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DIANTHRAMIDES (*N*-BENZOYL AND *N-PARA*COUMARYLANTHRANILIC ACID DERIVATIVES) FROM ELICITED TISSUES OF *DIANTHUS CARYOPHYLLUS*

MICHEL PONCHET, JEAN FAVRE-BONVIN,* MARCELLE HAUTEVILLE† and PIERRE RICCI

INRA—Station de Botanique et Pathologie Vegetale BP 2078-F-06606 Antibes Cedex; *Laboratoire de Mycochimie, ICBMC, Universite Claude Bernard, Lyon I-69622 Villeurbanne Cedex, France; †Laboratoire de Biochimie Vegetale, ICBMC Universite Claude Bernard, Lyon I 69622 Villeurbanne Cedex, France

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Key Word Index—*Dianthus caryophyllus*; Caryophyllaceae; dianthramides; elicitation; benzoic acid; salicylic acid; β-resorcylic acid; 4-methoxysalicylic acid; *p*-coumaric acid; anthranilic acid.

Abstract—The dianthramides, isolated from *Dianthus caryophyllus* L. elicited tissues, were identified from their spectral data (MS, ^{1}H NMR and UV). All these compounds were amides between a benzoic acid (benzoic, salicylic, β-resorcylic, 4-methoxysalicylic) or a cinnamic acid (*p*-coumaric) moiety, and an anthranilic acid moiety (anthranilic, 4-hydroxyanthranilic, 4-methoxyanthranilic). These dianthramides appeared during the elicitation of carnation as compounds of less importance compared with the amounts of the known dianthalexin and dianthramides A and B.

INTRODUCTION

During the elicitation of *Dianthus caryophyllus* cuttings by a mycelial wall extract from *Phytophthora parasitica* carnation strain, it has been shown that several substances accumulate in the plant tissues [1, 2]. The three major compounds: dianthalexin [3] and dianthramides A and B [4] have been already described. These phytoalexin-like substances, (e.g. dianthramide A) have been found to be fungitoxic towards a wide range of micromycetes [5], belong to the same structural type based on benzoic and anthranilic moities, linked together by an amide bond. The carboxylic function of the anthranilic acid can be free (dianthramide A), methylesterified (dianthramide B) or implicated in a benzoxazinone heterocycle (dianthalexin). In the present paper we report the purification and the identification of other *de novo* metabolites found in the carnation elicited tissues and previously described as highly fluorescent compounds [1].

These new substances have the same general structure combining a phenolic acid (i.e. benzoic or cinnamic acids) to an anthranilic acid in which the carboxylic function is free. They are all given the trivial name dianthramide, and in order to simplify their nomenclature we propose a new terminology where the previously named dianthramides A and B as in [4] are redundant (Table 1). The appellation of dianthalexin for the benzoxazinone structure is maintained.

