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Phytochemistry 68 (2007) 2415–2419
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Bufadienolides from *Drimia macrocentra* and *Urginea riparia* (Hyacinthaceae: Urgineoideae)

Nivan Moodley [a], Neil R. Crouch [a,b], Dulcie A. Mulholland [a,c,*]

[a] *School of Chemistry, University of KwaZulu-Natal, Howard College Campus, Durban 4041, South Africa*
[b] *Ethnobotany Unit, South African National Biodiversity Institute, P.O. Box 52099, Berea Road 4007, South Africa*
[c] *School of Biomedical and Molecular Sciences, University of Surrey, Guildford GU2 7XH, United Kingdom*

Received 24 January 2007; received in revised form 2 May 2007
Available online 2 July 2007

Abstract

The bufadienolides, rubellin and riparianin were isolated from the bulbs of *Drimia macrocentra* and *Urginea riparia* (Hyacinthaceae) respectively. Rubellin and riparianin contain a carbohydrate moiety doubly linked to the bufadienolide aglycone at the C-2 and C-3 positions. Riparianin showed moderate activity when tested against MCF7 (breast), TK10 (renal) and UACC62 (melanoma) cell lines.

Keywords: Hyacinthaceae; *Drimia macrocentra*; *Urginea riparia*; Bufadienolide; Rubellin; Riparianin; Anti-cancer screening

1. Introduction

Drimia macrocentra (Baker) Jessop (syn. *Urginea macrocentra* Baker) of the Hyacinthaceae (subfamily Urgineoideae) is called the Natal Slangkop, for young inflorescences unnervingly resemble an elevated snake's head. *D. macrocentra* is distributed in South Africa along the coast and uplands of KwaZulu-Natal, extending as far south as the Transkei of the Eastern Cape Province. It grows on the periphery of marshes or in damp ground at a wide range of elevations. This plant is known as *ujobo* or *injobo* to the Zulu, who value extracts of the fleshy bulbs as a vermifuge (Wood, 1902), particularly against tapeworms and roundworms (Gerstner, 1939). Plants may be encountered in the regional ethnomedicinal trade, sold under the name *isiklenama* (Tait and Cunningham, 1988).

Since early in the history of South African toxicology, ingestion of young leaves and inflorescences has been blamed as a cause of mortality in stock (Wood, 1914; Mitchell, 1926). Wood (1914) documented anecdotal evidence to indicate that the plant is either more poisonous during Spring, or alternatively more frequently ingested by stock during this period. Subsequent toxicity trials have shown that flowering stems are more poisonous that leaves (Mitchell, 1926), although all plant parts are toxic, whether fresh or dried (Watt and Breyer-Brandwijk, 1962). Constituents are toxic to a broad range of mammalian test subjects, including sheep, goats, guinea pigs and rabbits (Mitchell, 1926; Watt and Breyer-Brandwijk, 1962).

Watt and Breyer-Brandwijk (1962) reported on an attempt by C.F. Juritz (in 1923), the South African Government Analyst, to characterise phytochemically *D. macrocentra*; he managed only to isolate a crude though highly toxic principle from an alcoholic extract of the air-dried bulb. No phytochemical analyses have subsequently been reported for this species.

Urginea riparia Baker is a poorly understood bulbous species found both in the Midlands and coastal regions of KwaZulu-Natal and the Transkei of the Eastern Cape. It frequents rocky, exposed stream and riverside habitats, growing in clumped colonies with its bulbs submerged and leaves erect and emergent. Its relation to other urgineoids of the Hyacinthaceae remains to be properly elucidated, for at present its closest relative appears to be

* Corresponding author. Address: School of Biomedical and Molecular Sciences, University of Surrey, Guildford GU2 7XH, United Kingdom.
E-mail address: d.mulholland@surrey.ac.uk (D.A. Mulholland).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.05.023

Drimia calcarata (Baker) Stedje (syn. *Urginea calcarata* (Baker) Hilliard & Burtt) under which *U. riparia* has been synonymised by some authors (Manning and Goldblatt, 2003) via its prior sinking by Jessop (1977) in *Drimia modesta* (Baker) Jessop.

