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ISOLATION AND CHARACTERISATION OF (*E*)-4-HYDROXY-2-(HYDROXYMETHYL)BUT-2-ENYL β-D-ALLOPYRANOSIDE, THE MAJOR SOLUBLE CARBOHYDRATE IN LEAVES OF THE FERN *Cardiomanes reniforme*

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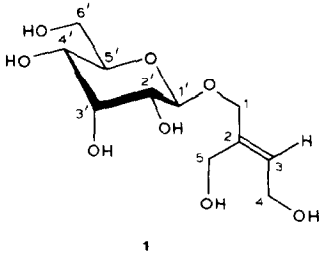
ABSTRACT

A novel glycoside, (*E*)-4-hydroxy-2-(hydroxymethyl)but-2-enyl β-D-allopyranoside (**1**, cardiomanol) has been identified as the major soluble carbohydrate in the leaves of *Cardiomanes reniforme*. The structure of **1** was established by chemical and spectroscopic methods.



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INTRODUCTION

This work was stimulated by the proposal[1] that endogenous polyols may assist plants to tolerate osmotic stress, whether by salinity, freezing, or desiccation. The question was also posed "do ferns contain polyols?" and led to a survey of the polyhydric alcohols in 5 ferns selected for their phylogenetic relationships and their ecological distribution in sites potentially subject to osmotic stress through salinity or desiccation.

The few studies of carbohydrates in ferns have indicated that, like most flowering plants, glucose, fructose, and sucrose are the major soluble carbo-

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hydrates, with erythrose, xylose, raffinose, and galactose also present in some species[2]. In our survey, a new glycoside was discovered as a major component in the sugar fraction of the New Zealand endemic filmy fern, *Cardiomanes (Trichomanes) reniforme* (family Hymenophyllaceae) and its structure has been established. The presence of the glycoside may account, in part, for the ability of *C. reniforme* to withstand periods of desiccation.

EXPERIMENTAL

Source of material. — Preliminary studies were on *C. reniforme* (Forst. f.) C. Presl obtained from Little Barrier Island near Auckland. Subsequently, *C. reniforme* and *Hymenophyllum demissum* (Forst. f.) Swartz were collected from the Waitakere Ranges, and *Trichomanes dentatum* Bosch was collected from a forest on Rarotonga, Cook Islands. Herbarium vouchers are lodged in the National Museum, Wellington.

General. — Chromatography was performed with *A*, 1-propanol–propyl acetate–water–acetic acid (120:40:50:4); *B*, ethyl acetate–1-propanol–water (57:32:13); and *C*, propyl acetate–formic acid–water (11:5:3). Preparative p.c. was performed on Whatman 3MM paper which had been pre-eluted with aqueous 10% acetic acid, aqueous 10% 2-propanol, and water. T.l.c. was performed on MN-300 cellulose. All solvents used for preparative p.c. were distilled before use. Compound **1** was detected with aqueous 95% ethanol–conc. H_2SO_4–*p*-anisaldehyde[3] (18:1:1) for 10–15 min at 110°. Reducing sugars were detected with *p*-anisidine[4] and radioactive compounds by autoradiography, with their radioactivity measured by the procedure of Redgwell *et al.*[5].

Sugars were quantified by g.l.c. of their trimethylsilyl derivatives, using a stainless steel column (2.0 m × 2 mm i.d.) packed with OV-101 on 5% Chromosorb WHP. Arabinitol was used as internal standard. The temperature programme was for 5 min at 170° then to 250° at 4°/min.

H.p.l.c. was performed using a column (25 cm × 4.6 mm) of Zorbax ODS. An aqueous solution (50 μL of a 40 mg/mL solution) of **1** was introduced through a Rheodyne 7125 injector (100-μL loop) and eluted with water at 1 mL/min at room temperature.

N.m.r. spectroscopy. — The ^{13}C-n.m.r. (50.3 MHz) and ^{1}H-n.m.r. spectra (200, 300, and 400 MHz) were obtained variously with Varian XL-200 and Bruker ACE 200, AM-300, and AM-400 spectrometers. The n.O.e. enhancements were made at 300 MHz, using the Bruker NOEMULT.AU programme.

F.a.b.-m.s. — A VG 70-SE spectrometer was used with argon as the bombarding gas. Samples were dissolved in methanol (4 μg/μL), and an aliquot (1–2 μL) was added to a drop of a 1:1 mixture of glycerol and thioglycerol on a stainless steel target.