RESULTS AND DISCUSSION

The positions of the substitutions on the aryl rings were determined by means of ^{1}H NMR (Table 2). Dianthramide B (DB) had a benzoyl ring (free of any substitution) as was demonstrated by ^{1}H NMR and MS. MS gave $[M]^+$ at *m/z* 241 and two strong fragment ions $[ArCO]^+$ (*m/z* 105) and $[Ar]^+$ (*m/z* 77). Dianthramide S (DS) had an OH on the benzoyl ring when compared with DB. MS showed $[M]^+$ at *m/z* 257 and typical fragment ions $[Ar(OH)–CO]^+$ at *m/z* 121 and $[Ar(OH)]^+$ at *m/z* 93. ^{1}H NMR showed the OH on the position 2′ (salicoyl ring). Dianthramide R (DR) presented an additional OH on the benzoyl ring with regard to DS: $[M]^+$ at *m/z* 273 and $[Ar(2OH)–CO]^+$ at *m/z* 137. The two OH functions were located in 2′ and 4′ (β resorcoyl ring) according to ^{1}H NMR. The dianthramide P (DP) structure was more difficult to define. EIMS on the natural product gave *m/z* 265 as the ion of higher mass, which was confirmed by CIMS, but CIMS on the TMSi derivative led to $[MH]^+$ at *m/z* 428 which demonstrated that the compound was easily dehydrated and that two free OH existed in the structure. ^{1}H NMR showed a cinnamoyl ring *p*-disubstituted with an OH in 4′ position. ^{1}H NMR of hydroxydianthramide B (HDB) showed a benzoyl ring free of substitution whereas MS on the TMSi derivative gave $[M]^+$ at *m/z* 401 that indicated two free OH in the structure. An OH in 4 position was confirmed by ^{1}H NMR. Hydroxydianthramide S (HDS) MS on the TMSi derivative gave $[M]^+$ at *m/z* 489 and $[MH]^+$ at *m/z* 490 (CI), so that it was obvious that three free OH existed in the structure. One OH was located on the benzoyl ring: $[Ar(OTMSi)–CO]^+$ at *m/z* 193 and placed on the 2′ position (salicoyl ring) by ^{1}H NMR. The MS data of hydroxydianthramide R (HDR) TMSi derivative exhibited $[M]^+$ at *m/z* 577 containing four OTMSi functions. Two of the four OTMSi were located on the benzoyl ring which was confirmed by the fragment ion $[Ar(2OTMSi)–CO]^+$ at *m/z* 281. These two OH (on the natural product) appeared clearly on the position 2′ and 4′ (β resorcoyl ring) by ^{1}H NMR. Methoxydianthramide B (MDB) ^{1}H NMR showed the presence of an OMe and of a benzoyl ring free of substitution. MS of the natural compound gave: $[M]^+$ at *m/z* 271, $[ArCO]^+$ at *m/z* 105 and $[Ar]^+$ at *m/z* 77 which confirmed the ^{1}H NMR data. MS of methoxydianthramide R (MDR) indicated the presence of two OH and one OMe in the structure.

Table 1. Structure and new nomenclature of the dianthramides found

Benzoic or cinnamic moiety \ Anthranilic acid moiety		Anthranilic acid $R^1=R^2=H$		4-Hydroxyanthranilic acid $R^1=OH$ $R^2=H$	
Benzoic (B) $R^{1'}=R^{2'}=H$	**1**	Dianthramide B (DB)	**II***	Hydroxydianthramide B (HDB)	
Salicylic (S) $R^{1'}=OH$ $R^{2'}=H$	**1**	Dianthramide S (DS)	**6c***	Hydroxydianthramide S (HDS)	6'*
β resorcylic (R) $R^{1'}=R^{2'}=OH$	**1**	Dianthramide R (DR)	**5'***	Hydroxydianthramide R (HDR)	**2***
4-Methoxysalicylic (M) $R^{1'}=OH$ $R^{2'}=OMe$	**1**	—‡		—	
p-Coumaric (P) $R^{1'}=H$ $R^{2'}=OH$	**2**	Dianthramide P (DP)	**3***	—	

*Number assignment in ref. [1]. †Denomination in ref. [4]. ‡Compound not yet confirmed.

Table 2. ^{1}H NMR of dianthramides

DB		DS		DR		DP		HDB	
δ	J	δ	J	δ	J	δ	J	δ	J
8.95 (*dd*)	*om*	8.81 (*dd*)	*om*	8.79 (*dd*)	*om*	8.87 (*dd*)	*om*	8.53 (*d*)	*m*
7.69 (*ddd*)	*oom*	7.72 (*ddd*)	*oom*	7.70 (*ddd*)	*oom*	7.6–7.7 *br* signal		OH	
7.23 (*ddd*)	*oom*	7.28 (*ddd*)	*oom*	7.25 (*ddd*)	*oom*	7.15 (*ddd*)	*oom*	6.67 (*dd*)	*om*
8.22 (*dd*)	*om*	8.22 (*dd*)	*om*	8.20 (*dd*)	*om*	8.13 (*dd*)	*om*	8.06 (*d*)	*o*
8.05 (*dd*)	*om*	OH		OH		7.57 (*d*)	*o*	8.03 (*dd*)	*om*
br signal		7 (*dd*)	*om*	6.41 (*d*)	*m*	6.9 (*d*)	*o*	*br* signal	
7.57–		7.52 (*ddd*)	*oom*	OH		OH		7.55–	
7.67		7.02 (*ddd*)	*oom*	6.52 (*dd*)	*om*	6.9 (*d*)	*o*	7.67	
8.05 (*dd*)	*om*	7.86 (*dd*)	*om*	7.71 (*d*)	*o*	7.57 (*d*)	*o*	8.03 (*dd*)	*om*
						7.64	15.5 Hz		
						6.61	15.5 Hz		

o; *ortho*, *ca* 8.5 Hz. *m*; meta, *ca* 2Hz. Multiplicities in parentheses. *Structure **2** only.

