Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 67 (2006) 2376–2379
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Malvone A, a phytoalexin found in *Malva sylvestris* (family Malvaceae)

Olga Veshkurova [a], Zamira Golubenko [a], Egor Pshenichnov [a], Irina Arzanova [a], Vyacheslav Uzbekov [a], Elvira Sultanova [a], Shavkat Salikhov [a], Howard J. Williams [b], Joseph H. Reibenspies [b], Lorraine S. Puckhaber [c], Robert D. Stipanovic [c,*]

[a] *A.S. Sadykov Institute of Bioorganic Chemistry, Academy of Science Uzbekistan, Tashkent, Uzbekistan*
[b] *Department of Chemistry, Texas A&M University, College Station, TX 77845, USA*
[c] *Southern Plains Agricultural Research Center, Agricultural Research Service, US Department of Agriculture, 2765 F&B Road, College Station, TX 77845, USA*

Received 30 March 2006; received in revised form 7 July 2006

Abstract

The isolation and structure of a phytoalexin, malvone A (2-methyl-3-methoxy-5,6-dihydroxy-1,4-naphthoquinone) is reported. Malvone A formation is induced in *Malva sylvestris* L. by the plant pathogen *Verticillium dahliae*. In a turbimetric assay for toxicity to *V. dahliae*, it had an ED_{50} value of 24 μg/ml. The structure of malvone A was determined by MS and NMR spectroscopy, and by X-ray crystallographic analysis. The X-ray analysis showed water molecules were located in channels that run along the *a*-axis.

Keywords: *Malva sylvestris*; Malvaceae; Gossypium; Cotton; *Verticillium dahliae*, Phytoalexin, Tetra-nor-sesquiterpene; 2-Methyl-3-methoxy-5,6-dihydroxy-1,4-naphthoquinone; Malvone A

1. Introduction

Antimicrobial compounds produced by plants when they are attacked by pathogens are termed phytoalexins. To increase the resistance of the cotton plant (*Gossypium*, Malvaceae) to pathogens, we are investigating phytoalexins produced in other members of the Malvaceae that are indigenous to Uzbekistan. During our search for compounds that are induced by the plant pathogen *Verticillium dahliae* in *Malva sylvestris* L., we observed the formation of several phytoalexins, the majority of them in very low concentrations. In view of the recent interest in *M. sylvestris*, as evidenced by the report by Cutillo et al. (2006), we now report the structure of a phytoalexin from this plant and its toxicity to *V. dahliae*.

2. Results and discussion

M. sylvestris was selected for study because it demonstrated resistance to the plant pathogen *V. dahliae*. The bark was removed from the stems of this plant and short pieces of stems were treated with a conidial suspension of the plant pathogen *V. dahliae*. After 72 h, the stems were extracted with an aqueous acetone solution containing ascorbic acid. These extracts were evaporated to near dryness and the extracts subjected to silica column chromatography. Fractions were spotted on silica TLC plates and the plates were bioassayed for their ability to reduce growth of the fungal plant pathogen *V. dahliae*. Those fractions that inhibited the growth of the pathogen were subjected to additional chromatographic procedures. Multiple spots were observed, but only one compound was separated in sufficient quantities for complete structural analysis.

The isolated compound was identified as 2-methyl-3-methoxy-5,6-dihydroxy-1,4-naphthoquinone (**1**) (Fig. 1)

* Corresponding author. Tel.: +1 979 260 9232; fax: +1 979 260 9319.
E-mail address: rdstip@cpru.usda.gov (R.D. Stipanovic).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.08.010

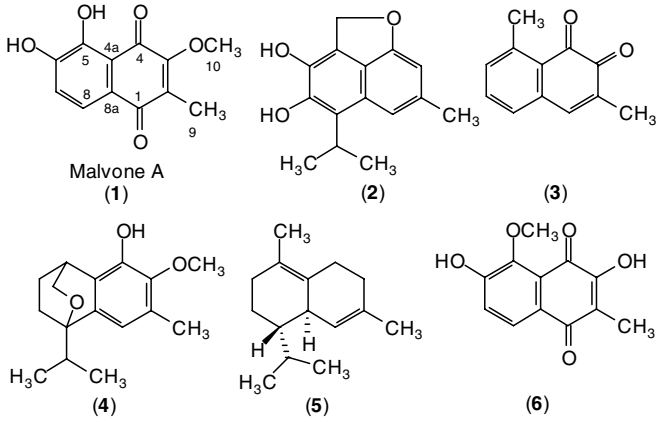

Fig. 1. Some terpenoids isolated from plants in the Malvaceae family.

