 1993; Fawe *et al*., 1998).

Plant Material

Randomly-selected healthy leaves from T-bar orchard-grown mature 'Hort16A' kiwifruit vines (Hamilton, New Zealand, 2002) were inoculated with 5 mm diam. 14-28 day old agar inoculum plugs (four plugs/leaf) of *B. cinerea*, which were secured face-down on the adaxial leaf surface with a piece of Danco 130 all-weather transparent tape (Danco (NZ) Ltd., Wellington, New Zealand). Six days after inoculation, leaf tissue was pooled from: (1) the diseased inoculation sites and (2) the ring of symptom-free leaf tissue immediately surrounding the diseased lesions. A total of 27.5 g of lyophilized leaf tissue (equivalent to approximately 110 g FW) was used for extraction and isolation of the phenolic compounds

Extraction and Isolation

The detailed extraction protocol is described by Wurms *et al*. (2003), except that the initial extraction comprised 2×24 h extractions in 80% v/v MeOH at 20 mL MeOH/g dry matter (DM). The acid-hydrolyzed phenolic extract (20 mL total of 1.5 g DM/mL MeOH) from the above extraction procedure was diluted 1:1 with water and then fractionated on 10 g Reversed-phase (RP) Solid Phase

Extraction (SPE) cartridge (High Load C18, Alltech Associates, Inc., Deerfield, IL) using a step-wise gradient of 0:100, 50:50, 80:20 and 100:0 MeOH-H_2O, where all solvents were acidified with 2.5% v/v glacial acetic acid to suppress compound ionization. TLC fungi-toxicity bioassay screening indicated that only the acidified 50:50 MeOH-H_2O fraction was markedly fungi-toxic and therefore of interest for further purification. Between each subsequent purification step described below, the fraction containing the compound(s) of interest was concentrated by rotary evaporation until the point of dryness, followed by re-dissolution in the smallest possible volume of 50:50 H_2O-MeOH (both solvents acidified with 2.5% v/v acetic acid). Resolution of the fungi-toxic fraction by RP-HPLC using initial solvent conditions of 14.5:85.5 CH_3CN-H_2O+0.1% TFA (trifluoroacetic acid) and a linear solvent gradient described previously (Wurms *et al*., 2003), with a flow rate of 1 mL min^{-1}, revealed the presence of 5 major peaks eluting at 8.3, 20.9, 22.5, 23.8 and 29.1 min. Co-chromatography with authentic standards and resulting matches in retention times, absorption spectra and mass spectral data indicated that the peaks at 8.3 and 29.1 min were caffeic acid (2) and quercetin (3), respectively. The other peaks did not match any of the known standards and LC-MS analysis indicated the presence of multiple compounds within each peak (data not shown). To further separate these compounds, RP-HPLC was employed using an initial mobile phase of 18:82 CH_3CN-H_2O+0.1% TFA and isocratic conditions for 10 min, followed by a linear gradient to 28:72 CH_3CN-H_2O+0.1% TFA over 10 min, held for 10 min before resetting to the original conditions over the next 5 min. Final separation by RP-HPLC was achieved isocratically using 27:73 MeOH-H_2O+0.1% TFA and yielded 2 mg of compound 1. This compound, which eluted at 22.5 min in the original RP-HPLC gradient, was then dried under a nitrogen stream, re-dissolved in 1 ml of DMSO and analyzed by NMR.

3,5-dihydroxy-2-(methoxycarbonylmethyl)phenyl 3,4-dihydroxybenzoate (1)

Beige needle-like crystals; UV (MeOH) λ_{max} 210, 268, 298 nm; ^{1}H NMR and ^{13}C NMR (Table 1); HRFABMS m/z 335.0760 (calcd for $C_{16}H_{15}O_8$, $[M+H]^+$, 335.0767); LC-MS m/z 333.0 $(M-H)^-$; 301.0 (MS^2 333); 165.1 (MS^3 333>301); 137.2 (MS^4 333>301>165); R_f 0.29 (Si gel 60 F_{234}, 35:65 CH_2Cl_2:EtOAc); 100 μg of pure compound was not fungi-toxic in the TLC bioassay.