$[Ar(2OH)-CO]^+$ at *m/z* 137 fragment ion and ^{1}H NMR allowed the assignment to the position 2' and 4' on the benzoyl ring (β resorcoyl ring) for these two OH. NOE experiments in ^{1}H NMR confirmed the 4 position for the OMe on the anthranilic ring. ^{1}H NMR of methoxydianthramide M (MDM) demonstrated the presence of an aromatic ring 1,2,4-trisubstituted and of two OMe in the structure. MS of the TMSi derivative with $[M]^+$ at *m/z* 461 (EI) or $[MH]^+$ at *m/z* 462 (CI) supported ^{1}H NMR results. The strong $[Ar(OMe, OTMSi)-CO]^+$ at *m/z* 223 fragment ion showed the presence of one OH and one OMe on the benzoyl ring. A NOE double assay in ^{1}H NMR clearly localized the two OMe in positions 4 and 4'.

All the purified dianthramides were clearly identified on the basis of their spectral data. In order to support these results all the purified compounds were compared with synthetic substances obtained as in [6]. The total identity between purified and synthetic products (MS, ^{1}H NMR, UV, chromatographic characteristics) was the final confirmation of their structure.

According to the data presented in this or previous papers [3, 4, 6] we can summarize the general spectral rules concerning the dianthramides. UV data: all the dianthramides with an OH or an OMe in position 4 on the anthranilic ring showed a strong absorption peak at about 250 nm, while the dianthramides with an anthranilic ring exhibited a less absorbant peak at about 270 nm. In addition, only the 2'-OH substituted dianthramides had an important absorption peak at about 310 nm. Fluorescence: the fluorescence under UV (312 nm) was typical of the benzoyl or *p*-coumaroyl ring: benzoic (pale silver blue), salicylic (strong green-yellowish), β resorcylic and 4-methoxysalicylic (strong violet) and *p*-coumaric

in carnation elicited tissues (structures **1** and **2**)

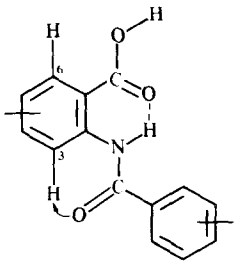
4-Hydroxyanthranilic acid Methylester $R^1=OH$ $R^2=Me$		4-Methoxyanthranilic acid $R^1=OMe$ $R^2=H$	
Hydroxydianthramide B methylester (HDBM)	1b*	Methoxydianthramide B (MDB)	I*
Hydroxydianthramide S methylester (HDSM)		Methoxydianthramide S (MDS)	
dianthramide B†	1*	dianthramide A†	6*
—‡		Methoxydianthramide R (MDR)	4*
—		Methoxydianthramide M (MDM)	5*
—		—‡	

solvent [$(CD_3)_2CO$ with *ca* 50 μl of TFA]

HDS		HDR		MDB		MDR		MDM		
δ	J	δ	J	δ	J	δ	J	δ	J	H
8.39 (*d*)	*m*	8.33 (*d*) *m*		8.70 (*d*)	*m*	8.46 (*d*)	*m*	8.46 (*d*)	*m*	3
OH		OH		3.94 (*s*) OMe		3.91 (*s*) OMe		3.90 (*s*) OMe		4
6.73 (*dd*)	*om*	6.66 (*dd*)	*om*	6.77 (*dd*)	*om*	6.79 (*dd*)	*om*	6.81 (*dd*)	*om*	5
8.10 (*d*)	*o*	8.03 (*d*)	*o*	8.13 (*d*)	*o*	8.14 (*d*)	*o*	8.12 (*d*)	*o*	6
OH		OH		8.06 (*dd*)	*om*	OH		OH		2′
7 (*dd*)	*om*	6.38 (*d*)	*m*	*br* signal		6.42 (*d*)	*m*	6.50 (*d*)	*m*	3′
7.50 (*ddd*)	*oom*	OH		7.57–		OH		3.80 (*s*) OMe		4′
7.02 (*ddd*)	*oom*	6.48 (*dd*)	*om*	7.67		6.52 (*dd*)	*om*	6.61 (*dd*)	*om*	5′
7.86 (*dd*)	*om*	7.68 (*d*)	*o*	8.06 (*dd*)	*om*	7.69 (*d*)	*o*	7.75 (*d*)	*o*	6′
										CO-CH*
										CH-Ar*

For nomenclature see Table 1.