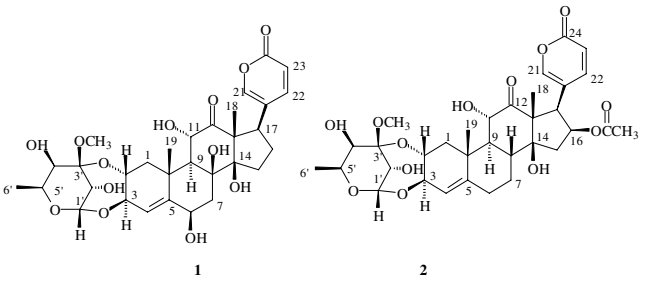
2. Results and discussion

The dichloromethane and methanol extracts of the plant bulbs were investigated. Although several bufadienolides were seen to be present in very minor quantities, only one major one could be isolated from each plant. Rubellin, **1**, was isolated from *D. macrocentra* and the novel ripariaanin, **2**, from *U. riparia*. It is of chemotaxonomic interest that no rubellin was isolated from *D. riparia* despite insistence by some systematists (Manning and Goldblatt, 2003) of its conspecificity with *D. calcarata*. The structure of rubellin, isolated originally from *D. calcarata* (as *Urginea rubella* Bak.) (Louw, 1949), was only much later elucidated using X-ray crystallographic techniques (Steyn et al., 1986). The structure was determined here using extensive 2D NMR studies and was found to be the same. There are some notable differences between the reported NMR data (run in d_6-acetone without access to 2D NMR methods) and ours, particularly in the assignment of several oxymethine carbon resonances which appear interchanged in the original report. Full NMR assignments are given in Table 1. The character of poisoning by *D. macrocentra* is consistent with bufadienolide cardiotoxicoses commonly noted for urgineoids (Kellerman et al., 1988) including *D. calcarata* (as *U. rubella*) (Van der Walt and Steyn, 1941). Rubellin is a well-documented potent cardiac glycoside capable of producing mortality in mammalian test subjects (Sapeika, 1950); as the main isolate from *D. macrocentra* it is likely responsible for the observed toxicoses.

Table 1
^{1}H, ^{13}C, HMBC, COSY and NOESY data for rubellin, 1 (CD_3OD)