Compounds 2-3

Caffeic acid (2) and quercetin (3) were identified by comparison of their spectral data with literature values (Mabry *et al*., 1970; Harborne and Mabry, 1982; Dey and Harborne, 1989) and unambiguously confirmed using RP-HPLC and LC-MS co-chromatography experiments with authentic standards.

Table 1: 1D and 2D NMR Spectral Data for Compound 1

Position	δ_C	δ_H (mult., J_{Hz})	HMBC (H→C)	LR 1D TOCSY	LR DQF COSY
1	119.5, s				
2	116.6, d	7.52 (d, 2.0)	1-CO-, 3, 4, 6		
3	145.2, s				
4	151.1, s				
5	115.4, d	7.50 (dd, 8.2, 2.0)	1, 3, 4		
6	122.5, d	6.97 (d, 8.2)	1-CO-, 2, 4		?
1'	150.7, s				
2'	105.1, s				
3'	156.7, s				
4'	99.8, d	6.35 (d, 2.0)	2', 3', 5', 6'		6'
5'	156.9, s				
6'	100.8, d	6.16 (d, 2.0)	1', 2', 4', 5'		4'
1-CO-	163.6, s				
2'-CH_2-	28.6, t	3.45 (s)	1', 2', 3', 2'-CH_2-CO-	4', 6', 2'-CH_2-CO-OCH_3	4', 6', 2'-CH_2-CO-OCH_3
2'-CH_2-CO-	171.2, s				
2'-CH_2-CO-OCH_3	51.3, q	3.57 (s)	2'-CH_2-CO-OCH_3		

Results and Discussion

A crude methanolic extract of 110 g fresh weight (FW) of *A. chinensis* Planch. var. *chinensis* 'Hort16A' leaf tissue was fractionated by reversed-phase solid phase (RP-SPE) extraction and the fractions were bioassayed (using a TLC spot bioassay described earlier) (Wurms *et al*., 2003) for fungi-toxicity. Fungi-toxic activity was predominantly associated with the fourth RP-SPE fraction (RP-SPE 4). This fraction became the focus of all subsequent purification work. Three of the major phenolic constituents in RP-SPE 4 were identified: 3,5-dihydroxy-2-(methoxycarbonylmethyl)phenyl 3,4-dihydroxybenzoate (1) a novel compound which to our knowledge has never before been characterized and two known phenolics-a hydroxycinnamic acid, caffeic acid (2) and a flavonol, quercetin (3) (Fig. 1). Approximately 80% of the total quantity of compound 1 was found in RP-SPE 4 and 2 mg of pure material was recovered from this fraction (*ca.* 20% recovery of the total yield in RP-SPE 4), while 2 and 3 were present at ca. 139.0 and 973.4 μg g^{-1} FW leaf tissue, respectively.

Identification of 1 was achieved via a combination of mass spectral and one and two-dimensional NMR studies. LC-MS using APCI ionization in negative ion mode afforded a strong $(M-H)^-$ ion at m/z 333. Fragmentation of this ion employing a data-dependent MS^n experiment gave the MS^2 daughter ion m/z 301, suggesting loss of a methoxyl group as a mole of methanol, followed by the MS^3 granddaughter ion m/z 165 and MS^4 ion m/z 137 (Fig. 2). HRFABMS gave $[M+H]^+$ m/z 335.0767 corresponding to a molecular formula of $C_{16}H_{14}O_8$, which was consistent with the results of ^{13}C NMR and DEPT experiments (Table 1). These experiments revealed 9 singlet resonances, 2 of which were consistent with chemical shifts expected for ester carbonyl groups (171.2 and 163.6 ppm), 5 doublet resonances, a quartet resonance consistent with an oxy methane group (51.3 ppm) and a triplet resonance (28.6 ppm). ^1H NMR, COSY and LRCOSY experiments showed 2 isolated aromatic spin systems comprising 2 doublets (7.52 ppm J = 2.0 Hz, 6.97 ppm J = 8.2 Hz) and a doublet of doublets (7.50 ppm J = 8.2, 2.0 Hz) in 1 ring and 2 doublets (6.35 ppm J = 2.0 Hz, 6.16 ppm J = 2.0 Hz) in a second ring. Coupling constants and chemical shift values suggested one aromatic ring with