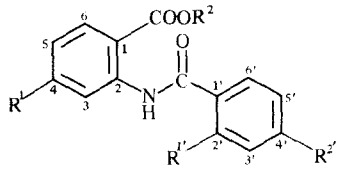
(pale silver blue and strong green at 366 nm + NH_3). MS data: MS of the natural compounds gave a strong [M $-H_2O$] fragment ion so it was obvious that dianthramides were easily dehydrated. ^{1}H NMR: all the results obtained by ^{1}H NMR were coherent, however the anthranilic protons shifts were characteristic. The most important shift (δ value) was expected for the H-6 proton, as was observed for the cyclic compound dianthalexin [3], but in fact the most deshielded proton was H-3. This observation was of interest in so far as it permitted the following spatial structure for dianthramides:



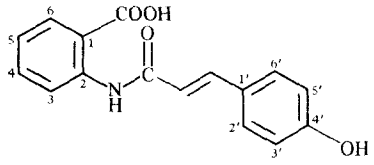
In this structure H-3 was influenced by the C=O from the benzoyl ring; meanwhile H-6 was less influenced by the COOH of the anthranilic ring which is engaged in a hydrogen bond with the NH.

The structures described in this paper are now of interest. It brings a new insight on the high diversity of dianthramide metabolism in carnation. In addition the structure of five or more other minor compounds from elicited tissues still remains unknown.

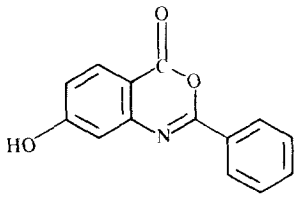
The biological importance of dianthramides is not yet well established. They were strongly associated with the resistance of carnation to fungal infections [1, 5, 7].



1 (for complete structure see Table 1)



2 DP



3 Dianthalexin

Moreover they were found to be closely related to some physiological mechanisms: rhizogenesis-scar process [1], phytotoxicity necrosis and hyperhydric stress (unpublished results). The new structures described above will contribute to a better knowledge of dianthramide metabolism not only in the entire plant but also in the plant tissue, organ and cell cultures. For example the production of dianthalexin and MDS was already described during the elicitation of cell cultures [8]. Are these substances related to the phytoalexin concept or involved in a larger response of the plant to the environmental stresses, such as a high ability to recover its normal physiological and morphogenetic functions after a stress?

The conjugation of anthranilic acid with a phenolic acid (i.e. benzoic or cinnamic acid) was earlier described for the avenalumins [9, 10] and the present results confirm the place of such amides in plant biochemistry. The presence of these amides in both oats (Monocot) [9, 10] and in the Diantheae (Dicot) [4] is remarkable: especially if we consider the singular position of the Caryophyllaceae in the dicotyledons. There is one other biochemical link between the Gramineae and the Caryophyllaceae; the presence of ferulic acid bound to the cell walls [11, 12].

EXPERIMENTAL

Preparation of dianthramides extract. The preparation of the extract containing the different dianthramides was achieved as in [4]. Analytical HPLC and 2D-PC as described in [4] were

