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Dianthramide glucosides from tissue cell cultures of *Delphinium staphisagria* L.

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Abstract

Tissue cell cultures of *Delphinium staphisagria* L. produced three dianthramide glucosides *N*-(2′-β-glucopyranosylsalicyl)-5-hydroxyanthranilic acid methyl ester, *N*-(2′-β-glucopyranosyl-5′-methoxysalicyl)-5-hydroxyanthranilic acid methyl ester and *N*-(2′-β-glucopyranosyl-5′-hydroxysalicyl)-5-hydroxy-6-methoxyanthranilic acid methyl ester, together with known methyl esters of *N*-salicylanthranilic acid and *N*-(2′-β-glucopyranosyl-5′-hydroxysalicyl)-5-hydroxyanthranilic acid. Structures of the glucosides were established by MS, 1-D and 2-D NMR techniques.
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Keywords: *Delphinium staphisagria*; Ranunculaceae; Tissue cell culture

1. Introduction

The literature contains numerous articles dealing with tissue cultures of plants producing bioactive alkaloids but references to such studies of *Aconitum* and *Delphinium* species, some of whose pharmacological properties have been known since ancient times, are sparse (Sawada et al., 1980; Hasegawa et al., 1983; Cervelli, 1987; Hatano, 1988). The alkaloid content of seeds of *Delphinium staphisagria* L. has been studied extensively (Pelletier et al., 1988, and references cited therein) but recent work from Tenerife on the aerial parts (Díaz et al., 2000) of this relatively accessible species which yielded a host of alkaloids, some of them new, suggested that this plant might also be suitable for a tissue culture study. In the event undifferentiated callus of *D. staphisagria* produced not the usual diterpenoid and norditerpenoid alkaloids characteristic of this species and the genus as a whole but several anthranilamides **1a–5** of a type trivially named dianthramides (Ponchet et al., 1988, Ponchet et al., 1984; Niemann, 1993) which have been implicated as phytoalexins.

2. Results and discussion

^{1}H and ^{13}C NMR spectra (Tables 1 and 2) of compound **1**, $C_{15}H_{13}NO_4$, indicated the presence of two vicinally disubstituted aromatic rings, one (ring A) an ortho-substituted methyl benzoate, the other (ring B) based on the chemical shifts of the four hydrogen and six carbon atoms, a salicylate moiety. The presence of a nitrogen atom and the chemical shifts made it likely that rings A and B were linked by an amide, i.e. that **1**

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Table 1
^{1}H NMR spectroscopic data of compounds **1–5a**[a]