for which we propose the trivial name malvone A. The structure was established based on spectroscopic data and ultimately on a single crystal X-ray analysis. In the mass spectrum, a parent ion was observed at m/z 234. The ^{1}H NMR spectrum was exceptionally simple with two coupled aromatic protons evident at δ 7.51 and 7.18, their coupling constant (8 Hz) indicated they were *ortho* with respect to one another. The only other peaks in the ^{1}H NMR spectrum were an O–CH_3 group on an sp^2 carbon (δ 4.08, s, 3H) and a CH_3 group on an sp^2 carbon (δ 1.98, s, 3H). The ^{13}C NMR spectrum indicated that there were 12 carbons in the molecule. Of these, two carbonyl groups were present probably as a quinone (δ 184.7 and 188.1). Additionally, three sp^2 carbons attached to oxygen (δ 158.4, 153.1 and 151.0) were evident; one of these was the carbon to which the O–CH_3 group was attached. Based on the molecular weight determined in the mass measurement, an additional two hydrogen atoms would give a molecular formula of $C_{12}H_{10}O_5$. The additional two hydrogens and two oxygens must be attached as hydroxyl groups. Considered *in toto*, the spectroscopic results indicated the molecule was a naphthoquinone. An HMBC experiment (see Table 1) established the position of the sp^2 carbon (δ 158.4, C-3) to which the O–CH_3 group was attached. It also showed that the CH_3 group was attached to the carbon at δ 135.3 (C-2), and that this carbon was between one of the carbonyl carbons (δ 184.7, C-1) and the sp^2 carbon to which the O–CH_3 group was attached (C-3). In the aromatic ring of the naphthoquinone, the proton at δ 7.51 was coupled to both of the quinone carbonyls (δ 188.1, C-4 and δ 184.7, C-1), with a stronger coupling to the carbon at 184.7 (C-1). These data indicate that the δ 7.51 proton was in a *peri* position and, since the two protons were *ortho* to one another, the two hydroxyl groups must also be *ortho* to one another with one in the *peri* position. Based on the spectroscopic results, structure **1** was a reasonable hypothetical structure. However, other structures such as structure **1** but with the hydroxyl groups at positions 7 and 8 instead of positions 5 and 6 could not be unequivocally excluded. Thus, a crystal was submitted for X-ray analysis. The X-ray analysis proved that the molecule had structure **1**. Furthermore, it showed that water molecules were located between molecules in channels that run along the *a*-axis (see Supplemental Material).

Table 1
NMR spectroscopic assignment of Malvone A (**1**) (acetone d_6)

Position	δ_H[a]	δ_C[b]	HMBC[c]
1		184.7	8, 9
2		135.3	9
3		158.4	9, 10
4		188.1	8
4a		116.2	8
5		151.0	7, 8
6		153.1	7, 8
7	7.18 *d* (8.0)	121.2	8
8	7.51 *d* (8.0)	121.4	7
8a		124.7	7
9	1.98 *s*	10.0	
10	4.08 *s*	61.8	

[a] ^{1}H chemical shift values are referenced to that of acetone δ 2.06, and are followed by multiplicity and coupling constants (J in Hz).
[b] ^{13}C chemical shifts are referenced to that of CH_3 in acetone δ 29.92.
[c] HMBC correlations are from ^{1}H to ^{13}C.

The toxicity of malvone A to *V. dahliae* conidia of isolate PM 7 was determined by a turbimetric assay previously described (Puckhaber et al., 1998). The ED_{50} value of malvone A was 24 μg/ml. Malvone A is not as toxic as desoxyhemigossypol (**2**) (ED_{50} 5.8 μg/ml), the most potent phytoalexin in cotton, or *o*-hibiscanone (**3**) (ED_{50}, 0.5 μg/ml) a phytoalexin found in kenaf (*Hibiscus cannabinus* L.) that is also in the Malvaceae family.

The biosynthetic origins of naphthoquinones in plants are intriguing. Govindachari et al. (1971) ascribed 1,8-dihydroxy-3-methylnaphthalene as the biosynthetic precursor of ancistroquinone (**4**) (Fig. 1) in *Ancistrocladus heyneanus* Wall. However, Davis and Essenberg (1995) showed that (+)-δ-cadinene (**5**) is the precursor of many of the terpenoid phytoalexins in *Gossypium* (Malvaceae). Naphthoquinones found in the Malvaceae such as *o*-hibiscanone from *H. cannabinus* and malvone A from *M. sylvestris* also may be derived from (+)-δ-cadinene by the oxidative loss of an isopropyl group in the case of *o*-hibiscanone or methyl and isopropyl groups in the case of malvone A. For example, compound **6**, which has also been isolated from *M. sylvestris* (Cutillo et al., 2006), may be biosynthetically related to malvone A.