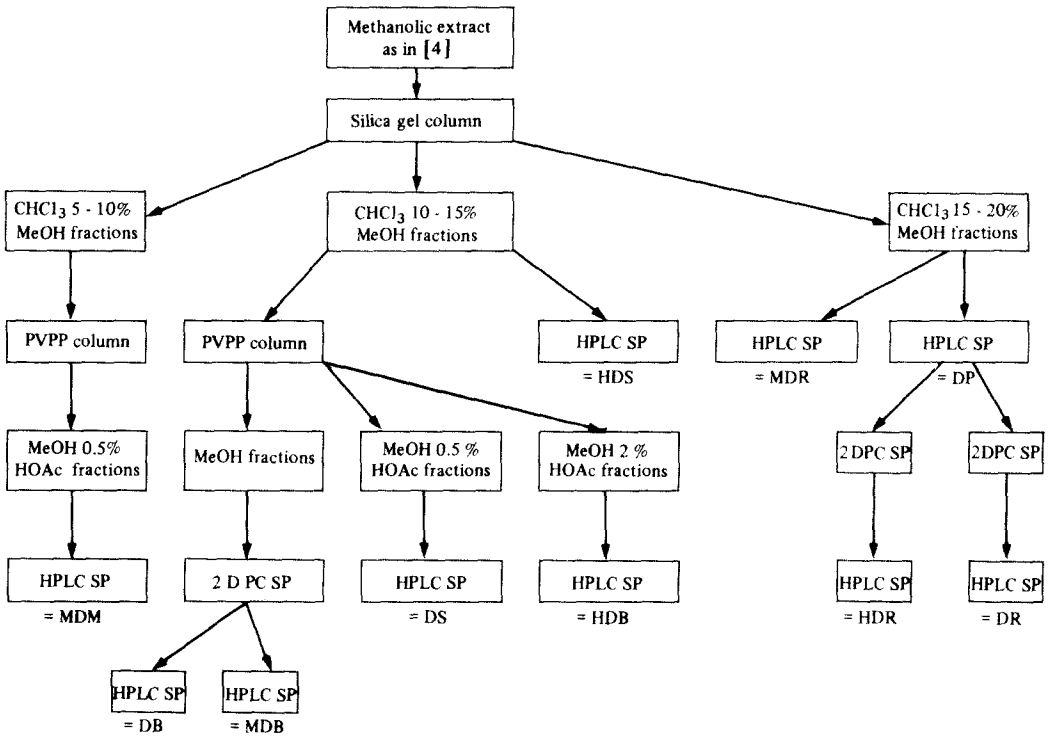


Fig. 1. General scheme of the isolation and purification of the dianthramides. (Technical details are given in the Experimental) SP = Semipreparative.

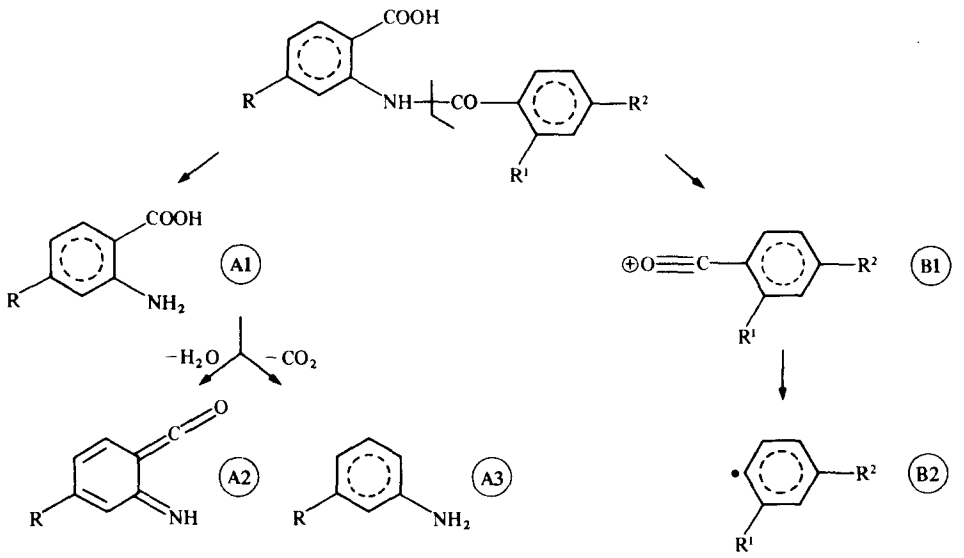
routinely used for the identification and visualization of the dianthramides throughout the purification.