Proton	**1**[**]	**2**[**]	**2a**[*]	**3**[**]	**3a**[*]	**4**[**]	**4a**[*]	**5**[**]	**5a**[*]
NH	12.21*s*	11.25 *s*	11.73 *s*	11.2 *s*	11.77 *s*	11.2 *s*	11.75 *s*	9.85 *s*	10.29 *s*
H-3	8.80 (8.5) *brd*	8.52 *brd* (9)	8.92 *d* (9)	8.51 *d*	(9) 8.88 *d* (9.7)	8.50 *d* (9)	8.92 *d* (9)	7.50 *d* (8.8)	8.20 *d* (9)
H-4	7.62 *td* (8, 1.6)	7.11 *dd* (9, 3)	7.30 *dd* (9, 3)	7.12 *dd* (9, 3)	7.30 *dd* (9.2, 2.8)	7.11 *dd* (9, 3)	7.30 *dd* (9, 2.8)	7.00 *d* (8.8)	7.20 *d* (9)
H-5	7.17 *td* (8, 1.0)								
H-6	8.10 *dd* (8, 1.6)	7.46 *d* (3.0)	7.83 *d* (3)	7.47 *d* (3)	7.82 *d* (2.8)	7.46 *d* (3.0)	7.83 *d* (2.8)		
H-3′	7.03 *brd* (8.5)	7.38 *d* (8.5)	7.24 *dd* (7.6, 1.6)	7.26 *d* (9)	7.26 *d* (8.9)	7.35 *d* (9.0)	7.20 *d* (9.0)	7.32 *d* (9.0)	7.2 *d* (9.0)
H-4′	7.45 *td* (8, 1.5)	7.49 *td* (8.6, 1.8)	7.45 *td* (7.6, 1.6)	6.96 *dd* (9, 3)	7.20 *dd* (8.9, 2.9)	7.06 *dd* (9, 3)	6.99 *dd* (9, 3)	6.98 *dd* (9, 3)	7.2 *dd* (9, 3)
H-5′	6.98 *td* (8, 1.0)	7.16 *brt* (7.5)	7.21 *td* (7.6, 1.6)						
H-6′	7.83 *dd* (8, 1.5)	7.91 *brt* (8)	7.95 *dd* (7.7, 1.6)	7.38 *d* (3)	7.70 *d* (2.8)	7.45 *d* (3)	7.46 *d* (3.2)	7.48 *d* (3)	7.82 *d* (3)
OMe[b]	3.99 *s*	3.88 *s*	3.98 *s*	3.90 *s*	3.99 *s*	3.90 *s*	3.99 *s*	3.89 *s*	3.97 *s*
OMe(5′)[b]					3.82 s	3.83 s			
OMe(6)						3.87 s	3.86 s		
H-1″		5.12 *d* (8)	5.15 *d* (8)	4.94 *d* (8)	5.13 *d* (8)	4.98 *d* (8)	5.03 *d* (8)	4.99 *d* (8)	5.22 *d* (8.2)
H-2″		3.62 *t* (8)	5.41 *dd* (9.2, 7.9)	3.57 *t* (9)	5.42 *dd* (9, 8)	3.59 *t* (8)	5.36 *t* (9.5, 8)	3.65 *t* (8.3)	5.48 *t* (8.5)
H-3″		3.51 *t* (9)	5.23 *t* (9)	3.49 *t* (9)	5.21 *t* (9)	3.50 *t* (9.8)	5.20 *t* (9.5)	3.51 *t* (9)	5.27 *t* (9.5)
H-4″		3.43 *t* (9)	5.17 *t* (9)	3.39 *t* (9)	5.16 *t* (9.5)	3.40 *t* (9.5)	5.14 *t* (9.5)	3.48 t (9)	5.20 *t* (9.5)
H-5″		3.54 *ddd* (9.5, 4.2, 2.6)	3.88 *ddd* (9.5, 5.6, 2.5)	3.46 *ddd* (9.5, 2.5, 2.5)	3.86 *ddd* (9.5, 5.6, 2.5)	3.66 *m*	3.80 *m*	3.52 *m*	3.85 *m*
H-6a″		3.85 *dd* (8.7, 2.6)	4.29 *dd* (12, 5.6)	3.84 *m*	4.27 *dd* (12.3, 5.6)	3.84 *m*	4.27 *dd* (12.3, 5.5)	3.89 *m*	4.25 *dd* (12.3, 5.3)
H-6b″		3.67 *dd* (8.7, 4.2)	4.20 *dd* (12.2, 2.5)	3.64 *m*	4.19 *dd* (12.3, 2.5)	3.60 *m*	4.17 *dd* (12.3, 2.4)	3.69 *m*	4.17 *dd* (12.3, 2.3)
ArOAc, 5[b]			2.29 *s*		2.29 *s*		2.30 *s*		2.33 *s*
ArOAc, 5′[b]					2.30 *s*				2.31 *s*
Glu-Oac 2″			1.74 *s*		1.74 *s*		1.74 *s*		1.83 *s*
Glu-Oac 3″[b]			1.94 *s*		1.94 *s*		1.94 *s*		2.01 *s*
Glu-Oac 4″[b]			2.03 *s*		2.03 *s*		2.03 *s*		2.04 *s*
Glu-Oac 6″[b]			2.05 *s*		2.05 *s*		2.05 s		1.97 *s*

[a] Chemical shifts are in ppm relative to TMS; coupling constants are in Hz.
[b] Intensity three protons.
[*] Run at 400 MHz in $CDCl_3$.
[**] Run at 500 MHz in acetone-d_6.