Carbon	δ_H (ppm)	δ_C (ppm)	δ_C* (ppm)	HMBC (C → H)	COSY	NOESY
1α	1.40 (d, 13.5 Hz)	47.75	47.33	9; 19	1β; 2	1β; 9
1β	2.86 (dd, 3.5, 13.5 Hz)				1α	1α; 2; 19
2	5.22 (brs)	72.39	74.99	1α; 3; 4	1β; 3	1β; 19
3	4.55 (dd, 1.8, 8.1 Hz)	79.87	73.78	1α, β	2; 4	4; 5′
4	5.47 (d, 1.8 Hz)	126.98	127.56	2; 6	3	3; 6
5		146.52	145.16	6; 19		
6	4.50 (m)	75.03	71.88	4; 7α	7α, β	4; 7β
7α	1.54 (dd, 4.6, 7.4 Hz)	38.67	40.94		6; 7α	6; 7β
7β						
	2.54 (dd, 4.6, 14.3 Hz)				6; 7β	6; 7α
8		78.98	78.72	6; 7; 9		
9	1.65 (d, 12.3 Hz)	54.48	48.37	11; 19	11	1α
10		41.35	38.51	1α; 4; 9; 11; 19		
11	5.21 (brs)	73.58	72.94	9	9	18; 19
12		214.95	214.73	9; 11; 17; 18		
13		63.84	63.15	16α, β; 17; 18		
14		86.69	86.26	16α, β; 17; 18		
15α	1.74 (m)	35.14	34.78		15β; 16α, β	16α
15β	1.40 (m)				15α; 16α, β	16β
16α	2.00 (m)	29.66	[b]	17	15α, β; 16β; 17	15α; 16β; 17
16β	1.76 (dd, 3.5, 8.4 Hz)				15α, β; 16α; 17	15β; 16α; 22
17	4.08 (t, 8.8 Hz)	42.99	42.64	18; 21	16α, β	16α; 18; 21; 22
18	1.09 (s)	20.57	18.00			11; 17; 21; 22
19	1.72 (s)	24.12	24.14	19		1β; 11
20		123.01	121.43	17; 21; 23		
21	7.54 (d, 2.6 Hz)	151.34	150.87	17; 22	22	17; 18
22	7.92 (dd, 2.6, 9.7 Hz)	148.95	47.32	17; 21	21; 23	16β; 17; 18; 23
23	6.34 (d, 9.7 Hz)	115.99	115.92		23	22
24		164.46	161.72	21; 22; 23		
1′	5.16 (d, 4.8 Hz)	98.86	98.23	2′	2′	2′
2′	4.39 (d, 4.8 Hz)	70.08	79.23	1′; 4′	1′	1′
3′		100.21	99.90	1′; 2′; 4; 3′-OCH_3		5′
4′	3.67 (d, 1.7 Hz)	71.80	70.15	6′	5′; 6′	5′; 6′
5′	4.66 (m)	74.83	71.80	1′; 6′	6′	3; 4′; 6′
6′	1.23 (d, 6.3 Hz)	17.94	20.44	5′; 6′	5′	4′; 5′
4′-OCH_3	3.34 (s)	[a]	[b]			

[a] Peak obscured by solvent.
[b] Carbon value not given in literature.
* Literature sample run in d_6-acetone (Steyn et al., 1986).



1 2

Compound **2**, riparianin, was isolated from the dichloromethane extract of *U. riparia*. The ^{1}H NMR spectrum showed the characteristic resonances of the lactone ring of a bufadienolide skeleton (H-21, δ 7.56 (brs), H-22, δ 8.35 dd (9.9, 2.6 Hz) and H-23, δ 6.23 d (9.9 Hz); δ 116.4 (C-20), δ 113.4 (C-23), δ 152.94 (C-21), δ 149.50 (C-22)). The H-17α resonance was assigned by means of correlations seen in the HMBC spectrum with the C-20, C-21 and C-22 resonances. This resonance also showed a correlation with a ketonic carbonyl resonance at δ 213.4, which was assigned to C-12 as in rubellin, and a correlation in the COSY spectrum with a multiplet resonance at δ 5.44 which was assigned to H-16. The ^{1}H NMR spectrum, showed the presence of an acetate group methyl proton resonance at δ 1.56, and this group was placed at C-16. This placement was confirmed by a correlation seen in the HMBC spectrum between the acetate carbonyl carbon resonance at δ 169.8 and the H-16 resonance. The C-17 resonance at δ 47.2 showed a correlation in the HMBC spectrum with the 3H-18 methyl group proton singlet resonance at δ1.09. The HMBC spectrum also showed a correlation between the H-16 resonance and the quaternary C-14 resonance at δ 84.4, the chemical shift of this resonance confirming the presence of a hydroxyl group at C-14. The 3H-19 remaining methyl group proton resonance which occurred at δ1.22 showed a correlation in the HMBC spectrum with a resonance at δ 1.44 which was assigned to H-9. The COSY spectrum indicated coupling between H-9 and H-11 (d, δ 4.81), the chemical shift of H-11 indicating the presence of a hydroxyl group at C-11, between H-9 and H-8 (δ 2.23), and then between H-8 and the 2H-7 resonance, which was, in turn, seen to be coupled to the 2H-6