Separation and purification of dianthramides. The general scheme of dianthramides purification is described in Fig. 1. The characteristics of the chromatographic methods used in this scheme are as follows: Silica gel column: the methanolic extract was chromatographed through a 40 × 3 cm silica gel column (Kieselgel 60, 0.065–0.2 mm, Merck). The elution was performed with $CHCl_3$ (2 l), $CHCl_3$–MeOH (98:2, 1.5 l), $CHCl_3$–MeOH (95:5, 1.5 l), $CHCl_3$–MeOH (85:15, 2 l) and $CHCl_3$–MeOH (80:20, 1 l). Fractions of 150 ml were collected, combined on the basis of their similar dianthramide content, evapd to dryness and dissolved in the appropriate solvent for the next chromatographic step. PVPP column: a 60 × 3 cm PVPP column (Polyvinylpolypyrrolidone, Fluka AG) was used. The PVPP was decanted in MeOH to eliminate fine particles prior to use. The column was equilibrated in $CHCl_3$ and after settling the sample, elution was carried out with $CHCl_3$ (1 l), MeOH (2 l) MeOH–HOAc (99.5:0.5, 2 l), MeOH–HOAc (99:1, 2 l) and MeOH–HOAc (98:2, 1.5 l). Fractions of 150 ml were collected and evapd to dryness after an eventual combination of the fractions showing the same dianthramide content. 2D-PC: the semiprep. 2D-PC was achieved as for the analytical one [4]. HPLC semiprep.: a Lichrosorb RP_{18} 7 μm 250 × 10 mm column protected by a 20 × 4 mm precolumn was used. The column was equilibrated with MeOH–H_2O–HOAc (70:29.4:0.6) at a flow rate of 3 ml/min and was allowed to remain at these isocratic conditions. UV detection was monitored at 254 nm. Peaks were collected during the successive injections, then evapd to dryness till HOAc was completely removed. The pure substances, obtained by means of HPLC, were dissolved in 1 ml of MeOH, then dried under N_2 to give white-yellowish crystals. Each compound was purified in amounts ranging from 1 to 5 mg. Note that all the chromatographic steps, described above, could be repeated twice in order to improve the separation and the compound purity.

Spectral data: UV spectral data were recorded in MeOH, ^{1}H NMR was performed at 350 MHz in $(CD_3)_2CO$ with *ca* 50 μl of TFA. TFA was necessary to obtain good resolution: without this, all the spectra remained very coarse, with broad signals in place of well resolved peaks. This demonstrated that traces of impurities co-occurred with the substances, probably as residual colloidal chromatographic adsorbents (^{1}H NMR of synthetic products did not need TFA [6]). NOE assays were required to localize the positions of OMe on the different rings and selective irradiations of proton signals were used to confirm the proton relationships. MS of the natural compounds was achieved in EI or CI (NH_3). When the $[M]^+$ or $[MH]^+$ was not clearly recognized, MS was carried out on the TMSi derivatives with direct introduction or through GC-MS either in EI and in CI(NH_3). High resolution (HR) MS was used on $[M]^+$ or on the principal fragment ions to determine their precise CHNO content. The spectral data of dianthramides were compared with those obtained for natural or synthetic compounds described in [3, 4].

Characterization of compounds. ^{1}H NMR data are given in Table 2. All the UV spectra were recorded in MeOH.

Dianthramide B (DB). *N*-Benzoylanthranilic acid $C_{14}H_{11}NO_3$ UV λ_{max} nm: 308, 267, 277. EIMS *m/z* (rel. int.): 241 $[M^+, C_{14}H_{11}NO_3]$ (21); 223 $[M-H_2O]$ (16); 197 $[M-COO]$ (3); 179 (8); 174 (8); 148 (13); 119 $[A_2, R=H]$ (13); 105 $[ArCO]^+$ (100); 77 $[Ar]^+$ (48); 51 (13); 44 (20). *Dianthramide S* (DS), *N-salicoylanthranilic* acid $C_{14}H_{11}NO_4$ UV λ_{max} nm = 316, 268, 234. EIMS *m/z* (rel. int.): 257 $[M^+, C_{14}H_{11}NO_4]$ (44); 239 $[M-H_2O]$ (34); 149 (16); 137 $[A_1, R=H]$ (100); 121 $[B_{11}, R_1=OH, R_2=H]$ (70); 120 (32); 119 $[A_2, R=H]$ (63); 93 $[B_2, R_1=OH, R_2=H]$ (15); 92 (17); 65 (26); 44 (26), *Dianthramide R* (DR), *N*-β-resorcoylanthranilic acid $C_{14}H_{11}NO_5$ UV λ_{max} nm = 317, 273. EIMS *m/z* (rel. int.): 273 $[M^+, C_{14}H_{11}NO_5]$ (19); 255 $[M-H_2O]$ (30); 137 $[B_1, R_1=R_2=OH]$ (100); 119 $[A_2, R=H, C_7H_5NO]$ (41); 109 (18); 92 (10); 69 (50); 51 (22); 44 (100). *Dianthramide P* (DP), *N*-*p*-coumarylanthranilic acid $C_{16}H_{13}NO_4$ UV λ_{max} nm = 329, 300. EIMS *m/z* (rel. int.): 265 $[M-H_2O, C_{16}H_{11}NO_3]$ (5); 238 $[M-45]$ (6); 137 $[A_1, R=H]$ (13); 119 $[A_2, R=H, C_7H_5N]$ (48); 119 [HO-Ar-CH=CH, C_8H_7O] (16); 107 (12); 94 $[C_6H_6O]$ (45); 44 (100). GC-EIMS TMSi *m/z* (rel. int.): 337 $[M-TMSiOH]$ (100); 322 $[M-TMSiOH-15]$ (22); 219 (25); 146 (12); 119 (9); 90 [TMSiOH] (14); 75 (16); 73 (67). CIMS TMSi *m/z* (rel. int.): 428 $[MH]^+$ (100). *Hydroxydianthramide B* (HDB). *N*-Benzoyl-4-hydroxyanthranilic acid $C_{14}H_{11}NO_4$ UV λ_{max} nm = 300, 272, 250. GC-EIMS TMSi *m/z* (rel. int.): 401 $[M^+, C_{20}H_{27}NO_4Si_2]$ (36); 386 $[M-15]$ (11); 311 $[M-90]$ (26); 296 $[311-15]$ (49); 284 $[M-COOTMSi]$ (13); 105 $[ArCO]^+$ (100); 77 $[Ar]^+$ (48); 75 (20); 73 (42). *Hydroxydianthramide S* (HDS). *N*-Salicoyl-4-hydroxyanthranilic acid $C_{14}H_{11}NO_5$ UV λ_{max} nm = 314, 252. EIMS *m/z*: 255 $[M-H_2O, C_{14}H_9NO_4]$. GC-EIMS TMSi *m/z*