Table 2
^{13}C NMR spectra of compounds **1–5a**

Carbon	**1****	**2****	**2a***	**3****	**3a***	**4****	**4a***	**5****	**5a***
CO(1)	169.3 *s*	167.8 *s*	167.2 *s*	167.7 *s*	167.2 *s*	167.7 *s*	167.1 *s*	166.5 *s*	166.3 *s*
C 1	115.7 s	118.6 s	117.3 s	118.8 s	117.4 s	118.9 s	117.3 s	122.6 *s*	118.4 s
C 2	140.9 *s*	133.2 *s*	138.8 *s*	133.2 *s*	138.5 *s*	133.0 *s*	138.7 *s*	128.2 *s*	135.7 *s*
C 3	120.9 *d*	123.6 *d*	122.1 *d*	123.6 *d*	122.2 *d*	123.7 *d*	122.7 *d*	121.2 *d*	118.7 *d*
C 4	134.8 *d*	120.9 *d*	127.3 *d*	120.9 *d*	127.3 *d*	120.9 *d*	127.3 *d*	118.1 *d*	126.6 *d*
C 5	123.2 *d*	152.8 *s*	145.3 *s*	152.8 *s*	145.4 *s*	153.0 *s*	145.3 *s*	147.5 *s*	140.5 *s*
C 6	131.1 *d*	116.3 *d*	123.6 *d*	116.3 *d*	123.7 *d*	116.4 *d*	123.7 *d*	145.4 *s*	151.1 *s*
CO(1′)	169.2 *s*	164.1 *s*	164.2 *s*	164.2 *s*	163.0 *s*	164.2 *s*	164.1 *s*	163.5 *s*	162.4 *s*
C 1′	115.1 *s*	125.4 *s*	126.3 *s*	126.8 *s*	127.2 *s*	126.6 *s*	127.3 *s*	125.1 *s*	126.6 *s*
C 2′	162.3 *s*	155.4 *s*	154.1 *s*	148.5 *s*	151.7 *s*	149.2 *s*	148.1 *s*	148.9 *s*	151.9 *s*
C 3′	118.8 *d*	116.6 *d*	116.7 *d*	119.0 *d*	117.9 *d*	118.9 *d*	119.3 *d*	119.1 *d*	118.2 *d*
C 4′	134.7 *d*	132.7 *d*	132.7 *d*	119.2 *d*	125.9 *d*	118.3 *d*	119.3 *d*	119.4 *d*	126.2 *d*
C 5′	119.3 *d*	122.5 *d*	123.9 *d*	152.8 *s*	146.5 *s*	155.1 *s*	155.9 *s*	152.8 *s*	146.6 *s*
C 6′	126.8 *d*	130.6 *d*	131.5 *d*	116.2 *d*	124.5 *d*	114.7 *d*	114.6 *d*	116.7 *d*	124.8 *d*
MeO[CO(1)]	52.7 *q*	52.0 *q*	52.5 *q*	52.0 *q*	52.5 *q*	52.0 *q*	52.6 *q*	51.9 *q*	52.8 *q*
MeO (5′)					55.2 *q*	55.8 *q*			
MeO (6)							60.89 *q*	62.1 *q*	
C 1″		102.1 *d*	100.2 *d*	103.2 *d*	100.5 *d*	103.1 *d*	101.3 *d*	103.6 *d*	100.7 *d*
C 2″		73.5 *d*	70.8 *d*	73.6 *d*	70.8 *d*	73.6 *d*	70.9 *d*	73.5 *d*	70.9 *d*
C 3″		76.7 *d*	72.9 *d*	76.8 *d*	72.9 *d*	76.9 *d*	73.0 *d*	77.0 *d*	72.7 *d*
C 4″		70.2 *d*	68.2 *d*	70.4 *d*	68.1 *d*	70.4 *d*	68.3 *d*	70.3 *d*	67.9 *d*
C 5″		77.1 *d*	72.2 *d*	77.1 *d*	72.2 *d*	77.1 *d*	72.1 *d*	77.3 *d*	72.4 *d*
C 6″		61.6 *t*	62.0 *t*	61.8 *t*	62.0 *t*	61.8 *t*	62.0 *t*	61.7 *t*	61.8 *t*
ArOAc, 5			20.9 *q*, 169.3 *q*		20.9 *q*, 169.3 *s*		20.9 *q*, 169.3 *s*		20.7 *q*, 168.7 *s*
ArOAc 5′			20.13 *q*, 169.1 *s*		20.9 *q*, 169.2 *s*		20.13 *q*, 169.1 *s*		21.0 *q*, 169.*3 s*
Gluc-OAc 2″			20.46 *q*, 170.1 *s*		20.13 *q*, 169.1 *s*		20.46 *q*, 170.1 *s*		20.3 *q*, 168.9 *s*
Gluc-OAc 3″			20.5 *q*, 169.4 *s*		20.46, 170.1 *s*		20.5 *q*, 169.4 *s*		20.5 *q*, 170.5 *s*
Gluc-OAc 4″			20.54 *q*, 170.4 *s*		20.50 *q*, 169.4 *s*		20.54 *q*, 170.4 *s*		20.5 *q*, 169.3 *s*
Gluc-OAc 6″			20.54 *q*, 170.4 *s*		20.5 *q*, 170.2 *s*				

* Run at 100 MHz in $CDCl_3$.
** Run at 100 MHz in acetone d_6.

was formed by a combination of two units, anthranilic and salicylic acid, and was the methyl ester of *N*-salicylic acid. Especially the downfield shifts of the amide and OH protons at δ 12.21 and 12.18 could be ascribed to intramolecular hydrogen bonding between the amide hydrogen and the carbomethoxy carbonyl on the one hand and the amide carbonyl and the phenolic hydroxyl on the other. Also the abnormally low chemical shift of H-3 at δ 8.80 could be attributed to its location in the same plane as that of the amide carbonyl. Assignments were verified by analysis of HSQC, COSY and HMBC (Table 3) experiments. Substance **1** is the methyl ester of dianthramide 5, the latter having been previously isolated from in vitro cultures of *Dianthus caryophyllus* (carnation) by Ponchet et al. (1988). Compound **1** itself has been reported in the patent literature without details as possessing anti-inflammatory and antifungal activity (Hsi, 1967).

