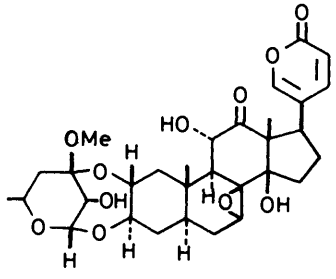
Bufadienolide Glycosides of the Crassulaceae. Structure and Stereochemistry of Orbicusides A—C, Novel Toxic Metabolites of *Cotyledon orbiculata*

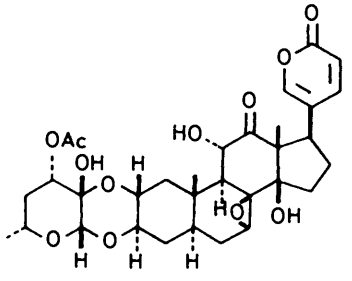
Pieter S. Steyn, Fanie R. van Heerden,* and Robert Vleggaar
National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria 0001, Republic of South Africa
Lourens A. P. Anderson
Veterinary Research Institute, Onderstepoort 0110, Republic of South Africa

Three new bufadienolide glycosides, orbicusides A—C, in addition to the known tyledoside C, have been identified as the toxic principles of *Cotyledon orbiculata* L. var. *orbiculata*. The structures (**3**), (**5**), and (**6**) for orbicusides A—C, respectively, with three ether bridges between the 4,6-dideoxyhexose moiety and the bufadienolide aglycone, were deduced from a detailed ^1H and ^{13}C n.m.r. study.

The poisoning of livestock by plants constitutes an important agricultural problem in Southern Africa. Some members of the Crassulaceae family are associated with cotyledonosis or nentapoisoning, an intoxication which affects the nervous and muscular systems of animals.[1] Investigations into the active principles of two members of the family, *Cotyledon wallichii*[2] and *Tylecodon grandiflorus*,[3] resulted in the characterization of bufadienolide glycosides, *e.g.* tyledoside D (**1**), containing a 3-hexosulose carbohydrate moiety doubly-linked to the aglycone by oxygen atoms in the C-2α and C-3β positions.[3] We now report the isolation and characterization of another series of novel bufadienolide glycosides, the orbicusides, toxic components of *Cotyledon orbiculata* L. var. *orbiculata*, a succulent which is widespread throughout Southern Africa.[5]



(1)



(2)

Extraction of fresh material of *C. orbiculata* with ethyl acetate, followed by solvent–solvent partitioning of the crude extract and subsequent column chromatography on silica gel, led to the isolation of tyledoside C (**2**), previously isolated from *T. grandiflorus*,[3] and three other bufadienolide glycosides, named orbicusides A—C.†

The structures of orbicusides A—C are based on a detailed study of their high-field ^1H and ^{13}C n.m.r. spectra. The ^1H and ^{13}C n.m.r. data of the orbicusides are collated in Tables 1 and 2, respectively.

Field desorption mass spectrometry confirmed the molecular formula of the main toxic component, orbicuside A, as $C_{30}H_{36}O_{10}$. The proton connectivity pattern of orbicuside A was determined by the application of two-dimensional (^1H, ^1H) correlation spectroscopy, using the COSY-45 sequence.[6,7] The assignment of the ^{13}C n.m.r. spectrum is based on the results obtained from two different two-dimensional (^1H, ^{13}C) chemical shift correlation experiments,[6,8,9] in which the delays Δ_1 and Δ_2 were adjusted to detect correlations *via* one-bond and long-range (^1H, ^{13}C) couplings, respectively. The results obtained from the latter experiment are summarised in Figure 1.

A comparison of the n.m.r. data of orbicuside A with those of tyledosides C (**2**) and D (**1**) revealed an identical substitution pattern and stereochemistry for the B, C, and D rings of these three compounds.