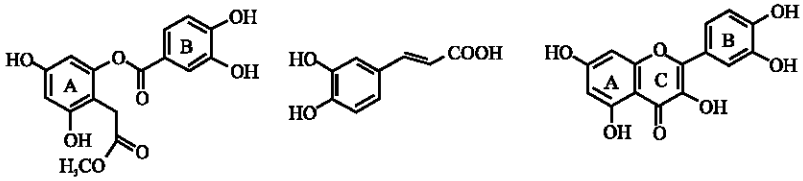

Fig. 1: Chemical structures for compounds 1, 3,5-dihydroxy-2-(methoxycarbonylmethyl)-phenyl 3,4-dihydroxybenzoate, 2 caffeic acid and 3 quercetin

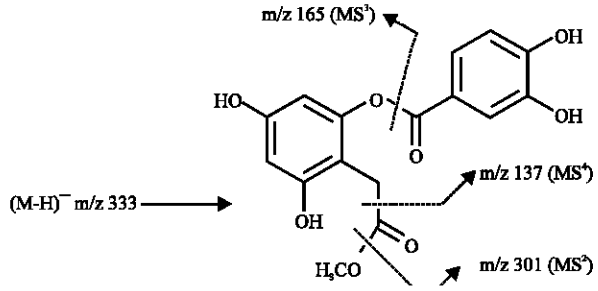

Fig. 2: LC-MS fragmentation data for compound 1

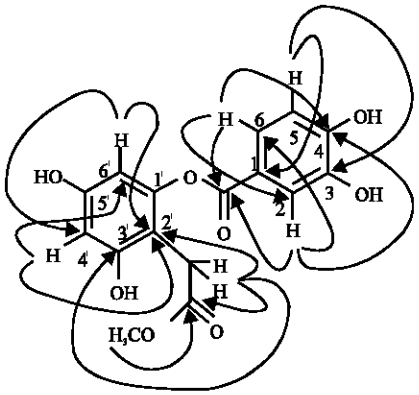

Fig. 3: Selected HMBC correlations for compound 1

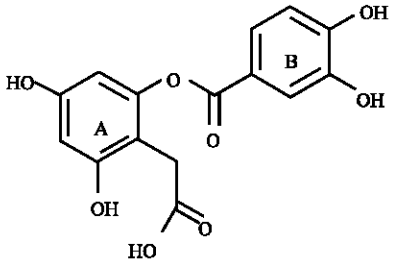

Fig. 4: Chemical structure of 2-*O*-(3,4-dihydroxy-benzoyl)-2,4,6-trihydroxyphenylacetic acid

substituents at the 1, 3 and 4 positions, the latter 2 oxygenated and a second ring with 4 substituents, 3 of which were oxygenated. This data was consistent with a penta-hydroxylated structure comprising A and B rings as for quercetin (Fig. 1), but without a C ring. The complete structure was finally elucidated using a combination of a long range 1D selective TOCSY experiment and HSQC and HMBC spectral data. In an experiment with a 250 msec mixing time, irradiation of the CH_2 group at 3.35 ppm showed weak but detectable correlations to the methoxy signal at 3.48 ppm and the aryl B ring protons at 6.16 ppm (H-6') and 6.35 ppm (H-4'). Selected HMBC correlations are shown in Fig. 3 and are consistent with the LC-MS fragmentation data previously described (Fig. 2). The structure of 1 was thus determined to be 3,5-dihydroxy-2-(methoxycarbonylmethyl)phenyl 3,4-dihydroxybenzoate.

To our knowledge, compound 1 represents a novel member of the depside family of compounds. Depsides are defined as substances derived from 2 or more molecules of the same or different phenolcarboxylic acids by esterification of the carboxyl group of one molecule with the hydroxyl group of a second molecule (Coffey, 1976; McNaught and Wilkinson, 1997). Most natural depsides occur in lichens, where they are biosynthesized via predominantly the acetate pathway and to a lesser extent via the shikimic acid pathway (Coffey, 1976), but they have also been isolated from higher plants including a number of commonly known medicinal herbs from the *Lamiaceae* family, including peppermint (*Mentha piperita* (L.) Hudson), marjoram (*Origanum majorana* L.), sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.) lavender (*Lavandula officinalis* Chaix), rosemary (*Rosmarinus officinalis* L.) and hyssop (*Hyssopus officinalis* L.) (Zgórka and Glowniak, 2001). Depsides are known to be active ingredients in European and Chinese folk medicines owing to their anti-oxidant, anti-inflammatory, anti-microbial and anti-pyretic activities (Hidalgo *et al*., 1994; Neamati *et al*., 1997; Li, 1998; Esimone and Adikwu, 1999; Zgórka and Glowniak, 2001;