The ^{1}H NMR spectrum of **2** (Table 1), $C_{21}H_{13}NO_{10}$, also exhibited two well differentiated aromatic spin systems, one similar to that of the salicylate moiety of **1** although the low field phenolic hydroxyl signal of **1** seemed to be replaced by the signal of an α-D-glucoside, the second that of a methyl anthranilate substituted at the C-4 or C-5 position by a hydroxyl function. The ^{13}C NMR spectrum is listed in Table 2. Three bond correlations (Table 3) between the anomeric proton of the glucoside unit and a singlet at δ 155.4 (C-2′ of the salicylate) and between H-6′ of the salicylate at δ 7.91 and carbon singlets at δ 164.1 (carbonyl of amide) and 155.4 confirmed that the glucose unit was linked to the former hydroxyl of the salicylate, while a correlation between the methoxy signal at δ 3.88 and a signal at δ 167.8 showed that the latter corresponded to the carbonyl of the ester function of the anthranilate whose signal also correlated with a doublet at δ 7.46 (J = 7.11, J = 9.3 Hz) at δ 7.11 and a *d* (J = 9 Hz) at δ 8.52. Hence the hydroxyl of the anthranilate portion was situated on C-5. Acetylation of **2** afforded a pentaacetate **2a** whose ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) displayed the expected chemical shifts following the introduction of acetate functions on C-5 of the anthranilate and C-2′, C-3′, C-4; and C-6′ of the glucoside portion.

The mass spectrum of **3**, $C_{21}H_{23}NO_{11}$, with a base peak at *m*/*z* 167 corresponding to $C_6H_9NO_3$, the methyl 5-hydroxyanthranilic acid fragment, indicated the presence of a new hydroxyl group in the salicylate moiety. This was confirmed by the ^{1}H and ^{13}C NMR spectra

Table 3
HMBC data for compounds **1** and **2a–5a**

H	**1**	**2a**	**3a**	**4a**	**5a**
NH	CO(1′), C-1, C-3	CO(1′), C-1, C-3	CO(1′), C-1, C-3	CO(1′), C-1, C-3	CO(1′), C-1, C-3
H-3	C-l, C-5	C-l, C-5	C-l, C-5	C-l, C-5	C-l, C-5
H-4	C-2, C-6	C-2, C-6	C-2, C-6	C-2, C-6	C-2, C-6
H-5	C-l, C-3				
H-6	CO(1), C-2, C-4.	CO(l), C-2, C-5	CO(l), C-2, C-5	CO(1), C-2, C-4	
H-3′	C-l′, C-5′	C-l′, C-5′	C-l′, C-5′	C-l′, C-5′	C-l′, C-5′
H-4′	C-2′, C-6′	C-2′, C-6′	C-2′, C-6′	C-2′, C-6′	C-2′, C-6′
H-5′	C-l′, C-6′	C-l′, C-3′			
H-6′	CO(l′), C-2′, C-4′	CO(l′), C-2′, C-4′	CO(l′), C-2′, C-4′, C-5′	CO(l′), C-2′, C-4′	CO(l′), C-2′, C-4′
MeO[CO(l)]	CO(l)	CO(l)	CO(l)	CO(l)	CO(l)
MeO(5′)				C(5′)	
OMe(6)					C-6
H-l″		C-2″, C-3″	C-2″, C-3″	C-2″, C-3″	C-2″, C-3″
H-2″		C-1″, C-3″, CO(2″)	C-l″, C-3″, CO(2″)	C-l″, C-3″, CO(2″)	C-1″, C-3″, CO(2″)
H-3″		C-2″, C-4″, CO(3″)	C-2″, C-4″ CO(3″)	C-2″, C-4″ CO(3″)	C-2″, C-4″, CO(3″)
H-4″		C-3″, C-6″, CO(4″)	C-3″, C-6″ CO(4″)	C-3″, C-6″ CO(4″)	C-3″, C-6″, CO(4″)
H-5″		C-6″	C-6″	C-6″	C-6″
H-6a″		C-4″, C-5″, CO(6″)	C-4″, C-5″, CO(6″)	C-4″, C-5″, CO(6″)	C-4″, C-5″, CO(6″)
H-6b″	C-5″	C-5″	C-5″	C-5″	
ArOAc, 5	CO(5)	CO(5)	CO(5)	CO (5)	
ArOAc, 5″		CO(5″)		CO (5″)	
Glu-Oac 2″	CO(2″)	CO(2″)	CO(2″)	CO(2″)	
Glu-Oac 3″	CO(3″)	CO(3″)	CO(3″)	CO(3″)	
Glu-Oac 4″	CO(4″)	CO(4″)	CO(4″)	CO(4″)	
Glu-Oac 6″	CO(6″)	CO(6″)	CO(6″)	CO(6″)	