The A-ring of orbicuside A differs substantially from those of tyledosides C (**2**) and D (**1**). The absence of protons at C-2 of orbicuside A is evident from the observation that the C-1 protons show only geminal coupling [δ_H 1.483 p.p.m. (J 14.8 Hz, 1α-H) and 2.602 p.p.m. (J 14.8 Hz, 1β-H)], whereas 3-H (δ_H 3.663 p.p.m., J 11.5 and 5.7 Hz) is coupled only to 4α-H and 4β-H. The only unaccounted for carbon signal in the ^{13}C n.m.r. spectrum of orbicuside A was a singlet at δ_C 105.07 p.p.m., characteristic of the carbon atom of an acetal function. The assignment of this resonance to C-2 followed from the observed correlation between the resonances assigned to 1α-H and 1β-H

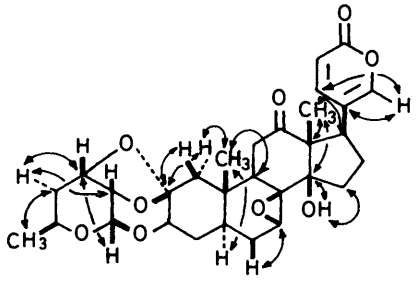


Figure 1. Long range (^1H, ^{13}C) connectivity pattern observed for orbicuside A (**3**) in a heteronuclear chemical shift correlation experiment. The delays Δ_1 and Δ_2 (45 and 30 ms, respectively) were adjusted to allow the detection of correlation *via* long range (^1H, ^{13}C) couplings.

† Orbicusides A, B, and C were referred to as cotyledosides A, C, and D, respectively, and tyledoside C as cotyledoside B in a preliminary toxicological report.[4]

Table 1. ^{1}H N.m.r. data for orbicusides A (**3**), B (**5**), and C (**6**)

	(3)		(5)		(6)	
Proton	δ_H (p.p.m.)	*J*/Hz	δ_H (p.p.m.)	*J*/Hz	δ_H (p.p.m.)	*J*/Hz
1α	1.483 d	14.8	1.468 d	14.9		
1β	2.602 d	14.8	2.747 d	14.9		
3	3.663 dd	11.5, 5.7	3.659 dd	11.5, 5.7	3.693 dd	12.4, 5.8
4α	1.604 ddd	12.9, 5.7, 3.1	1.557 ddd	12.9, 5.6, 2.9		
4β	1.461 ddd	12.9, 12.9, 11.5	1.407 ddd	12.9, 12.9, 11.5		
5α	1.304 dddd	12.9, 12.9, 5.2, 3.1	1.204 dddd	12.9, 12.9, 5.0, 2.9		
6α	1.838 ddd	15.3, 6.0, 5.2	1.752 ddd	15.2, 5.9, 5.9	3.946m	
6β	1.547 dd	15.3, 12.9	1.452 dd	15.0, 13.0		
6β-OH					4.564 d	5.9
7	3.373 d	6.0	3.167 d	6.1	3.460 d	5.4
9	1.844 d	12.6	1.670 d	11.1	1.782d	12.4
11β	4.617 dd	12.6, 5.1	3.580 ddd	11.1, 9.3, 4.4	4.637 dd	12.6, 5.3
11α-OH	4.846 d	5.1	4.472 d	4.4	4.917 d	5.2
12α			3.154 dd	9.3, 5.0		
12β-OH			3.927 d	5.0		
14β-OH	4.745 d	1.3	3.815 d	1.6	4.732 d	1.5
15	1.715 m					
15	1.604 m					
16	1.925 m					
16	1.715 m					
17α	3.907 dd	7.9, 7.9	3.010 dd	9.0, 9.0	3.932 dd	8.4, 8.4
18	0.824 s		0.564 s		0.838 s	
19	1.103 s		1.011 s		1.211 s	
21	7.568 d	2.6	7.414 d	2.6	7.574 d	2.6
22	7.650 dd	9.7, 2.6	7.696 dd	9.7, 2.6	7.675 dd	9.7, 2.6
23	6.296 d	9.7	6.294 d	9.7	6.302 d	9.7
1′	5.117 d	5.1	5.106 d	5.2	5.140 d	5.1
2′	4.244 dd	5.1, 4.0	4.236 dd	4.6, 4.6	4.257 dd	4.7, 4.7
3′	3.933 ddd	4.0, 2.5, 2.5	3.922 ddd	3.6, 3.0, 3.0	3.913 m	
4′α	1.993 ddd	14.7, 2.5, 2.5	1.985 ddd	14.8, 2.6, 2.6	1.986 ddd	14.8, 2.4, 2.5
4′β	1.473 ddd	14.7, 11.3, 2.5	1.461 ddd	14.8, 11.6, 3.0		
5′	4.048 qdd	6.3, 11.6, 2.5	4.037 qdd	6.2, 11.6, 2.4	4.046 qdd	6.1, 11.9, 2.6
6′	1.061 d	6.3	1.054 d	6.3	1.057 d	6.3

