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DIANTHRAMIDES A AND B, TWO *N*-BENZOYLANTHRANILIC ACID DERIVATIVES FROM ELICITED TISSUES OF *DIANTHUS CARYOPHYLLUS*

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Key Word Index—*Dianthus caryophyllus*, Caryophyllaceae; dianthramides A and B; elicitation; phytoalexins; phenolic derivatives; amides.

Abstract—Two new phenolic derivatives, dianthramide A and B, were isolated from *Dianthus caryophyllus* tissues elicited with mycelial extracts of *Phytophthora parasitica*. The purified substances were identified on the basis of their spectral data and were characterized as *N*-salicyl-4-methoxyanthranilic acid (dianthramide A) and *N*-salicyl-4-hydroxyanthranilic acid methyl ester (dianthramide B). Dianthramides A and B co-occur in carnation tissues with the known phytoalexin dianthalexin.

INTRODUCTION

A previous study [1] of the response of *Dianthus caryophyllus* to the elicitation by a mycelial extract from *Phytophthora parasitica*, has shown that several highly fluorescent compounds accumulate as *de novo* metabolites. Only the two most important fluorescent compounds have been purified and given trivial names dianthramides (substance 6 in ref. [1] = dianthramide A, substance 1 in ref. [1] = dianthramide B). They have now been identified respectively as *N*-salicyl-4-methoxyanthranilic acid **1** and *N*-salicyl-4-hydroxyanthranilic acid methyl ester **2**.

Dianthalexin, a benzoxazinone phytoalexin **3**, has been already described [2, 3] as a major (non-fluorescent) component of carnation response to elicitation and to infection by *P. parasitica*.

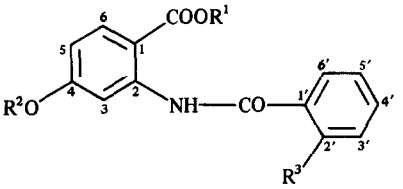
It has been demonstrated that dianthalexin and dianthramides exhibit *in vitro* antifungal activity against *P. parasitica* [unpublished results], so that these substances are considered as phytoalexins. Many different classes of antifungal compounds have been identified: pterocarpans, sesquiterpenes and polyacetylenes [4] and more recently biphenyls [5], stilbene-carboxylic acids [6], alkadienals [7] and isocoumarin [8], but nitrogen-containing substances, such as the phenolic amides dianthalexin and dianthramides, have been found only in oat (avenalumin I) [9, 10] and in barley (hordatines) [11]. The biological importance of dianthramides is discussed.

RESULTS AND DISCUSSION

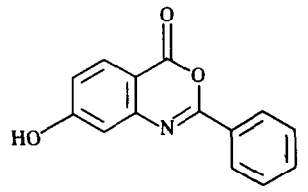
Dianthramides A and B were clearly identified on the basis of their spectral data. They had the same molecular weight (287) and MS showed typical fragment ions ArCOOH (*m/z* 121) and $C_8H_9NO_3$ (*m/z* 167). Analysis of ^{1}H NMR spectra (CD_3OD) confirmed the presence of two aromatic systems: first with δ6.90, 7.37 and 7.97 and second with δ6.65, 8.10 and 8.35 (dianthramide A); in fact, selective irradiation of the proton signal δ6.65 (in dianthramide A) resulted in a simplification of the proton signals δ8.10 and 8.35, so it was obvious that these three protons were part of the same aromatic system. Methylation of dianthramide A and B gave the same product **5**. In addition, a comparative study of synthetic **4** and **1–2** revealed quite similar spectra, and led to the formulae **1** and **2** for dianthramides A and B. These compounds differed only by the position of a methyl function on the anthranilic ring. IR spectra showed important absorption bands in the νC=O region, especially for the 1620 cm^{-1} band which could be explained by a carbonyl group strongly bound to an hydrogen bond. When the compound was deuterated, all these strong hydrogen bond interactions disappeared from the IR spectra.

The identification of these new compounds from *Dianthus caryophyllus* is of interest for the study of host–pathogen interactions: they were always found to be very closely related to the resistance of carnation to *Phytophthora parasitica* [1, 12]. They are chemically similar to dianthalexin [3], and as they appear during the carnation response to infection, we can consider that *N*-benzoylanthranilic acid metabolism is important for the general understanding of resistance to parasitism in carnation. Considering their antifungal properties against *P. parasitica* [unpublished results], they could be compared with classical phytoalexins, especially avenalumin [9, 10], but they may also be compared with the phenolic amides, e.g. the hydroxycinnamoyl amides, found in other families [13–17].

