PERGAMON

Phytochemistry 56 (2001) 717–721

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Kaempferide triglycoside: a possible factor of resistance of carnation (*Dianthus caryophyllus*) to *Fusarium oxysporum* f. sp. *dianthi*

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Received 26 July 2000; received in revised form 21 November 2000

Abstract

A kaempferide triglycoside has been found as a constitutive component in an uninfected carnation (*Dianthus caryophyllus*) of the cultivar Novada. The chemical structure has been determined mainly by the use of spectroscopic methods, including 2D NMR experiments. It showed a strong activity in restricting fungal parasite development, which could contribute to the known ability of carnation cv. Novada to resist to *Fusarium oxysporum* f. sp. *dianthi* infection. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Dianthus caryophyllus*; Caryophyllaceae; Carnation; Kaempferide glycoside; NMR; Fungitoxic activity

1. Introduction

Fusarium oxysporum f. sp. *dianthi* represents the most important fungal parasite affecting carnation (*Dianthus caryophyllus* L., Caryophyllaceae) cultivated areas, where it causes severe crop losses all over the world (Garibaldi and Gullino, 1987). Carnation of the cultivar Novada has a commercial interest because of its high resistance to the parasite (Baayen, 1986; Baayen et al., 1988). Species of this cultivar have been, therefore, extensively studied to elucidate the mechanism of this high resistance degree at a biochemical level. An adequate explanation seems to reside in the production, after a *F. oxysporum* f. sp. *dianthi* attack, of nitrogen-containing phytoalexins effective against the pathogen (Niemann et al., 1992). These molecules have been identified as anthranilic acid derivatives, dianthalexins and dianthramides (Reinhard and Matern, 1989). However, within the tissues of the same carnation a further compound, which does not contain nitrogen and displays fungitoxic properties, has been detected but not identified (Niemann, 1992; Niemann and Baayen, 1988). Since in another highly resistant carnation cultivar a constitutive phenol is responsible for the fungitoxic properties of the tissue extract (Curir et al., 1996), the possibility that the unidentified fungitoxic compound from cv Novada is a phenol derivative could not be excluded. Therefore, a research has been undertaken to assess if Novada uninoculated tissues contain pre-formed compounds effective against *F. oxysporum* f. sp. *dianthi*, besides the already recognized nitrogen-containing phytoalexins.

As a result of such a study, herein we describe the isolation and structural elucidation of a new kaempferide triglycoside (**1**), a factor of resistance of carnation to *F. oxysporum* f. sp. *dianthi*, as evidenced by preliminary fungitoxic data.

2. Results and discussion

Compound **1**, a yellow powder with $[\alpha]_D^{25} = -16°$ (MeOH; $c = 0.002$), was obtained in pure form only after repeated chromatographic steps based on C-18 reverse-phase and Sephadex LH-20 gel filtration (see Section 3). It gave a pseudomolecular ion peak at m/z 769.2170 $(M–H)^-$ in the negative-ion high-resolution FAB mass spectrum, consistent with the molecular formula $C_{34}H_{42}O_{20}$ (calc. m/z 769.2191). The UV spectrum (MeOH, Table 1) showed absorptions at λ_{max} 264, 316 and 350 nm, in accordance with a C-3 substituted flavonol

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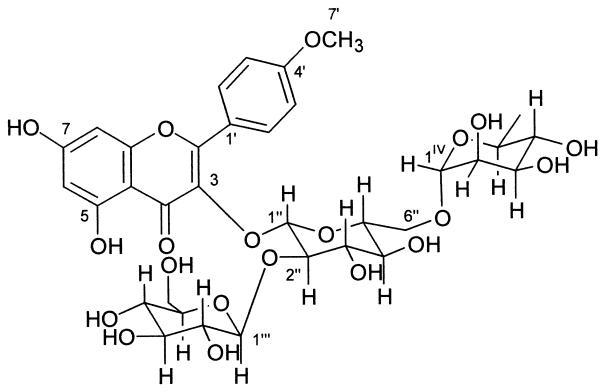
0031-9422/01/$ - see front matter
PII: S0031-9422(00)00488-X

skeleton (Mabry et al., 1970). The bathochromic shifts obtained by addition of several diagnostic reagents (Table 1) were in good agreement with the data reported for the kaempferide skeleton (Mabry et al., 1970).



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The 500 MHz ^{1}H and ^{13}C NMR spectra (Table 2) confirmed these conclusions and, in addition, revealed that a triglycosidic chain should account for the remaining atoms of the molecular formula. The combined interpretation of these spectra was aided by the 2D HMQC spectrum, which allowed us to associate all the protons with the relevant carbon signals, and by the 2D HMBC spectrum, which was essential to interconnect the different spin systems.