To determine if malvone A was a phytoalexin, a time course investigation was performed. That is, 10-week-old *M. sylvestris* plants were inoculated between the first and second internode with either sterile water (control) or with the pathogen *V. dahliae*. Tissue was collected and frozen immediately after inoculation and after 1, 3, 5, 8, 15, 24, 48, 72 and 96 h. The tissues were extracted and subjected to HPLC analysis. Three different plants were sampled at each time period. The mean concentrations and standard errors at each time are shown in Fig. 2. Between 0 and 8 h after inoculation, both the control and the inoculated

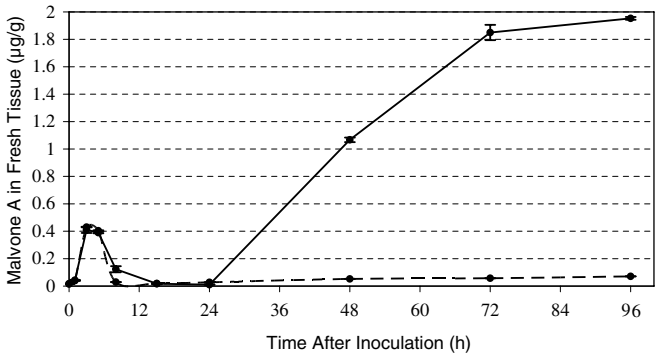

Fig. 2. Induced formation of malvone A (**1**) synthesis in plants inoculated with *Verticillium dahliae* (—) and with sterile water (----).

plants showed a sharp increase in Malvone A and then a return to baseline levels. After 8 h, the control plants maintained the baseline level of malvone A while the *V. dahliae* inoculated plants continually produced malvone A up to 96 h. These results indicate that malvone A is produced by the plant in response to the initial tissue injury from the inoculation. The return to baseline in both the control and inoculated plants indicates malvone A is metabolized by the plant to other unidentified compounds. This demonstrates the dynamic process occurring in the plant.

3. Experimental

3.1. General

Mps are uncorr. ^{1}H and ^{13}C NMR spectra were run on a Bruker ARX 500 instrument in acetone-d_6. EI-MS were obtained with a Thermo Electron DSQ [ion source 200 °C, scan rate 500 amu/s; scan range 50–550 amu] using a direct insertion probe. UV were recorded on a Hewlett–Packard 8453 diode array spectrometer, whereas IR spectra was obtained using on a Fourier transform Nicolet Magna IR 550 spectrometer. Turbidity measurements were performed on a Biotech Instruments Inc. ILX 808 Ultramicroplate Reader. Plants were grown outside in a lysimeter in soil that had been sterilized before planting.

3.2. Induction and extraction of phytoalexins for structure determination

Plants were grown for three months, then the stems were cut off just above the roots, and the bark was removed. The canes were cut into short sections (~20 cm), dipped into a conidial suspension of the plant pathogen *V. dahliae* (10^7 spores/ml) and then kept at room temperature in the dark. Non-inoculated stems served as a control. After 72 h, the stems were cut into 2 cm pieces, placed in acetone: 1% aqueous ascorbic acid (9:1, vol/vol), and soaked for 1 h with vigorous swirling every 15 min. The acetone solution was filtered through No. 42 Whatman filter paper and the acetone was evaporated under reduced pressure at 30 °C. The aqueous suspension was extracted three times with one-third volume of EtOAc. The organic phase was evaporated to near dryness.

3.3. Isolation of phytoalexins and bioassay for relative fungitoxicity

The crude extract (4 g) from the canes was applied to a silica gel 60 (Fluka) column (300*g*, 800 mm × 25 mm) and eluted with hexane:EtOAc in 10 increments beginning at 95:5 and ending at 50:50 with 5% step increases of EtOAc (500 ml/step). Fractions (75 ml) were collected and were spotted on Silufol UV 254 (200 mm × 200 mm; Kavalier, Czech Republic) TLC plates. The plates were developed with $CHCl_3$:MeOH:HOAc (96:3:1). Compounds were detected with UV light and bands containing the same components were combined to yield 28 samples to be tested for fungitoxicity. Fungitoxic activity was assayed by spotting the 28 samples on TLC plates. The plates were over sprayed first with a layer of potato dextrose agar then with a conidial suspension of *V. dahliae*. The plates were placed in a damp chamber at 23 °C for 48 h. Inhibition of fungal growth marked compounds with fungitoxic activity. Fraction 19 possessed the highest activity. This fraction was evaporated to dryness, dissolved in MeOH, and applied to a Zorbax Eclipse XDB C_8HPLC column (4.6 mm × 250 mm; Agilent Technologies). The column was developed with a linear 0.1% water H_3PO_4:MCCN gradient (10–100%, flow rate 1 ml/min). The effluent was monitored at 235 nm, fractions were collected, and their fungitoxicity evaluated. Active fractions were further purified by re-injecting them on the HPLC column using the same conditions.