Phenolic amides were previously described as physiological indicators of flowering [13–17], of tuberization [18, 19] and of the hypersensitive response of tobacco to tobacco mosaic virus [20, 21]. In this respect, it is interesting that we have already observed that dianthramides accumulate in carnation tissues during the rhizogenesis of cuttings *in vivo* [1]. In addition, we have observed the accumulation of these compounds (after elicitation) in other species of Caryophyllaceae (*Dianthus*, *Saponaria*, *Gypsophila*), but never in other dicot families such as the Solanaceae [unpublished results].



1 R^1 = H, R^2 = Me, R^3 = OH dianthramide A
2 R^1 = Me, R^2 = H, R^3 = OH dianthramide B
4 R^1 = Me, R^2 = R^3 = H
5 R^1 = R^2 = Me, R^3 = OMe



3 dianthalexin

Table 1. ^{1}H NMR of dianthramides A and B (δ values)

Dianthramide A		Dianthramide B		
δ (CD_3OD)	J (cpm)	δ (CD_3OD)	δ (DMSO)	Proton n°
8.35 (*d*, 1H)	2.5	8.37 (*d*, 1H)	8.22	H-3
8.10 (*d*, 1H)	9	8.10 (*d*, 1H)	7.85	H-6
7.97 (*dd*, 1H)	1.5, 8	7.95 (*dd*, 1H)	7.90	H-6′
7.37 (*ddd*, 1H)	1.5, 8.8	7.55 (*ddd*, 1H)	7.35	H-4′
6.90 (*m*, 2H)	—	7.15 (*m*, 2H)	6.95 6.85	H-3′ H-5′
6.65 (*dd*, 1H)	2.5, 9	6.70 (*dd*, 1H)	6.57	H-5
3.90 (*s*, 3H)	—	4.00 (*s*, 3H)	3.80	OMe
			12.5 (*s*, 1H)	OH

Selective irradiation of 6.65 proton results in a simplification of 8.35 and 8.10 protons (dianthramide A). *s*, Singlet; *d*, doublet; *m*, multiplet.

Table 2. 62.9 MHz ^{13}C NMR of dianthramide B (δ values)*

δ (CD_3OD)	Carbon n°
170.9 and 171.2 (*s*)	2C=O
166.0 (*s*)	C-4
163.4 (*s*)	C-2′
144.8 (*s*)	C-2
136.5 (*d*)	C-4′
135.2 (*d*)	C-6
129.2 (*d*)	C-6′
121.3 (*d*)	C-5′
120.2 (*d*)	C-3′
118.2 (*s*)	C-1′
113.1 (*d*)	C-5
109.4 (*d*)	C-3
109.3 (*s*)	C-1
53.6 (*q*)	OMe

Run in CD_3OD. *s*, Singlet; *d*, doublet; *q*, quartet (OR data).
*Relative to TMS.

EXPERIMENTAL

Extraction of the compounds. Dianthus caryophyllus L. var Scania cuttings were used for elicitation. Elicitor was obtained as in [1]. Halves of cuttings (longitudinal sections from the apical to the basal part of cuttings) were dipped in elicitor (100 mg/l for 3 days (1 kg fr. wt), then homogenized in MeOH (2.5 l), filtered and washed twice with the same volume of MeOH. The total MeOH fraction was evaporated under vacuum to 50 ml, then diluted with H_2O (1 l) and extracted with EtOAc (3 × 1 l.). The H_2O fraction was discarded and EtOAc fractions were evaporated to dryness, then dissolved in MeOH (100 ml). Quantification of dianthramides A and B was performed by HPLC as in [12], in this system retention times were respectively 980 sec and 770 sec for the two substances, and by 2D-PC as in [1]. These two methods were regularly used for the recovery of substances throughout the purification.

Separation and purification of dianthramides A and B. The preceding MeOH extract was evaporated to dryness and dissolved in $CHCl_3$ (100 ml), then passed through a 5 cm × 30 cm silica gel column (Kieselgel 60, 0 2–0 5 mm, Merck). Elution was carried out with $CHCl_3$ (2 l.), $CHCl_3$–MeOH (98 2, 1 l.), $CHCl_3$–MeOH (95:5, 2 l.), then $CHCl_3$–MeOH (85 15, 1 l.) The successive fractions (200 ml) contained lipid material ($CHCl_3$), dianthramide B (end of $CHCl_3$, $CHCl_3$–MeOH, 98:2) and dianthramide A ($CHCl_3$–MeOH, 95.5) Dianthramides A and B were easily separated but still remained contaminated with chlorophyll. The fractions containing each substance were combined and evaporated to dryness, dissolved in $CHCl_3$ (100 ml), then chromatographed separately on a 5 cm × 30 cm PVPP column (polyvinylpolypyrrolidone, Fluka AG) equilibrated with $CHCl_3$. The extract containing dianthramide B was eluted with $CHCl_3$ (1 l.) then MeOH (3 l.); as dianthramide B is strongly

retained on PVPP, prolonged elution with MeOH is necessary, but provided a good purification. The fraction containing dianthramide B was evaporated to dryness, dissolved in MeOH (20 ml), then rechromatographed on a 2.5 × 40 cm PVPP column with MeOH (1.5 l.) as single developing solvent. Peak containing dianthramide B was evaporated to dryness and dissolved in MeOH (20 ml)