In particular, two broad singlets at δ 6.24 and 6.42 in the ^{1}H NMR spectrum were attributed to H-6 and H-8, respectively, while the two 2H integrating doublets at δ 6.92 and 8.06 fitted well with data reported for H-3′ and H-2′, respectively. The singlet at δ 3.39 (3H) was assigned to the 7′-OMe group by the following series of evidences: (i) the downfield shift of the ^{13}C NMR signal of C-4′ (δ 162.8) suggested the presence of an ether linkage at that position (Bilia et al., 1993); (ii) a correlation between the methoxy protons (δ 3.39) and the signal at δ 6.92 (H-3′ and H-5′) was evident in the ROESY spectrum; (iii) the presence of a key HMBC cross peak between H-7′ and C-4′. Finally, the HMBC spectrum allowed us to unambiguously assign all the ^{13}C resonances of this aglycone.

Going on, three anomeric carbon signals at δ 101.4, 104.5 and 101.8 were identified in the ^{13}C NMR spectrum and associated with the relevant protons (δ 5.38, 4.80 and 4.50, respectively) through the HMQC experiment.

Table 1
UV spectral absorptions of **1**

λ_{max} in nm				
MeOH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc
350 *sh*	384	400 *sh*	400 *sh*	350
316	316	350	350	272
264	272	308	306	
		270	272	

Table 2
^{13}C and ^{1}H NMR data of compound **1** in CD_3OD

Pos.	δ_C (mult.)	δ_H (mult., *J* in Hz)
2	159.0 (C)	
3	134.1 (C)	
4	180.0 (C)	
5	163.1 (C)	
6	99.8 (CH)	6.24 (*s*)
7	166.1 (C)	
8	95.0 (CH)	6.42 (*s*)
9	158.1 (C)	
10	105.1 (C)	
1′	124.0 (C)	
2′-6′	132.8 (CH)	8.06 (*d*, 8.5)
3′-5′	115.8 (CH)	6.92 (*d*, 8.5)
4′	162.8 (C)	
7′	50.5 (CH_3)	3.39 (*s*)
1″	101.4 (CH)	5.38 (*d*, 7.5)
2″	81.5 (CH)	3.77 (*t*, 7.5)
3″	77.5 (CH)	3.61[a]
4″	71.9 (CH)	3.31 (*t*, 9.5)
5″	77.7 (CH)	3.50 (*ddd*, 9.5, 5.5, 3.5)
6″	63.3 (CH_2)	3.67[a]
		3.60[a]
1‴	104.5 (CH)	4.80 (*d*, 7.0)
2‴	74.5 (CH)	3.43[a]
3‴	77.1 (CH)	3.37[a]
4‴	71.5 (CH)	3.72 (*dd*, 8.5, 7.5)
5‴	77.5 (CH)	3.61[a]
6‴	62.8 (CH_2)	3.86[a]
		3.77[a]
1^{IV}	101.8 (CH)	4.50 (*bs*)
2^{IV}	72.6 (CH)	3.62 (*bd*, 3.0)
3^{IV}	71.8 (CH)	3.74 (*dd*, 3.0, 8.5)
4^{IV}	73.8 (CH)	3.29 [a]
5^{IV}	71.5 (CH)	3.42 (*m*)
6^{IV}	17.1 (CH_3)	1.12 (*d*, 6.0)

[a] Overlapped with other signals.

Although the mid-field region of the ^{1}H NMR spectrum of **1** contained several overlapping signals, analysis of homonuclear COSY and HOHAHA 2D experiments allowed us to assign all the proton resonances of the sugar moieties. In particular, the 1D subspectra of the 2D HOHAHA experiment relative to each spin system allowed a better evaluation of most coupling constants, and this was very useful to elucidate the monosaccharide relative stereochemistry.

Hence, when the anomeric proton at δ 5.38 (H-1″) was used as a starting point, a sequence of four oxymethines and one oxymethylene (Table 2) was identified from the above spectra. The large coupling constants observed for all the protons, in accordance with axial–axial relationships, indicated the β-glucopyranose nature of this sugar. In addition, the ROESY couplings of H-1″ with H-3″ and H-5″, and of H-2″ with H-4″ further supported the above conclusion. This residue should be linked to the C-3 of the aglycone, as indicated by the key HMBC correlation peak between the anomeric proton H-1″ and C-3 (δ 134.1).

The HMBC cross peaks of H-2″ (δ 3.77) with the anomeric carbon at δ 104.5 and of H_2-6″ (δ 3.60 and 3.67) with the anomeric carbon at δ 101.8 identified position 2 and 6 of the inner glucose as glycosidic linkage sites. This was also confirmed by the spatial couplings of H-2″ with H-1‴ (δ 4.80) and of H_2-6″ with H-1^{IV} (δ 4.50), evidenced in the ROESY spectrum.