3.4. Malvone A (*1*)

Red crystals, m.p. 169.5–172 °C; UV λ_{max}^{EtOH} nm (log ε): 201 (4.20), 223 (4.23), 270 (3.98), 295 (3.90), 448 (3.43), 598 (2.99); IR ν_{max}^{KBr} cm^{-1}: 1605, 1448, 1353, 1282, 1209, 1112; EIMS (direct insertion probe) 70 eV, *m/z* (rel. int.): 234 [M^+] (100), 219 (35), 204 (18), 191 (45), 188 (24), 176 (31), 173 (26), 163 (30), 161 (25), 160 (17), 145 (20), 137 (37), 136 (19), 135 (26), 108 (23), 89 (29), 79 (21), 77 (22), 55 (17), 51 (19); for ^{1}H and ^{13}C NMR (acetone-d_6), see Table 1.

3.5. X-ray analysis of Malvone A (*1*)

Crystal data for as$_{12}$: $C_{12}H_{12}O_6$, $M = 252.22$, red needle, $0.30 \times 0.01 \times 0.01$ mm, orthorhombic, space group $P2_12_12_1$ (No. 19), $a = 3.7095(11)$, $b = 13.568(4)$, $c = 41.337(9)$ Å, $V = 2080.5(10)$ Å^3, $Z = 8$, $D_{calc} = 1.610$ g/cm^3, $F(000) = 1056$, MWPC area detector, Cu Kα radiation, $\lambda = 1.54178$ Å, $T = 110(2)$ K, $2\theta_{max} = 120.0°$, 15 548 reflections collected, 2826 unique ($R_{int} = 0.5195$). The structure was solved and refined using the programs SHELXS-97 (Sheldrick, 1997a) and SHELXL-97 (Sheldrick, 1997b),

respectively. The program XSHELL was used as an interface to the SHELX programs, and to prepare the figures. Final GoF = 1.020, $R_1 = 0.0563$, $wR_2 = 0.0643$, R indices based on 616 reflections with $I > 2\sigma(I)$ (refinement on F^2), 328 parameters, 101 restraints. Lp and absorption corrections applied, $\mu = 1.119\ mm^{-1}$. Absolute structure parameter = 0(4) (Flack, 1983; Flack and Bernardinelli, 2000).

3.6. Induction of phytoalexins

Plants were grown in soil that had been sterilized, but in the open air. Plants that were approximately 10 weeks old were either mock inoculated between the 1st and 2nd internodes with sterile water or with a conidial suspension of the plant pathogen *V. dahliae* (10^7 spores/ml). At intervals from 0 h to 96 h, plant tissue (approximately 5 g) was harvested 1 cm above the inoculation site and the tissue was immediately frozen. Immediately prior to analysis, the tissue was cut into small pieces and extracted with acetone:H_2O (4:1) containing 1% ascorbic acid. The acetone was removed under vacuum and the aqueous phase was extracted 3 times with an equal volume of EtOAc. The EtOAc was evaporated under vacuum and the residue was dissolved in ACN and subjected to HPLC analysis.

Acknowledgement

This work was supported by a Grant from USDA/ARS Project # P-115.

Appendix A. Supplementary data

Crystallographic data have been deposited at the Cambridge Crystallographic Data Center (CCDC) (CCDC 603412). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.08.010.

References

Cutillo, F., D'Abrosca, B., DellaGreca, M., Fiorentino, A., Zarrelli, A., 2006. Terpenoids and phenol derivatives from *Malva silvestris*. Phytochemistry 67, 481–485.

Davis, G.D., Essenberg, M., 1995. (+)-δ-Cadinene is a product of sesquiterpene cyclase activity in cotton. Phytochemistry 39, 553–567.

Flack, H.D., 1983. On enantiomorph-polarity estimation. Acta Crystallogr. Sect. A 39, 876–881.

Flack, H.D., Bernardinelli, G., 2000. Reporting and evaluating absolute-structure and absolute-configuration determinations. J. Appl. Crystallogr. 33, 1143–1148.

Govindachari, T.R., Parthasarathy, P.C., Modi, J.D., 1971. Chemical investigations of *Ancistrocladus heyneanus* Wall.: Part IV – Structure and synthesis of ancistroquinone. Indian J. Chem. 9, 1042–1043.

Puckhaber, L.S., Stipanovic, R.D., Bell, A.A., 1998. Kenaf phytoalexins: toxicity of *o*-hibiscanone and its hydroquinone to the plant pathogens *Verticillium dahliae* and *Fusarium oxysporum* f. sp. *vasinfectum*. J. Agric. Food Chem. 46, 4744–4747.

Sheldrick, G., 1997a. SHELXL-97 program for crystal structure refinement, Institüt für Anorganische Chemie der Universität, Tammanstrasse 4, Gottingen, Germany.

Sheldrick, G., 1997b. SHELXS-97 program for crystal structure solution, Institüt für Anorganische Chemie der Universität, Tammanstrasse 4, Gottingen, Germany.