Table 2
^{1}H, ^{13}C, HMBC, COSY and NOESY data for riparianin, 2 (C_5D_5N)

Carbon	δ_H (ppm)	δ_C (ppm)	HMBC (C → H)	COSY	NOESY
1α	1.92 (d, 13.4 Hz)	45.54	19	1β; 2	1β; 11
1β	3.18 (d, 13.4 Hz)			1α; 2	1α; 19
2	5.66 (brs)	72.53	4	1α, β; 3	19
3	4.85 (d, 7.7 Hz)	79.17	1′; 2	1; 2; 4	4
4	5.41 (brs)	122.56		3	3
5		144.42	19		
6	2.41 (m) (2H)	28.92		7	7
7	1.12 (m) (2H)	29.93		6; 8	6; 8
8	2.23 (m)	41.07	19	7; 9	7; 11
9	1.44 (m)[a]	53.95	11; 19	8; 11	
10		41.28	1; 4; 19		
11	4.81 (d, 11.7 Hz)	74.81	9	9	8; 19
12		213.40	11; 17; 18		
13		63.90	15; 17; 18		
14		84.37	15; 16; 17; 18		
15	1.98 (m)	40.34	16	16	16
16	5.44 (m)	73.55	15; 17	15; 17	15; 17
17	4.67 (d, 8.8 Hz)	47.19	15; 18	16	16; 21
18	1.09 (s)	17.73			21; 22
19	1.22 (s)	19.90	9		1β; 2; 11
20		116.38	17; 21; 23		
21	7.56 (brs)	152.94	17	22	17; 18
22	8.35 (dd, 9.9, 2.6 Hz)	149.50	17; 21	21; 23	18; 23
23	6.23 (d, 9.9 Hz)	113.37		22	22
24		161.63	21; 23		
1′	5.71 (d, 4.8 Hz)	98.81	2′	2′; 3	2′
2′	5.21 (d, 4.8 Hz)	69.75	1′; 4′	1′	1′
3′		100.33	2, 1′; 2′; 4′; 3′-OCH_3		
4′	4.01 (brs)	71.66	6′	5′	5′
5′	4.97 (m)	73.90	6′; 1′	4′; 6′	4′; 6′
6′	1.46 (d, 6.3 Hz)	18.44		5′	5′
3′-OCH_3	3.47 (s)	48.29			
16-$\underline{C}OCH_3$		169.78	16; 16-CO$\underline{C}H_3$		
16-CO$\underline{C}H_3$	1.58 (s)	20.60			

[a] Peak obscured.

resonances. A double bond was placed at Δ^4due to a correlation seen in the HMBC spectrum between the 3H-19 methyl group proton singlet (δ 1.22) and the C-5 resonance at δ 144.42. The COSY spectrum showed coupling between the H-4 (δ 5.41) and H-3 resonances (δ4.85). The H-3 resonance was seen to be coupled to the H-2 resonance at δ 5.66 and the H-2 resonance showed coupling to the 2H-1 resonances. The chemical shifts of H-2 and H-3 indicated oxygen substituents at these positions.

Apart from the 26 carbon resonances required for the acetylated bufadienolide skeleton, seven resonances ascribable to a methylated sugar were present in the ^{13}C NMR spectrum. The HMBC spectrum indicated correlations between the C-3 resonance at δ 79.17 and the H-1′ resonance at δ 5.71 and between the C-3′ resonance at δ 100.3 and the H-2 resonance at δ 5.66. The H-1′ resonance was seen to be coupled to the H-2′ doublet at δ 5.21 which was not further coupled. The quaternary C-3′ resonance showed correlations in the HMBC spectrum with the H-1′, H-2′ H-4′ and a methoxy group proton resonance at δ 3.47. Thus a methoxy group was placed at C-3′. The H-4′ resonance at δ 4.01 was seen to be coupled to the H-5′ resonance (δ 4.97), which was, in turn, seen to be coupled to the 3H-6′ methyl group proton doublet at δ 1.46.