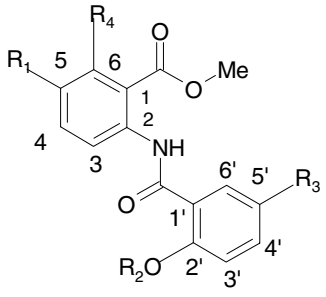
(Tables 1 and 2) which differed from those of **2** only in the absence of signals for the former H-5′, the conversion of the former C-5′ doublet to a singlet at δ 152.8 and the appearance of H-3′, H-4′ and H-6′ as an AMX system at δ 7.29 (*d*, 9 Hz), 6.96 (*dd*, $J = 9.3$ Hz) and 7.38 (*d*, $J = 3$ Hz). The attachment of the glucose unit to C-2′ was confirmed by three bond correlations (Table 3) between the anomeric proton at δ 4.94 and C-2′ at δ 148.5, the latter correlating with the signals of H-4′ and H-6′. H-6 further correlated with the amide carbonyl at δ 164.2 and with C-5′ at δ 152.8. The 6.9 ppm downfield shift of the latter relative to its shift in **2** is due to the newly present C-5′-OH group. Acetylation of **3** furnished a hexaacetate whose ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) confirmed that acetylation had resulted in esterification of the C-5- and C-3′-hydroxyls of the parent dianthramide as well as the four hydroxyls of the glucoside portion. A Japanese patent (Murayama, 1995) has claimed the synthesis of **3**, without details, as one of a large number of benzanilides which can stimulate hair growth.

The molecular formula of **4**, $C_{22}H_{25}NO_{11}$, and the NMR spectra (Tables 1 and 2) indicated that one of the two hydroxyl groups of **3**, that on C-5′, had been replaced by a methoxide. This was confirmed by HMBC and ROESY (Table 3) experiments. Thus one of the two –OMe signals, that at δ 3.82, correlated with the *dd* of H-4′ at δ 7.06 and the *d* of H-6′ at δ 114.7 and C-2′ at δ 149.2 while H-6′ was further correlated with signals at δ 164.0 (CO-1′), 118.3 (C-4′) and 149.2 (C-2′), the last exhibiting a three bond correlation with the anomeric proton of the glucose unit. Acetylation afforded a pentaacetate one of whose acetate units exhibited a signal at δ 2.30 characteristic of an acetate on an aromatic ring. Chemical shifts of C-2, C-4 and C-6 in pentaacetate **4a** when compared with the shifts in **4** confirmed that the hydroxyl group in ring A of **4** was located on C-5.

The ^{1}H NMR spectrum (Table 1) of the remaining dianthramide **5**, $C_{22}H_{22}NO_{12}$, was at first glance very similar to that of **4** as was its behavior on TLC in different solvent systems. However closer examination indicted that one half on the anthramide portion was 1,2,5,6-tetra-substituted with an AB system of H-3 and H-4 at δ 7.47 and δ 7.04 ($J = 8.5$ Hz). Presence in the mass spectrum of a fragment at *m*/*z* 197 (22% $C_9H_{11}NO_4$) confirmed that ring A contained an extra methoxy group evidenced in the ^{1}H NMR spectrum by a signal at δ 3.87 and in the ^{13}C NMR spectrum by a signal at δ 60.8. HMBC experiments (Table 3) showed a three bond correlation between this –OMe frequency and C-6 at δ 7.49 while C-3 at δ 121.2 exhibited a three bond correlation with the amide proton at δ_H 9.8, thus confirming the substitution pattern in ring A. Acetylation of **5** afforded a hexaacetate **5a** whose ^{1}H NMR spectrum exhibited signals of two acetates on an aromatic ring and four aliphatic acetates. As in the case of compounds **4** and **4a**, changes on acetylation in the

chemical shifts of C-2, C-4 and C-6 in ring A and changes on acetylation in the chemical shifts of C-2′, C-4′ and C-6′ confirmed the location on C-5 of the free hydroxyl group in ring A and locations of the free hydroxyl group and the glucoside on C-5′ resp. C-2′ of ring B.