The extract containing dianthramide A was eluted with $CHCl_3$ (1 l.), MeOH (2 l.), MeOH–HOAc (99 5:0.5, 2 l.) and MeOH–HOAc (99·1, 1 l) The MeOH–HOAc (99:1) fraction contained dianthramide A and was evaporated to dryness to remove HOAc, and then dissolved in MeOH (20 ml). Before analysis (spectral data), dianthramide A and B were passed through a 3 × 40 cm Sephadex LH-20 column eluted with MeOH; peaks were collected, concentrated and dried under N_2 giving crystals of dianthramide A (25 mg) and dianthramide B (10 mg).

Spectral data. Spectral data of dianthramide A and B were compared with those of synthetic **4** [3]. Note that **4** is a degradation product of dianthalexin **3** by methanolysis [3]; this compound was found during the purification of dianthramide B, in the PVPP first MeOH fraction. UV spectral data were recorded in MeOH (with and without NaOH); IR were obtained either from KBr discs or by multiple reflection (FMIR). ^1H and ^{13}C NMR were achieved at 250 MHz in CD_3OD or DMSO. MS were carried out for $[M]^+$ determination, CHNO assay and MS chemical desorption with NH_3 Methylation of dianthramide A and B with CH_2N_2 gave the same compound **5** which was identified by MS and ^1H NMR.

Characterization of compounds. Dianthramide A, *N*-salicyl-4-methoxyanthranilic acid $C_{15}H_{13}NO_5$ UV λ_{max}^{MeOH} nm: 314, 245, 207, $\lambda_{max}^{MeOH+NaOAc}$ nm. unchanged; $\lambda_{max}^{MeOH+NaOH}$ nm: 319, 281, 239. IR ν_{max} cm^{-1}: 600–900 (aromatic ring), 1600 (C=O), 3450 (OH), 1375, 1280, MS at *m/z*· 287 $[M]^+$, 243 $[M-COO\cdot]$, 269 $[M-H_2O]$, 225 $[M-H_2O-COO\cdot]$, 167 $[M-ArCOO\cdot]$, 121 $[ArCOO\cdot]$. ^1H NMR (CD_3OD): δ8.35 (*d*, 1H), 8.10 (*d*, 1H), 7.97 (*dd*, 1H), 7.37 (*ddd*, 1H), 6 90 (*m*, 2H), 6.65 (*dd*, 1H), 3.90 (*s*, 3H). Table 1.

Dianthramide B, *N*-salicyl-4-hydroxyanthranilic acid methyl ester $C_{15}H_{13}NO_5$. UV λ_{max}^{MeOH} nm· 313, 277, 252, 206; $\lambda_{max}^{MeOH+NaOH}$ nm. 302, 269, 239, 219. IR ν_{max} cm^{-1}: 600–900 (aromatic ring), 1680, 1650, 1620 (C=O); 3250 (OH); 1255 (C_6H_5–O–). MS *m/z*: 287 $[M]^+$; 255 $[M-MeOH]$, 167 $[M-ArCOO]$, 135 $[M-ArCOO-MeOH]$, 121 $[ArCOOH]$. ^1H NMR (CD_3OD): δ8.37 (*d*, 1H), 8.10 (*d*, 1H), 7.95 (*dd*, 1H), 7.55 (*ddd*, 1H), 7.15 (*m*, 2H), 6.70 (*dd*, 1H), 4.00 (*s*, 3H); (DMSO): δ12.5 (*s*, 1H), 8.22 (*d*, 1H), 7.90 (*dd*, 1H), 7.85 (*d*, 1H), 7.35 (*ddd*, 1H), 6.95 and 6 85 (*m*, 2H), 6.57 (*dd*, 1H), 3.80 (*s*, 3H). Table 1. ^{13}C NMR (CD_3OD): δ170.9 (*d*), 171.2 (*s*), 166 (*s*), 163.4 (*s*), 144.8 (*s*), 136.5 (*d*), 135 2 (*d*), 129.2 (*d*), 121.3 (*d*), 120 2 (*d*), 118.2 (*d*), 113 1 (*d*), 109.4 (*d*), 109.3 (*s*), 53.6 (*q*). Table 2.

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