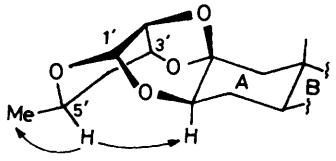


Figure 2. Nuclear Overhauser effects observed upon irradiation of 5′-H (δ_H 4.048 p.p.m.) of orbicuside A [solvent: $CDCl_3$–5% $(CD_3)_2SO$].

and the resonance at δ_C 105.07 p.p.m. in the two-dimensional (^{1}H, ^{13}C) chemical shift correlation experiment (Figure 1).

The ^{1}H n.m.r. data of the sugar moiety of orbicuside A (as summarized in Table 1) define it as a 4,6-dideoxy carbohydrate moiety with oxygen functions at C-1′, C-2′, and C-3′.

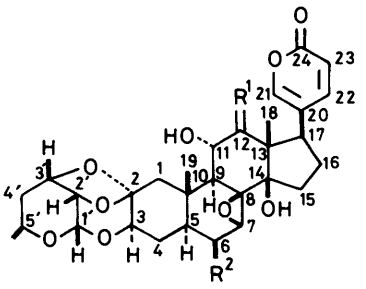
An evaluation of all these data enabled us to propose structure (**3**) for orbicuside A. The stereochemistry of orbicuside A, as indicated in (**3**), was derived from difference n.O.e. experiments. No effect was observed at 3-H upon irradiation of 1′-H and *vice versa*. These negative results suggested the β-orientation for the C-1′ proton. This assignment was confirmed by irradiation of the signal at δ_H 4.048 p.p.m. (5′-H) which resulted in a n.O.e. enhancement of 3-H. This effect defines the stereochemistry of the carbohydrate moiety unambiguously, since only the stereoisomer (**3**) permits the proximity of 3-H and 5′-H, as indicated in Figure 2.

Structure (**4**) also fits the available data and was considered as an alternative structure for orbicuside A. The detection of a three-bond (^{1}H, ^{13}C) connectivity across the ether bridge would allow us to differentiate between structures (**3**) and (**4**). However, the only correlation corresponding to long-range (^{1}H, ^{13}C) coupling between the carbohydrate moiety and the aglycone observed in the delayed two-dimensional (^{1}H, ^{13}C) chemical shift correlation experiment, was between 2′-H and C-2 (Figure 1), and does not differentiate between structures (**3**) and (**4**). The application of SPI experiments[10] to detect three-bond (^{1}H, ^{13}C) coupling also gave negative results. Since the magnitude of three-bond (^{1}H, ^{13}C) coupling constants is dependent on the torsion angle between the corresponding ^{1}H and ^{13}C nuclei,[11] the absence of three-bond (^{1}H, ^{13}C) coupling may be due to a C–O–CH torsion angle of 90°.

Structure (**4**) was discarded since its formation would imply the unlikely participation of the anomeric hydroxy function in acetal formation and the attachment of C-3′ to the aglycone.

Orbicuside B, $C_{30}H_{38}O_{10}$, and orbicuside C, $C_{30}H_{36}O_{11}$, both contain the same carbohydrate moiety as orbicuside A. Orbicuside B (**5**) differs by two mass units from orbicuside A (**3**). A comparison of the ^{13}C n.m.r. data of these two compounds indicates that the 12-oxo function of orbicuside A (**3**) (δ_C 212.05 p.p.m.) is replaced by a 12-hydroxy function (δ_C 79.61 p.p.m.) in orbicuside B (**5**). The stereochemistry of the 12-hydroxy group followed from the coupling constant for 11-H and 12-H (*J* 9.3 Hz) which indicates an antiperiplanar relationship between these two protons.