The monosaccharide linked at position 2″ has been identified as a further β-glucose basing on the same arguments as those used for the characterization of the previous sugar unit. On the other hand, the spin system starting with H-1^{IV} extended to four oxymethine and one methyl group (see Table 2). This sugar moiety was identified as a rhamnopyranose due to the axial-axial couplings H-3^{IV}/H-4^{IV} and H-4^{IV}/H-5^{IV} and the axial-equatorial relationship between H-2^{IV} and H-3^{IV}. In accordance with data reported in the literature (Sang et al., 1999), the α-anomeric configuration of this sugar was judged by the chemical shift of C-3^{IV} (δ 71.8) and C-5^{IV} (δ 71.5).

The nature of the sugar residues was confirmed by chemical analysis. To this aim, compound **1** was subjected to acid methanolysis followed by silylation with TRISIL-Z. GC analysis of the released saccharides showed a sugar composition of glucose and rhamnose in the relative ratio 2:1. Therefore, if the hexopyranoses are assumed to belong to the most commonly found series, D for glucose and L for rhamnose, the stereostructure of **1** is completely defined as kaempferide 3-*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside.

Compound **1** exhibited a good fungitoxic activity towards *F. oxysporum* f. sp. *dianthi* conidia germination, as shown in Table 3. Its inhibitory activity is statistically appreciable, at 50 and 100 μM on potato dextrose liquid medium, throughout all the observation periods, while the effectiveness of the lowest tested dosage becomes statistically significant after 2 days of culture. However, when the fungitoxic activity is assayed on a gelled medium slab, the concentration requested to induce the inhibition of conidia germination is consistently lower: 1, 2 and 5 μM are enough to determine a visible, a clearly present or a strong inhibitory effect, respectively (Table 4).

Table 3
Fungitoxic activity of **1** towards *F. oxysporum* f. sp. *dianthi* conidia germination in liquid medium[a]

μM of **1**	% Of conidia germination	Days of observation
0	85a	1
10	70a	
50	20b	
100	0c	
0	95a	2
10	73b	
50	25c	
100	0d	
0	98a	4
10	75b	
50	30c	
100	10d	

[a] Each value is the mean of 50 observations. Values in each column and for each period of observation followed by the same letter are not significantly different for $P = 0.05$, according to the Student–Newmann–Keuls statistical test. Values were arcsin-transformed before the analysis.

The concentration of the investigated flavonoid (**1**) within the plant fresh tissues is about 3.9 μmol/g for the stem and 0.8 μmol/g for roots, these values being calculated from ε, measured at λ_{max} 347.5 in EtOH, as indicated by Mabry et al. (1970). These values fall within the range of concentrations effective in vitro. Compound **1** could therefore play a role in determining the Novada resistance ability (Curir et al., 1993; Baayen et al., 1997). To the best of our knowledge, this is the first report of a carnation constitutive flavonol glycoside with fungitoxic properties. Further investigations are required to ascertain if the biosynthesis of this constituent increases as a consequence of an infection attempt by *F. oxysporum* f. sp. *dianthi*.

3. Experimental

3.1. General experimental procedure

FABMS (glycerol matrix, CsI) were measured on a VG Prospec (FISONS) mass spectrometer. Optical rotation was determined on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10-cm microcell. ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer. Chemical shifts were referenced to the residual solvent signal (CD_3OD: $\delta_H = 3.34$, $\delta_C = 49.0$). ^1H connectivities were determined by using COSY and HOHAHA experiments. The 2D HOHAHA experiments were performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time 125 ms) sequence for mixing. One-bond heteronuclear ^1H–^{13}C connectivities were determined with 2D HMQC experiments with a BIRD pulse sequence followed by a delay of 0.5 s before each scan to suppress the signal from proton not directly bonded to ^{13}C. The interpulse delays were adjusted for an average value of $^1J_{CH}$ of 125 Hz. Two- and three-bond

Table 4
Fungitoxic activity of **1** after 4 days of observation[a]

μmol of **1**	Fungitoxic activity
0	−
1	±
2	+
5	++

[a] The compound was spotted on TLC plates, further covered by a layer of potato dextrose agar containing a *F. oxysporum* f. sp. *dianthi* conidial suspension.

heteronuclear ^{1}H–^{13}C connectivities were determined with 2D HMBC experiments optimized for a $^{2,3}J_{CH}$ of 8 Hz. Nuclear Overhauser effect (NOE) measurements were performed by 2D ROESY experiments.