Methylation analysis. — The method of Ciucanu and Kerek[6] was used. Methylated compounds were hydrolysed in 0.4M CF_3CO_2H for 30 min at 121°.

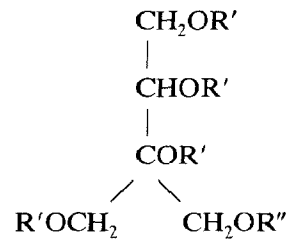
Partially methylated products were acetylated in acetic anhydride (100 μL) at 120° for 3 h, using sodium acetate as a catalyst[7]. G.l.c.–m.s. of the methylated alloside and of partially methylated alditol acetates was done with a VG 70-SE mass spectrometer (70 eV), using an SP-2330 fused-silica column (30 m × 0.32 mm) programmed for 2 min at 70°, then to 150° at 25°/min, and to 220° at 4°/min.

Isolation and purification of (E)-*4-hydroxy-2-(hydroxymethyl)but-2-enyl* β-D-*allopyranoside* (**1**). — A terrestrial clump of *C. reniforme* was collected intact with its soil substrate and enclosed in a plastic bag containing 37 MBq of $NaH^{14}CO_3$. The $^{14}CO_2$ was released[8] and the leaves were allowed to photosynthesise for 1 h. The bag was removed, the plants were left overnight, the labelling was repeated, and the plants were then left for 48 h. The leaves (30 g) were harvested, the stipes were removed and discarded, the laminas were immersed in liquid nitrogen, and 12:5:3 MeOH–$CHCl_3$–H_2O (80 mL) was added. The material was stored overnight at −20°, and the water-soluble fraction (2.5 g) was extracted[9] and eluted from columns of QAE and SP Sephadex[10], in sequence, in order to remove the charged fractions and phenolic compounds. The neutral fraction (1.5 g) was dissolved in aq. 10% 2-propanol (7 mL), and aliquots (0.4 mL) were subjected to preparative p.c. with solvent *A* (18 h). Compound **1**, located by autoradiography and its reaction on marker strips with the anisaldehyde spray ($R_{Sucrose}$ 1.1), was eluted from appropriate parts of the chromatogram with aq. 10% 2-propanol. The eluate was concentrated, a solution of the residue in aq. 10% 2-propanol (1.5 mL) was re-chromatographed in solvent *B* (16 h), and **1** was detected and recovered as before.

A column (1.8 × 60 cm) of LH20 Sephadex was equilibrated with aq. 80% methanol. A solution of partially purified **1** (0.1 g) in aq. 80% methanol (1 mL) was layered on top of the column and then eluted with aq. 80% methanol at 20 mL/h. The first 70 mL of eluate was discarded, and 2-mL fractions were then collected and monitored for **1** by t.l.c. (solvent *C*). The appropriate fractions were combined, and re-run on the LH20 column twice more in order to obtain pure **1** (0.255 g from 0.3 g of crude **1**), which was stored over P_2O_5 at 4° and had $[\alpha]_D^{20}$ −43° (*c* 1, water). The product contained no N, P, or S (Found: C, 46.81; H, 7.16. $C_{11}H_{20}O_8$ calc.: C, 47.14; H, 7.14%). Acetylation of **1** (acetic anhydride–pyridine, 1:1; at room temperature for 2 days) yielded the hexa-acetate, $[\alpha]_D^{20}$ −29.6° (*c* 1.9, chloroform). (Found: C, 51.51; H, 5.92. $C_{23}H_{32}O_{14}$ calc.: C, 51.87; H, 6.01%).

Hydroxylation of **1**. — A solution of ^{14}C-labelled **1** (30 mg) in aq. 30% H_2O_2 (1 mL) and aq. 20% HCO_2H (300 μL) was left overnight at room temperature, then concentrated in a stream of air, and the oxidation was repeated on the residue. P.c. (solvent *A*) of the product on a single sheet of pre-washed 3MM paper for 24 h gave two bands detected by autoradiography. The slower running component was eluted from the paper to give **7** (11.1 mg).

Isolation of D-*allose from* **1**. — Compound **1** (200 mg) was hydrolysed in 2M CF_3CO_2H for 1 h at 100°. The solution was filtered and co-concentrated several times with water. Aliquots (0.5 mL) of a solution of the residue in aq. 10% 2-propanol (2 mL) were subjected to p.c. (solvent *C*). The allose was located by the



7 R′ = H, R″ = allosyl
8 R′ = R″ = H
9 R′ = Me, R″ = Ac

p-anisidine spray and eluted from the chromatogram with aq. 10% 2-propanol. The eluate was concentrated and freeze-dried, and the residue (79 mg) was recrystallised twice from ethanol–water to yield D-allose (25 mg), m.p. 131°, mixture m.p. 141° with authentic D-allose[11], $[\alpha]_D^{20}$ +15° (*c* 1, water).