The stereochemistry of the aglycone was determined by the use of the NOESY spectrum in conjunction with a model and assuming the normal β-configuration of the lactone ring and C-18 and C-19 methyl groups. A correlation was seen between the H-17α resonance and the H-16 resonance implying H-16 was α and the acetate group was in the usual β-orientation. Correlations were seen between the H-11, 3H-19 and H-8 resonances, and between the 3H-19 and H-2 resonances, indicating these substituents were in the β orientation. Riparianin was found to be closely related to rubellin, **1**, with an acetoxy group occurring at C-16β, and lacking the hydroxy groups at C-6β and C-8β. NMR data are given in Table 2.

The structure derived from 2D NMR experiments suggested a molecular formula of $C_{33}H_{42}O_{13}$. Considerable difficulty was experienced obtaining a mass spectrum for this compound and FAB MS showed a highest peak at m/z 604 corresponding to the loss of a CH_2CO fragment that can occur for acetate groups. Difficulties were also experienced obtaining the molecular ion for rubellin using FAB MS and other techniques for rubellin and for the related lydenbergenin (Crouch et al., 2006).

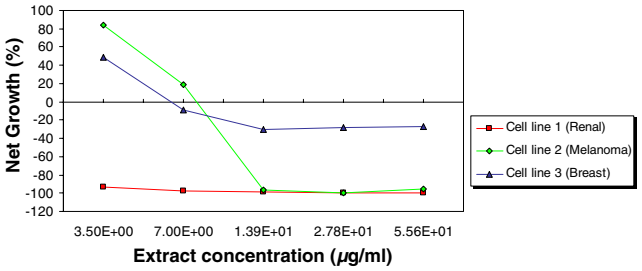


Fig. 1. Dose-response curves of compound 2.

Riparianin, **2**, was tested for its anti-cancer activity against the MCF7 (breast), TK10 (renal) and UACC62 (melanoma) cell lines and displayed moderate activity, with mean total growth inhibition (TGI) values of <3.5, <7 and <10.5 ppm in the three cell lines respectively, indicating moderate anti-cancer activity (Fig. 1). Etoposide was used as a control and gave a TGI value of 0.3 ppm.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded at room temperature on a 400 MHz Varian UNITY-INOVA spectrophotometer. Chemical shifts (δ) are expressed in ppm relative to tetramethylsilane (TMS) as internal standard and coupling constants are given in Hz. UV spectra were obtained on a Varian DMS 300 UV-visible spectrometer with CH_2Cl_2 as solvent. IR spectra were recorded on a Nicolet Impact 400D Fourier-transform infrared (FT-IR) spectrometer, using NaCl windows with CH_2Cl_2 as solvent against an air background. FAB MS were recorded on a VG 70-70E mass spectrometer instrument, with electron impact and chemical ionisation, by Dr. Louis Fourie, at University of Potchefstroom, RSA.

3.2. Plant material

U. riparia Baker was collected during April 2002 from Lupitana Gorge in the Transkei and a voucher specimen (*Crouch, Styles and Van den Bergh 921*, NU) retained for verification purposes. *D. macrocentra* (Baker) Jessop was collected at Bushman's Nek in the KwaZulu-Natal Drakensberg during April 2002, and a voucher specimen (*N. Crouch 938*, NH) lodged.

3.3. Extraction and isolation of compounds

The white bulbs (0.65 kg) of *D. macrocentra* (Baker) Jessop were air-dried, chopped into smaller pieces and extracted successively with dichloromethane and methanol by agitation on a Labcon Mechanical shaker at 140 rpm. The extracts obtained were then filtered and the solvent removed under reduced pressure to yield a dichloromethane extract (3.00 g) and methanol extract (27.00 g). TLC analysis and ^{1}H NMR spectroscopy of the dichloromethane and methanol extracts showed them to be similar and they were combined.