(rel. int.): 489 $[M]^+$ (1); 474 $[M-15]$ (20); 399 $[M-90]$ (1); 384 $[399-15]$ (59); 193 $[B_1, R_1=OH, R_2=H]$ (100); 151 (10); 75 (15); 73 (54). *Hydroxydianthramide R* (HDR). *N*-β-Resorcoyl-4-hydroxyanthranilic acid $C_{14}H_{11}NO_6$ UV λ_{max} nm = 313, 287, 258. GC-EIMS TMSi *m/z*: 577 $[M^+, C_{26}H_{43}NO_6Si_4]$. GC-CIMS TMSi *m/z*: 578 $[MH]^+$. *Methoxydianthramide B* (MDB). *N*-Benzoyl-4-methoxyanthranilic acid $C_{15}H_{13}NO_4$ UV λ_{max} nm = 306, 272, 250. EIMS *m/z* (rel. int.): 271 $[M^+, C_{15}H_{13}NO_4]$ (27); 253 $[M-H_2O]$ (5); 227 $[M-COO^-]$ (3); 149 $[A_2, R=OMe]$ (26); 131 (8); 105 $[Ar-CO]^+$ (100); 77 $[Ar]^+$ (43). *Methoxydianthramide R* (MDR). *N*-β-Resorcoyl-4-methoxyanthranilic acid: $C_{15}H_{13}NO_6$ UV λ_{max} nm = 316, 262. EIMS *m/z* (rel. int.): 303 $[M^+, C_{15}H_{13}NO_6]$ (2); 285 $[M-H_2O, C_{15}H_{11}NO_5]$ (13); 259 $[M-COO^-]$ (18); 216 (5); 167 $[A_1, R=OMe]$ (10); 149 $[A_2, R=OMe]$ (30); 137 $[B_1, R_1=R_2=OH]$ (40); 123 $[A_3, R=OMe, C_7H_9NO]$ (100); 122 (11); 94 (17); 93 (12); 81 (19). *Methoxydianthramide M* (MDM). *N*-4-Methoxysalicoyl-4-methoxyanthranilic acid $C_{16}H_{15}NO_6$ UV λ_{max} nm = 315, 263. GC/EIMS TMSi *m/z* (rel. int.): 461 $[M^+, C_{22}H_{31}NO_6Si_2]$ (1); 446 $[M-15]$ (12), 371 $[M-90]$ (2); 356 $[371-15]$ (24); 223 $(B_1, R_1=OTMSi, R_2=OMe]$ (100); 180 (14); 149 $[A_2, R=OMe]$ (10); 75 (15); 73 (34). CIMS *m/z*: 318 $[MH]^+$. CIMS TMSi *m/z*: 462 $[MH]^+$.

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