To the best of our knowledge dianthramides which are generally considered to be phytoalexins have been isolated previously only from infected members of the Caryophyllaceae, primarily from carnations (Niemann, 1993), and naturally occurring dianthramide glycosides are so far unknown. Dianthramides **3–5** also differ from previously described members of this group by carrying a hydroxyl or methoxyl group at C-5′ rather than at C-2′ or C-4′ (Niemann et al., 1991, 1992). The formation of dianthramide glucosides in callus tissue of a *Delphinium* species appears to be unprecedented and may be a response to unknown pathogens.



1 R_1, R_2, R_3, R_4 = H
2 R_1= OH, R_2 = Glc, R_3, R_4 = H
3 R_1, R_3 = OH, R_2 = Glc, R_4 = H
4 R_1 = OH, R_2 = Glc, R_3 = OMe, R_4 = H
5 R_1, R_3 = OH, R_2 = Glc, R_4 = OMe

3. Experimental

3.1. General experimental procedures

Optical rotations were determined using a Perkin–Elmer-241 polarimeter with a 1 dm cell. UV spectra were measured in MeOH using a Perkin–Elmer-550 SE spectrophotometer. IR spectra were recorded on a Bruker IFS-5 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX-400 or AMX-500 spectrometers in $CDCl_3$, or $CD_3{\cdot}COCD_3$; δ values in ppm relative to internal TMS, J values in Hz. EIMS and exact mass measurements were determined on a Micromass Autospec instrument at 70 eV. SiO_2 Merck (art. 7734) and Maacherey–Nagel (Polygram® Sil G/uv 254) was used for column chromatography (CC) and TLC, respectively. Sephadex LH-20, Pharmacia (ref. 17-0090-01). HPLC separations were performed on a JASCO Pu-980 series pumping system equipped with a JASCO UV-975 ultraviolet detector and with a Waters Kromasil® Si 5 μm (10 × 250 mm) column; flow rate of mobile phase 2 ml/min with EtOAc–MeOH, 49:1. Spots on chromatograms were detected with Dragendorff's reagent.

3.2. Plant material

D. staphisagria L plants used for callus induction were grown in the greenhouse of the Department of Biología Vegetal of the Universidad de La Laguna. Optimal growth was observed with a photoperiod of 13/11 h (day/night), at 71.6% relative humidity and in a range of 28 and 16 °C during the day and night periods. The authenticity of plant material was certified by Professor Julian Molero Briones, Botany Department, Faculty of Pharmacy, Universidad de Barcelona. The callus was induced from leaves of *D. staphisagria* in 1999 by using Murashige and Skoog's (MS) medium, supplemented with ANA (2.0 mg/l) and kinetin (0.5 mg/l). Callus typically appeared within 3 weeks when the explants were cultured at 25 ± 1 °C with 16 h light and 8 h dark periods. Young and healthy callus was subcultured at 4 week intervals, four sequential subcultures being made. If differentiated structures such as roots and shoots were observed after 27 ± 1 days, those were manually separated, dried at 60 °C for 72 h and milled separately.

3.3. Extraction and isolation of dianthramides

Undifferentiated callus tissues (dry weight 48.4 g) were extracted with EtOH–H_2O (4:1) at room temperature for 7 days. Filtration and removal of solvent under reduced pressure afforded a crude extract (5.6 g) which was adsorbed on Si gel (30 g 70–230 mesh) and subjected to flash chromatography using hexane (3 l), EtOAc (3 l) and MeOH (3 l) to furnish 350 mg, 2.7 g and 1.4 g of residues in the respective eluates. The residues from the hexane and MeOH frs were discarded. The EtOAc fraction was applied to a Sephadex LH-20 column (eluent hexane–CH_2Cl_{12}–MeOH 1:2:3) to afford 30 frs of 25 ml each which were grouped into 4 subfrs F-1 (frs 1–10), F-2 (fr 11), F-3 (frs 12–17) and F-4 (frs 18–30). The residues from F-1 (450 mg) and F-4 (1.5 g) consisted of polar mixtures which were discarded when attempts at further purification failed to yield homogeneous material. Fraction F-2 (60.9 mg) on rechromatography over Si gel column (2 × 14 cm) using EtOAc–MeOH, 24:1 as eluent furnished in subfrs 6–8, of **1** (5 mg) and in subfrs 17–26, of **2** (12 mg). HPLC of F-3 (280.7 mg) gave **2** (16 mg, R_t 30 min), **3** (60.5 mg, R_t 24 min,), **4** (20.3 mg, R_t 15 min) and **5** (14.8 mg, R_t 18 min).