RESULTS

The glycoside **1** was discovered first in the water-soluble neutral fraction of the leaves of *C. reniforme* as a major unknown peak on a g.l.c. trace of the trimethylsilylated sugar derivatives (Fig. 1). It was isolated subsequently from leaves which had been allowed to photosynthesise $^{14}CO_2$ and was obtained as a radioactive, non-crystalline, glassy solid in ~25% yield after chromatography. Amounts and specific activities of the main carbohydrate components in the leaves of *C. reniforme* are given in Table I, and **1** accounted for 73% of the sugars determined. However,

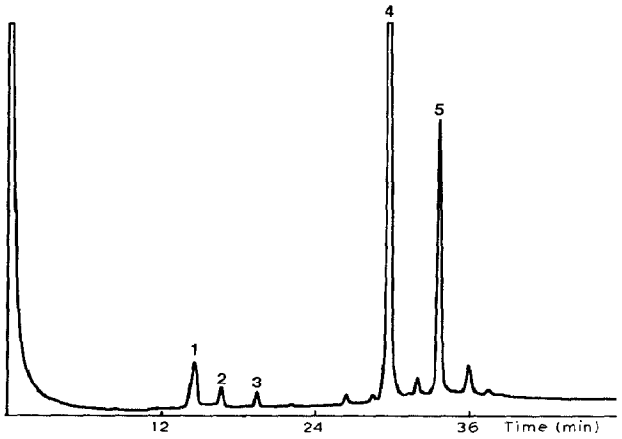


Fig. 1. G.l.c. of the sugars (Me_3Si derivatives) extracted from the leaves of *C. reniforme*: 1, fructose, 2, α-glucose; 3, β-glucose; 4, **1**; 5, sucrose. Unnumbered peaks were not identified.

TABLE I

AMOUNTS AND RADIOACTIVITIES OF SUGARS IN LEAVES OF *Cardiomanes reniforme* AFTER PHOTOSYNTHESIS WITH $^{14}CO_2$

Compound	*Tissue content (mg/g f.wt.)*[a]	*Radioactivity (kBq/mg)*
1	40.5	0.5
Sucrose	10.2	46.3
Fructose	2.9	15.2
Glucose	1.0	24.1
Raffinose[b]	0.9	20.5

[a]1 g fresh weight (f.wt.) = 0.4 g dry weight. [b]Tentative assignment based on the identification of component monosaccharides by t.l.c. and g.l.c.

the low specific activity of **1** compared to that of sucrose showed that sucrose was the primary photosynthetic product.

The $[\alpha]_D^{20}$ value (−43°) of **1** was indicative of a β-glycoside, and the fact that **1** decolourised a solution of $KMnO_4$ indicated the presence of unsaturation. In t.l.c., **1** did not react as a conventional reducing sugar, but gave a strong reaction with the anisaldehyde reagent[3] (black colour).

F.a.b.-m.s. indicated the molecular weight of **1** to be 280, and an ion at *m/z* 561, believed to correspond to $(2M + 1)^+$, reflected dimerisation.

Hydrolysis of **1** in M CF_3CO_2H gave a sugar with properties in t.l.c. and g.l.c. of the trimethylsilylated and acetylated derivatives, indistinguishable from those of D-allose, and the $[\alpha]_D^{20}$ value of +15° confirmed the D configuration.

Further evidence of the *allo* structure of the sugar moiety of **1** was provided by the ^1H- and ^{13}C-n.m.r. spectral data (Tables II and III). Thus, the resonances assigned to C-2′/6′ were within 0.5 p.p.m. of the values for methyl β-D-allopyranoside[12] (the deviation for C-1′ was expected as a consequence of the different aglycon). In the ^1H-n.m.r. spectrum, the coupling constants ($J_{1',2'}$ 8, $J_{2',3'}$ 2.9, $J_{3',4'}$ 2.9, and $J_{4',5'}$ 10.0 Hz) were consistent with a β-allopyranoside.