The extract was loaded onto a 5 cm diameter column packed with silica gel (Merck 9385) and eluted with a step gradient solvent system collecting 50 ml fractions (100% dichloromethane fractions 1–3; 5% ethyl acetate

in dichloromethane fractions 4–14; 10% ethyl acetate in dichloromethane fractions 15–27; 50% ethyl acetate in dichloromethane fractions 28–63; 5% methanol in dichloromethane fractions 64–83; 10% methanol in dichloromethane fractions 84–107). Purification of fractions 64–83 using a 2% methanol in dichloromethane solvent mixture afforded partially pure compound **1**. Final purification of compound **1** was obtained using a Sephadex column (LH 20) eluted with 100% methanol in a yield of 0.3% (15 mg).

The bulbs (3 kg) of *U. riparia* Baker were air-dried, chopped into smaller pieces and extracted successively with dichloromethane and methanol by agitation on a Labcon Mechanical shaker at 140 rpm for 24 h. The extracts obtained were then filtered and the solvent removed under reduced pressure to yield a dichloromethane extract (1.20 g) and methanol extract (15.00 g). Analysis of the crude methanol extract showed the presence of sugars.

The dichloromethane extract was loaded onto a 2 cm diameter column and eluted with a step gradient solvent system collecting 100 ml fractions (100% dichloromethane fractions 1–3; 10% ethyl acetate in dichloromethane fractions 4–17; 20% ethyl acetate in dichloromethane fractions 18–25; 50% ethyl acetate in dichloromethane fractions 26-40; 100% ethyl acetate in dichloromethane fractions 41–51). Purification of fractions 26–40 using a 20% ethyl acetate in dichloromethane solvent system afforded partially pure compound **2**. Final purification was obtained using a Sephadex column (LH 20) eluted with 100% methanol in a yield of 1.1% (13 mg).

Rubellin (**1**): yellow amorphous material (15 mg); FAB MS: [M^+] not seen; EIMS *m/z*: [M^+ 620.24689 calcd. not seen, $C_{31}H_{40}O_{13}$], 586, 530, 458, 373, 191, 149, 129, 109; IR: ν_{max} (NaCl) cm^{-1} 3420 (O–H stretch), 2923 (C–H stretch), 1708 (C=O stretch); ^1H NMR data (400 MHz, CD_3OD) Table 1; ^{13}C NMR data (100 MHz, CD_3OD) Table 1.

Riparianin (2): brown amorphous material (13 mg); $[\alpha]_D = +18.18$ (c, 0.022 g/100 ml, MeOH); FAB MS: [M^+] not seen, EIMS *m/z*: [M^+ 646.26254 calcd. not seen, $C_{33}H_{42}O_{13}$], 604 [M^+–CH_2CO]$^+$, 430, 390, 191, 149, 136, 123; IR: ν_{max} (NaCl) cm^{-1} 3418 (O–H stretch), 2935 (C–H stretch), 1706 (C=O stretch); ^1H NMR data (400 MHz, C_5D_5N:) Table 2; ^{13}C NMR data (100 MHz, C_5D_5N:) Table 2.

3.4. Anti-cancer assay

The three cell lines utilised were MCF7 (breast), TK10 (renal) and UACC62 (melanoma). The method employed was National Cancer Institute method (http://dtp.nci.nih.gov/branches/btb/ivclsp.html).

Acknowledgements

We thank Mr Dilip Jagjivan and Dr. Louis Fourie for the running of NMR and mass spectra, respectively, Mr Bret Parel and the late Mr Ernest Makhaza for their technical assistance, the CSIR Biochemtek for anti cancer assaying and the University of KwaZulu-Natal and the National Research Foundation for financial aid. Prof. T. Edwards of the Herbarium of UKZN (NU) provided useful taxonomic insights. Mrs Tessa Dean kindly facilitated access to *D. macrocentra* bulbs, and the Mary Gunn Library (National Herbarium) to obscure literature.

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