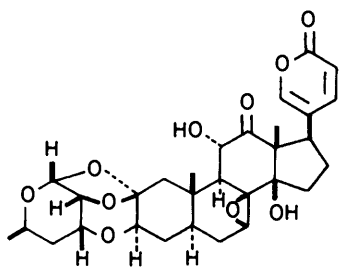
Orbicuside C was assigned the 6β-hydroxyorbicuside A structure (**6**). An evaluation of the ^{13}C n.m.r. data of orbicuside C enabled us to determine the location and stereochemistry of



(3) R^1 = O, R^2 = H

(5) R^1 = β-OH, H, R^2 = H

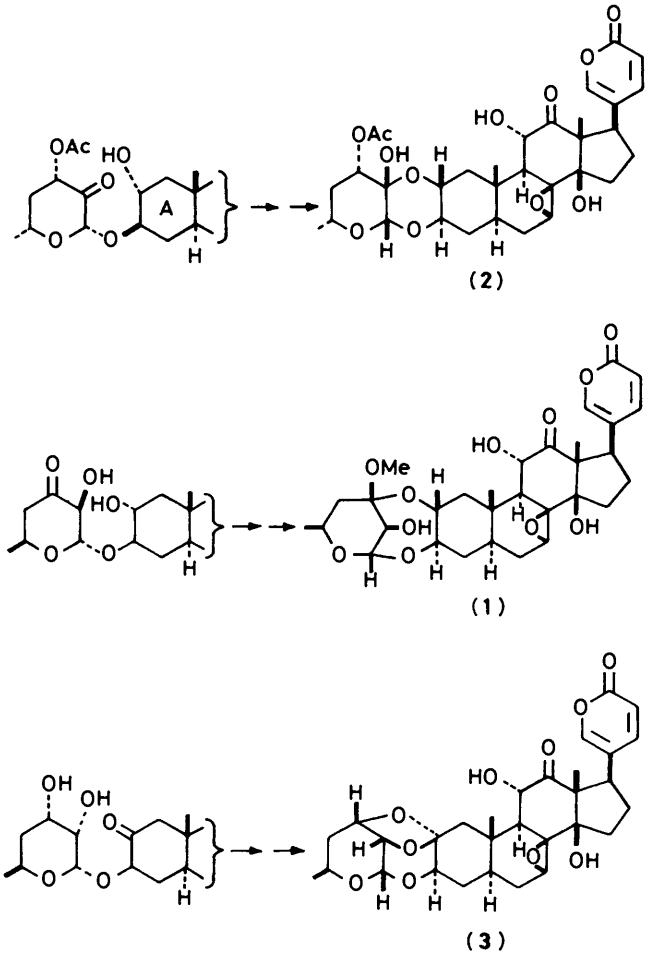
(6) R^1 = O, R^2 = OH



(4)

Table 2. ^{13}C N.m.r. data of orbicusides A (3), B (5), and C (6)

Carbon	(3) δ_C (p.p.m.)	(5) δ_C (p.p.m.)	(6) δ_C (p.p.m.)
1	45.84 t	47.44	47.69
2	105.07 s	105.25	104.91
3	78.33 d	78.53	79.08
4	34.07 t	34.10	30.40
5	37.92 d	37.87	43.65
6	26.39 t	26.79	64.99 d
7	52.35 d	51.03	54.12
8	62.21 s	62.90	62.81
9	47.14 d	47.01	47.61
10	36.40 s	35.12	36.33
11	73.34 d	70.78	73.15
12	212.05 s	79.61 d	211.88
13	63.14 s	55.56	63.00
14	80.15 s	79.67	79.98
15	35.38 t	34.72	35.31
16	28.93 t	29.65	28.57
17	41.00 d	46.53	40.93
18	18.56 q	13.01	18.21
19	12.69 q	12.15	15.55
20	120.14 s	121.92	119.95
21	150.11 d	149.41	149.96
22	146.71 d	147.13	146.48
23	146.68 d	114.27	114.46
24	160.98 s	161.12	160.71
1′	89.26 d	89.31	89.20
2′	70.45 d	70.47	70.39
3′	69.51 d	69.48	69.32
4′	34.07 t	34.10	34.02
5′	57.48 d	57.47	57.30
6′	20.28 q	20.29	20.10



(2)

(1)

(3)

Scheme

the hydroxy group. The resonance assigned to C-6 in orbicuside A (δ_C 26.39 p.p.m.) is absent in the spectrum of orbicuside C, which contains an additional doublet resonance at δ_C 64.99 p.p.m. The change in chemical shifts of C-4 ($\Delta\delta$ −3.67 p.p.m.) and C-5 (δ_C +5.73 p.p.m.) are also in agreement with a C-6 hydroxy substituent.