The 50.3-MHz ^{13}C-n.m.r. spectrum of **1** contained 11 signals, two of which were coincident (Table III), and was indicative of a C_5 aglycon. Comparison of the spectrum with that of methyl β-D-allopyranoside, together with off-resonance DEPT measurements, indicated this C_5 moiety to be a trisubstituted alkene that carried three CH_2O substituents. The chemical shifts of these methylene resonances suggested that one was the site of glycosidation (δ 71.9) and two were hydroxylated (δ 57.4 and 57.8). The conclusion was substantiated by the ^1H-n.m.r. spectrum of the hexa-acetate of **1** (Table II). When compared to that of the alloside, the spectrum also showed that two of the three resonances for CH_2 groups in the aglycon were shifted downfield[13]. The formulation of **1** as an alloside of 4-hydroxy-2-(hydroxymethyl)but-2-ene was also in accord with the molecular weight of 280 determined by f.a.b.-m.s.

None of the possible isomers (**1–3**) of this structure appear to have been

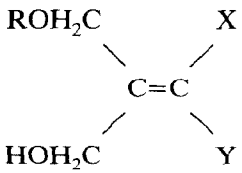
TABLE II

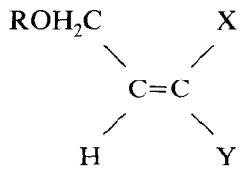
^{1}H-N.M.R. DATA FOR **1** AND ITS HEXA-ACETATE[a]

Proton	*Chemical shift (δ in p.p.m.; J in Hz)*	
	1	*Hexa-acetate*[b]
1A	4.22 d ($J_{1A,1B}$ 12)	4.13 d ($J_{1A,1B}$ 12.6)
1B	4.38 d	4.37 d
3	5.85 t ($J_{3,4}$ 6.5)	5.84 t ($J_{3,4}$ 6.6)
4	4.16 d	4.73 d
5A	4.22[c]	4.64 d ($J_{5A,5B}$ 15)
5B	4.20[c]	4.68 d
1′	4.67 d ($J_{1',2'}$ 8.2)	4.80 d ($J_{1',2'}$ 8.2)
2′	3.42 dd ($J_{2',3'}$ 2.9)	4.91 dd ($J_{2',3'}$ 3.0)
3′	4.11 dd ($J_{3',4'}$ 2.9)	5.66 dd ($J_{3',4'}$ 2.9)
4′	3.56 dd ($J_{4',5'}$ 10.0)	4.98 dd ($J_{4',5'}$ 10.2)
5′	~3.71 ddd ($J_{5',6'A}$ 1.5)	4.06 dt ($J_{5',6'}$ 3.4)
6′A	3.63 dd ($J_{6'A,6'B}$ 12.0)	
6′B	3.85 dd	4.22 d

[a]At 400 MHz. Solvents: D_2O for **1** (internal MeOH, δ 3.30); $CDCl_3$ for the hexa-acetate (internal $CHCl_3$, δ 7.27). [b]AcO signals: δ 2.00, 2.04, 2.06 (2), 2.09, 2.16. [c]These are presumably the inner lines of an almost coalesced AB quartet, the outer lines of which are lost in the base-line noise.

described, but the glucosides sarmentosin[14] (**4**), sutherlandin[15] (**5**), and (**6**)[16] have been reported.





1 R = β-D-allopyranosyl, X = H, Y = CH_2OH
2 R = β-D-allopyranosyl, X = CH_2OH, Y = H
5 R = β-D-glucopyranosyl, X = CN, Y = H
6 R = β-D-glucopyranosyl, X = H, Y = CN

3 R = β-D-allopyranosyl, X = Y = CH_2OH
4 R = β-D-glucopyranosyl, X = CH_2OH, Y = CN

The ^{1}H-n.m.r. data in Table II revealed that one of the CH_2OH groups in the aglycon was geminal to the vinylic-H, so that structure **3** was eliminated. Irradiation of the vinyl proton (H-3) in the hexa-acetate resulted in small n.O.e.s on the signals for H-4 (6%) and H-1A,1B (~2%). Significantly, irradiation of the H-1A,1B

TABLE III

^{13}C-N.M.R. DATA FOR **1** AND ITS HEXA-ACETATE[a]

Carbon	*Chemical shift (δ) in p.p.m.*	
	1	*Hexa-acetate*[c]
1	71.9	70.6
2	131.1	134.6
3	132.0	127.2
4	57.8[b]	60.1
5	57.4[b]	59.5
1′	99.6	97.9
2′	71.9	69.0
3′	71.0	68.5
4′	67.6	66.2
5′	74.3	70.1
6′	61.9	62.2

[a]Solvents: D_2O for **1** (internal MeOH, δ 49.6); $CDCl_3$ for the hexa-acetate (internal $CDCl_3$, δ 77.0). [b]Assignments may be reversed. [c]AcO signals: δ 20.83, 20.75 (2), 20.68, 20.57 (2); 170.71, 170.55, 169.72, 169.09, 169.05.