3.2. Extraction and isolation

Carnation plants of the cultivar Novada were obtained by courtesy of Professor J. De Jong, CPLO-DRO Institute, Wageningen, Holland. A voucher specimen has been deposited at the Orto Botanico of Università di Torino, Italy. Leafy stems (40 cm) and plant roots were separately harvested and extracted as follows. Plant fresh tissues (100 g) were homogenized with a Waring Blender in 2 l of bidistilled H_2O; the homogenates were extracted for 6 h under N_2 atmosphere in a Soxhlet apparatus, and then filtered through cheesecloth and nylon net. The finest debris was removed by centrifugation at 3000 *g* for 0.5 h. To 1 l of clear liquid, put into a separating funnel, 100 ml diethyl ether were added and the upper phase, containing large amounts of fat, was discarded; this step was repeated at least 3 times. Then, 10 ml HCOOH per litre of water phase were added, and the solution loaded onto a glass column (100×5 cm) containing cellulose packed with EtOH-1% HCOOH; the elution was carried out using EtOH–H_2O (2:8, v/v). The hydroalcoholic fractions containing the investigated compound were pooled, concentrated under reduced pressure, loaded on a column (100×5 cm) filled with Polyamide 6 (Riedel-de Haën, Germany), and eluted with a linear gradient from H_2O to H_2O–EtOH (8:2, v/v). The hydroalcoholic fractions containing the investigated compound, pooled and concentrated as above, were re-chromatographed onto a column (50×1.5 cm) filled with silica gel 60 packed and eluted with H_2O–EtOH (linear elution gradient from 9:1 to 1:9, v/v). The further purification phase was carried out by a column (50×0.8 cm) filled with silica gel 100 C_{18}-reversed phase packed with EtOH, and loading the samples in 5% HCOOH-containing solutions. The column was first eluted with H_2O, and then with isoamyl alcohol. The alcoholic fractions were finally purified by gel-filtration through a Sephadex LH-20 column (30×1 cm) eluted with EtOH, to give **1** (15 mg).

3.3. Kaempferide 3-O-β-D-glucopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (*1*)

$[\alpha]_D^{25} = -16^\circ$ (MeOH; $c = 0.002$); FABMS (negative ion): m/z 769 $[M\text{-}H]^-$; HRFABMS: found m/z 769.2170, calc. for $C_{34}H_{42}O_{20}$ m/z 769.2191. UV spectra: Table 1; ^{1}H NMR and ^{13}C NMR spectra: Table 2.

3.4. Methanolysis of *1*: sugar analysis

A solution of **1** (1 mg) in anhydrous 2M HCl in MeOH (0.5 ml) was heated at 80°C in a stoppered reaction vial for 8 h. After cooling, neutralization with Ag_2CO_3 and centrifugation, the supernatant was evaporated to dryness under N_2. The residue was trimethylsilylated with TRISIL Z (Pierce Chemical Co.) for 15 min at room temp. GLC analysis gave peaks that co-eluted with those of the authentic methyltrisilglucoside [R_t (min): 37.6 and 42.8 (2 mol)] and methyltrisilrhamnoside [R_t (min): 9.3 (1 mol)]. GLC analyses were run using a Hewlett-Packard 5890 gas chromatograph equipped with a fused-silica column SBB-I Supelco (30 m×0.32 mm; i.d. 0.25 μm film) and a dual FID detector. Column temperature: 140°C; Injector and detector temperature: 200°C

3.5. Fungal material

Fusarium oxysporum f. sp. *dianthi*, race 2, isolate P 1121, was obtained by courtesy of Professor Garibaldi, Dipartimento VA.P.R.A., Università di Torino, Italy. Fungus was sub-cultured in Petri dishes on potato dextrose agar while, to obtain conidia production, explants were kept on liquid potato dextrose medium for 1 week as a shaken culture. Mycelium was removed from the liquid medium by filtration through nylon net and glass wool and the conidia, harvested by centrifugation, were transferred into fresh liquid medium to obtain a concentration of about 1×10^7 conidia/ml.

3.6. Evaluation of the fungitoxic activity

Known amounts (1, 2 and 5 μmol, respectively) of compound **1** were dissolved in EtOH, spotted on silica gel TLC plates, 20×20 cm and dried. The TLC plates were then sprayed with a potato dextrose agar solution, containing the *F. oxysporum* f. sp. *dianthi* conidial suspension, and incubated for 4 days in a moist chamber. At the end of the incubation period, the fungitoxic activity was evaluated and expressed, according to an already published evaluation scale (Niemann et al., 1992), as — (not present), ± (just visible), + (clearly present) and ++ (strong). A further assay was performed by adding 10, 50 and 100 μM of **1**, respectively, to the liquid potato dextrose medium containing the *F. oxysporum* f. sp. *dianthi* conidia. After 1, 2 and 4 days of incubation, the percentages of germinated conidia were respectively recorded. Before the statistical analysis, to normalize the percentage data, their transformation in angular values has been performed (arcsin x).

Acknowledgements

Financial support by Università del Molise (ex quota 60%) and by Ministero per le Politiche Agroalimentari are gratefully acknowledged. We thank Mr. Raffaele Troiano for skilful technical assistance.

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