The 6-H resonance (δ_H 3.932 p.p.m.) in the ^{1}H n.m.r. spectrum of orbicuside C (6) is obscured by the resonances of 17-H and 3′-H, and therefore excluded analysis of this signal in order to determine the stereochemistry at C-6. Analysis of the ^{1}H n.m.r. spectrum of orbicuside A (3) has shown that 7α-H is coupled only to 6α-H. The absence of coupling between 6β-H and 7α-H is due to a dihedral angle of 90° between these two protons. The appearance of 7-H as a doublet (δ_H 3.460 p.p.m., J 5.9 Hz) therefore defines the stereochemistry of the 6-hydroxy function as β. The assignment of the stereochemistry at C-6 was confirmed by the pronounced deshielding of C-19 in orbicuside C ($\Delta\delta_C$ 2.86 p.p.m.) as compared to orbicuside A (3), an effect characteristic of 6β-hydroxy substituted steroids.[12]

Bufadienolide glycosides containing multiple ether bridges between the aglycone and carbohydrate moieties have so far only been isolated from members of the Crassulaceae family. These compounds can be divided into three different classes according to their biosynthetic origins (see Scheme). The first class represented by tyledoside C (2), which is closely related to a large group of cardenolides, *e.g.* gomphoside,[13] is derived from a 2α,3β-dihydroxybufadienolide and a 2-oxo carbohydrate

moiety. The second class includes cotyledoside [2] and tyledosides A, B, D (**1**), F, and G,[3] and originates from a 2α,3β-dihydroxybufadienolide and a 3-oxo carbohydrate moiety. The third class of compounds, comprising orbicusides A, B and C, differs from the first two classes in that it is derived from a 3β-hydroxy-2-oxobufadienolide and a 4,6-dideoxy carbohydrate moiety.

An evaluation of the biological activity of these compounds is presently being carried out. The availability of these series of compounds with a fixed conformation of the carbohydrate moiety should contribute towards a better understanding of the function of the carbohydrate moiety in the biological activity of these compounds.

Experimental

M.p.s were determined on a Kofler hot-stage apparatus and are uncorrected. U.v. absorptions were measured for solutions in methanol on a Unicam SP 8-100 spectrometer, while i.r. spectra were recorded for KBr discs on a Perkin-Elmer 237 spectrometer. Mass spectra were taken on a Varian MAT 212 double focussing spectrometer. N.m.r. spectra of $(CD_3)_2SO$ solutions were recorded on a Bruker WM-500 spectrometer operating at 500.13 MHz for ^1H and 125.76 MHz for ^{13}C nuclei. Chemical shifts are reported in p.p.m. relative to tetramethylsilane. Optical rotations were measured at 22 °C on a Perkin-Elmer 241 polarimeter for solutions in pyridine.

Merck silica gel 60 (particle size 0.063—0.200 mm) was used for column chromatography. R_F values are indicated for silica gel t.l.c. plates developed with chloroform–acetone–methanol (70:30:1, v/v/v).

The toxicity of the plant extracts and fractions was monitored by dosing to weaned guinea-pigs as described earlier.[4]

Isolation of the Toxic Principles.—Fresh plants of *C. orbiculata* (212 kg) were minced and extracted with ethyl acetate in a Waring blender (3 times). The solvent was evaporated under reduced pressure and the resultant syrup was partitioned between 95% aqueous methanol (3.5 l) and light petroleum (b.p. 60—80 °C). Only the residue obtained by evaporation of the methanol extract (89 g) was toxic to weaned guinea-pigs. This material was chromatographed on silica gel (2 kg) using consecutively chloroform, chloroform–acetone (7:3, v/v), and chloroform–acetone–methanol (8:2:0.5, v/v/v) as eluant. The toxic material was in the latter two fractions and recombination of the fractions gave a yield of 17 g.