Piovano *et al*., 2002). The most closely related previously-reported structure to compound 1 is the depside, 2-*O*-(3,4-dihydroxy-benzoyl)-2,4,6-trihydroxyphenylacetic acid, shown in Fig. 4, which was isolated from the herbaceous plant, *Papaver rhoeas* L. (Hillenbrand *et al*., 2004). The difference between the 2 compounds is that the carboxyl group attached to ring A in 2-*O*-(3,4-dihydroxy-benzoyl)-2,4,6-trihydroxyphenylacetic acid is replaced by a methyl ester in compound 1 (Figure 1 c.f. Figure 4). However it is extremely unlikely that compound 1 is a derivative of 2-*O*-(3,4-dihydroxy-benzoyl)-2,4,6-trihydroxyphenyl-acetic acid, created artifactually by extraction in methanol, since the initial stages of the extraction process involving methanol were identical for both compounds, i.e., in both cases powdered plant material was extracted in 80% methanol, followed by evaporative removal of the methanol under vacuum, leaving the aqueous residue, which was then partitioned against various solvents (Hillenbrand *et al*., 2004). Methanol is a commonly used solvent in the initial stages of extraction of phenolic compounds, including depsides (Elix *et al*., 1999; Zgórka and Glowniak, 2001; McNally *et al*., 2002; Hillenbrand *et al*., 2004) and methanolic degradation of phenolic compounds to form depsides does not occur spontaneously, but rather requires irradiation of the methanol solvent to generate reactive free radicals that initiate redox reactions of flavonoids leading to depside formation (Marfak *et al*., 2002, 2003). Moreover, based on the mass and spectral data for 2-*O*-(3,4-dihydroxy-benzoyl)-2,4,6-trihydroxyphenylacetic acid (Hillenbrand *et al*., 2004), this compound was not present as a major component in our fungi-toxic RP-SPE 4 phenolics fraction.

Compounds 1-3 are unlikely to be anti-fungal *in planta*, since 100 μg quantities of the pure compounds (equivalent to>0.5g FW of leaf tissue) were not fungi-toxic in the TLC fungi-toxicity bioassay, whereas replicates of RP-SPE 4 extract (equivalent to>0.5g FW of leaf tissue) produced fungal growth inhibition zones with a mean diameter of 17 mm. However, compounds 1-3 accounted for 3 of 5 of the major components in the fungi-toxic RP-SPE 4 phenolics fraction. Wurms *et al*. (2003) demonstrated that the degree of anti-fungal activity of 'Hort16A' leaf extracts (as quantified in the fungitoxicity bioassay by diameter of fungal growth inhibition zones) was correlated positively with concentrations of three phenolic compounds-caffeic acid and two other compounds of unknown identity. A comparison of spectral and mass data and retention times indicates that one of these previously observed compounds can now be characterized as novel compound 1 described in the current study. Although not inherently fungi-toxic, these phenolics may contribute indirectly to defense in a number of ways: by acting as precursors or co-factors of directly anti-fungal compounds, as signal molecules, as elicitors of other plant defenses, or as inhibitors of key pathogenesis enzymes. For example, caffeic acid is central to plant secondary metabolism because it acts as a building block in the phenylpropanoid pathway (Dey and Harbourne, 1989; Harbourne, 1998), while quercetin is one of the most ubiquitous flavonols and functions ascribed to flavonols include UV and disease protection, pollination attractants, enzyme inhibitors, antioxidants, plant hormone controllers and allelopathic agents (Dey and Harbourne, 1989). Indeed, many consider the indirect contributions of various endogenous compounds to host resistance to be more important than direct effects (Kuc, 1995).

Future research will investigate the applicability of compound 1 as a marker of host resistance for screening novel germplasm arising from breeding trials.

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