3.4. N-salicylanthranilic acid methyl ester (*1*)

Amorphous material; HREIMS *m/z* 271.0855 (calc. for $C_{15}H_{13}NO_4$, 271.0844); EIMS *m/z* 271 (M^+, 54.6), 239 (19), 151 (100), 121 (29), 119 (41), 105 (5), 93 (7).

3.5. N-(2′-β-glucopyranosylsalicyl)-5-hydroxyanthranilic acid methyl ester (*2*)

Amorphous; $[\alpha]_D^{25}$ −22.9° (*c* 0.25, MeOH); UV (MeOH) λ_{max} nm 207 (log ε 4.31), 236 (log ε 4.0), 281 (log ε 3.82), 355 (log ε 3.5); IR ν_{max}^{NaCl} 3344, 1693, 1648, 1600, 1525, 1447, 1307, 1234 1073 cm^{-1}; for ^1H, and ^{13}C NMR spectra, see Tables 1 and 2; HREIMS *m/z* 449.1340 (calc. for $C_{21}H_{23}NO_{10}$, 449.1322); EIMS *m/z* 449 (M^+, 0.2), 287 (M^+–$C_6H_{10}O_5$, 29), 256 (M^+–$C_6H_{10}O_5$–OMe, 13), 167 (100), 135 (44.7), 121 (63), 107 (12), 93 (7).

Acetylation of (**2**) (4 mg) with Ac_2O (1 ml) in pyridine (0.5 ml) at rt overnight, work-up in the usual fashion and chromatography of the product over Si gel (hexane-EtOAc, 1:1) afforded the pentaacetate **2a** (3.5 mg) as an amorphous solid, $[\alpha]_D^{25}$ −109.5 (*c* 0.44, MeOH); UV (MeOH) λ_{max} nm 212 (log ε 4.55), 231 (log ε 4.39), 271 (log ε 4.1), 316 (log ε 3.9); IR ν_{max}^{NaCl} 1758, 1663, 1600, 1525, 1449, 1370, 1301, 1227, 1074, 1041, 757 cm^{-1}; for ^1H, and ^{13}C NMR spectra, see Tables 1 and 2; HREIMS *m/z* 659.1816 (calc. for $C_{31}H_{33}NO_{15}$, 659.1850); EIMS *m/z* 659 (M^+, 0.91), 617 ($M^+ + H$–CH_3CO, 1.9), 451 (0.45), 368 (0.3), 331 (32), 287 (7.4) 255 (2), 169 (100), 167 (28), 145 (6.4), 139 (7.1), 127 (18.5), 121 (40), 109 (58).

3.6. N-(2′-β-glucopyranosyl-5′-hydroxysalicyl)-5-hydroxyanthranilic acid methyl ester (*3*)

Amorphous material; $[\alpha]_D^{25}$ −11.2° (*c* 1.9, MeOH); UV (MeOH) λ_{max} (log ε) nm 215 (4.39), 237 (4), 280 (3.8), 319 (3.7); IR ν_{max}^{NaCl} 1611, 1531, 1309, 1233, 1074 cm^{-1}; for ^1H, and ^{13}C NMR spectra, see Tables 1 and 2; HRFABMS *m/z* 488.1198 (calc. for $C_{21}H_{23}NO_{11}Na$, 488.1168), 466.1332 (calc. for $C_{21}H_{24}NO_{11}$, 466.1349, $M^+ + H$); EIMS *m/z* 303 (M^+–$C_6H_{10}O_5$, 42), 271 (M^+ –$C_6H_{10}O_5$–MeOH, 85), 167 (100), 137 (54.8), 135 (45), 107 (14).

Acetylation of **3** (14 mg) with Ac_2O (4 ml) and pyridine (2 ml) as above, with chromatography of the product over Si gel (hexane-EtOAc, 3:2) afforded the hexaacetate (12.5 mg) as an amorphous solid, $[\alpha]_D^{25}$ −88.1 (*c* 2.8, MeOH); UV (MeOH) λ_{max} (log ε) nm: 213 (4.6), 231 (4.4), 271 (4.1), 315 (3.9); IR ν_{max}^{NaCl} 3282, 2955, 1755, 1668, 1594, 1519, 1417, 1369, 1208, 1033, 908, 834, 790 cm^{-1}; for ^1H, and ^{13}C NMR spectra, see Tables 1 and 2; HRFABMS *m/z* 740.1897 (calc. for $C_{33}H_{35}NO_{17}Na$, 740.1802), 718.1931 (calc. for $C_{33}H_{36}NO_{17}$, 718.1983); EIMS *m/z* 387 (M^+–$C_{14}H_{19}O_9$, 12), 345 (17), 331 (40), 313 (7), 312 (6), 303 (8), 271 (16), 229 (3.5), 211 (3.6), 209 (7), 169 (100), 167 (47), 137 (18), 127 (16), 109 (49).