Repeated chromatography of this toxic material on silica gel using the abovementioned solvent systems yielded four colourless crystalline compounds, orbicuside A (390 mg), tyledoside C (180 mg), orbicuside B (58 mg), and impure orbicuside C (48 mg). Orbicuside C was purified by chromatography on a prepacked Merck Lobar Lichoprep RP-8 column using methanol–water (9:1, v/v), followed by chromatography on silica gel with benzene–methanol (9:1, v/v).

Orbicuside A (**3**) crystallized from acetone–ether as white needles, m.p. 317 °C, $[\alpha]_D$ +32.0 (*c* 0.312); $\lambda_{max.}$ 298 nm (log ε 3.39); $\nu_{max.}$ 1 740, 1 720, and 1 640 cm^{-1}; R_F = 0.48 (Found: M^+, 556.231. $C_{30}H_{36}O_{10}$ requires *M* 556.230).

Orbicuside B (**5**) was obtained as white needles (acetone), m.p. 285—287 °C, $[\alpha]_D$ +14.97 (*c* 0.334); $\lambda_{max.}$ 298 nm (log ε 3.69); $\nu_{max.}$ 1 710 and 1 640 cm^{-1}; R_F = 0.20 (Found: M^+, 558.245. $C_{30}H_{38}O_{10}$ requires *M* 558.246).

Orbicuside C (**6**) was isolated as white crystals (acetone–ether), m.p. 308 °C (decomp.); $\lambda_{max.}$ 298 nm (log ε 3.39); $\nu_{max.}$ 1 720 and 1 635 cm^{-1}; R_F = 0.14; M^+, 572.

Tyledoside C (**2**) crystallized from acetone–ether as white needles, m.p. 210 °C (lit.,[3] 210—212 °C), $[\alpha]_D$ −16.86 (*c* 0.334); $\lambda_{max.}$ 298 nm (log ε 3.40); $\nu_{max.}$ 1 740, 1 720, and 1 640 cm^{-1}; R_F = 0.37; M^+, 616.

References

1 J. Vahrmeijer, 'Poisonous plants in Southern Africa,' Tafelberg, Cape Town, 1981.
2 P. S. Steyn, F. R. Van Heerden, and A. J. van Wyk, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1573.
3 P. S. Steyn, F. R. van Heerden, R. Vleggaar, and L. A. P. Anderson, *J. Chem. Soc., Perkin Trans. 1*, 1986, 429.
4 L. A. P. Anderson, R. A. Schutte, T. S. Kellerman, S. M. Kotze, L. Prozesky, G. L. Erasmus, and L. Labuschagne, *Onderstepoort J. Vet. Res.*, 1985, **52**, 21.
5 A. Fabian and G. Germishuizen, 'Transvaal Wild Flowers,' Macmillan SA, Johannesburg, 1982, p. 118.
6 A. Bax, 'Two-dimensional Nuclear Magnetic Resonance in Liquids,' Delft University Press, Delft, 1982.
7 A. Bax, R. Freeman, and G. Morris, *J. Magn. Reson.*, 1981, **42**, 164; A. Bax and R. Freeman, *ibid.*, 1981, **44**, 542.
8 G. Bodenhausen and R. Freeman, *J. Magn. Reson.*, 1977, **28**, 471; A. Bax and G. Morris, *ibid.*, 1981, **42**, 501.
9 A. Bax, 'Topics in Carbon-13 NMR Spectroscopy,' vol. 4, ed. G. C. Levy, John Wiley and Sons, New York, 1984, p. 197.
10 K. G. R. Pachler and P. L. Wessels, *J. Magn. Reson.*, 1973, **12**, 337; *ibid.*, 1977, **28**, 53.
11 R. Aydin, J.-P. Loux, and H. Gunther, *Angew. Chem., Int. Ed. Engl.*, 1982, **21**, 449.
12 J. W. Blunt and J. B. Stothers, *Org. Magn. Reson.*, 1977, **9**, 439.
13 H. T. A. Cheung and T. R. Watson, *J. Chem. Soc., Perkin Trans. 1*, 1980, 2162.

Received 14*th November*, 1985; *Paper* 5/2003