3.7. N-(2′-β-glucopyranosyl-5′-methoxysalicyl)-5-hydroxyanthranilic acid methyl ester (*4*)

Amorphous solid; $[\alpha]_D^{25}$ −14.2° (*c* 0.22, MeOH); UV (MeOH) λ_{max} (log ε) nm: 216 (4.6), 241 (4.17), 281 (3.95), 322 (3.88); IR ν_{max}^{NaCl} 1691, 1653, 1603, 1498, 1438, 1306, 1235, 1071, 935, 885, 789 cm^{-1}; for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRFABMS *m/z* 502.1319 (calc. for $C_{22}H_{25}NO_{11}Na$, 502.1325, $M^+ + Na$); 480.1581 (calc. for $C_{22}H_{26}NO_{11}$, 480.1551, $M^+ + H$); FABMS *m/z* 502 ($M^+ + Na$, 10.9), 480 ($M^+ + H$, 18), 317 (M^+–$C_6H_{11}O_5$, 16.5), 307 (19.5), 289 (10), 281 (8), 176 (16), 167 (11.5), 151 (24).

Acetylation of **4** (10 mg) with Ac_2O (3 ml) and pyridine (1.5 ml), as for the hexaacetate above furnished the pentaacetate **4a** (9 mg) as an amorphous solid, $[\alpha]_D^{25}$ −67.8 (*c* 0.17, MeOH); UV (MeOH) λ_{max} nm (log ε) 218 (4.5), 240 (4.17), 269 (4.32), 313 (3.8), 340 (3.2); IR ν_{max}^{NaCl} 1757, 1661, 1601, 1526, 1441, 1370, 1227, 1070, 1039, 983, 756 cm^{-1}; for ^1H, and ^{13}C NMR spectra, see Tables 1 and 2; HREIMS *m/z* 689.2034 (calc. for $C_{32}H_{35}NO_{16}$, 689.1955), EIMS *m/z* 689 (M^+, 0.1), 647 (0.2), 615 (0.2), 359 (M^+–$C_{14}H_{19}O_9$, 10), 331 (23), 317 (7.7), 285 (19), 270 (13.2), 209 (5.8), 169 (100), 167 (37.5), 151 (24).

3.8. N-(2′-β-glucopyranosyl-5′-hydroxysalicyl)-5-hydroxy-6-methoxyanthranilic acid methyl ester (*5*)

Amorphous solid; $[\alpha]_D^{25}$ −27.9° (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.5), 240 (4.0), 305 (3.8); IR ν_{max}^{NaCl} 1718, 1642, 1588, 1492, 1451, 1302, 1207, 1071, 810 cm^{-1}; ^1H, ^{13}C NMR Tables 1 and 2; HRFABMS *m/z* 518.1271 (calc. for $C_{22}H_{25}NO_{12}Na$, 518.1274; $M^+ + Na$). FABMS *m/z* 518 ($M^+ + Na$, 100), 496 ($M^+ + H$, 10.8), 334 (57.6), 302 (59.4), 197 (22), 176 (35), 137 (57).

Acetylation of **5** (6 mg) using Ac_2O (2 ml) and pyridine (1 ml) at rt overnight as above gave after preparation the hexaacetate **5a** (5.5 mg) as an amorphous solid, $[\alpha]_D^{25}$ −87.8 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.4), 211 (4.3), 230 (4.0), 262 (3.7). IR ν_{max}^{NaCl} 1758, 1671, 1605, 1526, 1486, 1417, 1370, 1214, 1045, 756 cm^{-1}; for ^1H, and ^{13}C NMR spectra, see Tables 1 and 2; HREIMS *m/z* 747.2007 (calc. for $C_{34}H_{37}NO_{18}$, 747.2019) EIMS *m/z* 747 (M^+, 0.4), 705 (0.9), 417 (4.4), 375 (7), 343 (5.7), 331 (20), 301 (20), 197 (14), 169 (100), 165 (8.5), 139 (8), 137 (13), 127 (17), 109 (55.8).

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