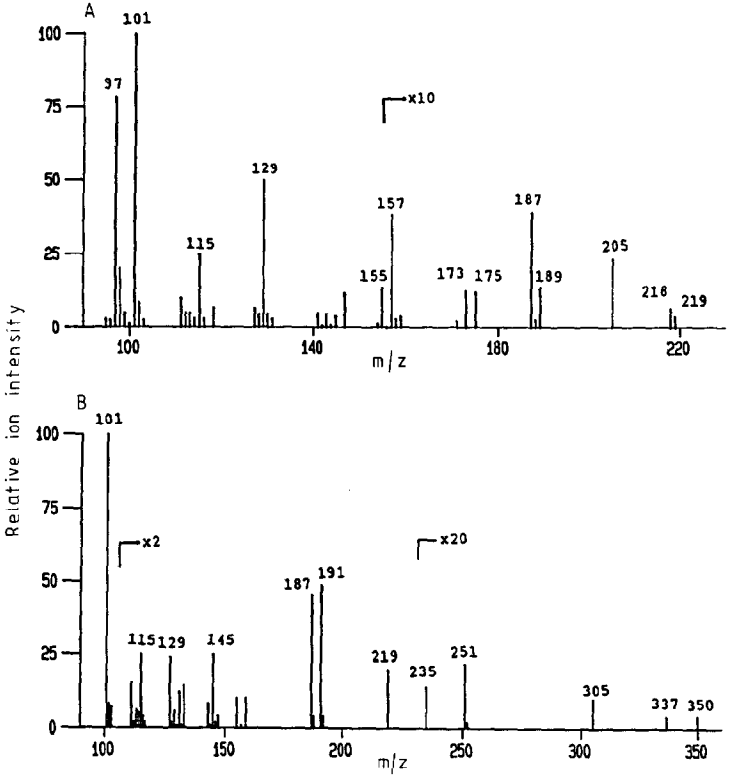


Fig. 2. E.i.-mass spectra of A, methylated **1**; and B, methylated **7**.

multiplet enhanced the signals due to H-3 (18%) and H-1′ (16%), whereas irradiation of the H-4 doublet resulted only in a small n.O.e. on the signal for H-3 (~2%). These results confirmed the structure **1**.

Acid hydrolysis of **1** gave (t.l.c., solvent *C*) allose and two unidentified compounds which gave violet (R_{Allose} 2.0) and black (R_{Allose} 1.89) colours, respectively, with the anisaldehyde spray.

Hydroxylation of **1** with performic acid gave a major product (**7**), isolated by p.c., which, on hydrolysis with 2M CF_3CO_2H (100°, 1 h), gave (t.l.c.) allose and apiitol (**8**). G.l.c. of their acetate derivatives confirmed these assignments. Apiitol is the product expected from **1–3**.

F.a.b.-m.s. of methylated **1** gave a molecular weight of 364, and the e.i.-mass spectrum (Fig. 2), interpreted according to established principles[17], contained the expected ions at *m/z* 219 (aA1 0.25%), 129 (bA1 30), and 189 (abJ1 1.0). Secondary fragment ions were formed by elimination of methanol (32 mass units). Ions at *m/z* 205 and 218 were not accounted for.

F.a.b.-m.s. of methylated **7** gave an ion at *m/z* 427 corresponding to $[M + 1]^+$ for a hexosyl-pentitol. E.i.-m.s. (Fig. 2) gave ions at *m/z* 191 (bA1 25.1%), 219 (aA1 12), 251 (abJ1 1.5), and 337 ($M^+ - 89$ 0.2). Application in sequence to methylated **7** of hydrolysis with CF_3CO_2H, borohydride reduction, acetylation, and g.l.c.-m.s. identified the derivatives 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylallitol and 4-*O*-acetyl-1,2,3,4′-tetra-*O*-methylapiitol (**9**). The fact that **9** was 4′- and not 1-acetylated is consistent with n.m.r. data that eliminated structure **3** and showed that methylated **7** was derived from D-All*p*-(1→4)-apiitol.

The unusual nature of the aglycon in **1** prompted a check of the extraction procedure. Samples of fern leaf were extracted with MeOH–$CHCl_3$–H_2O (12:5:3), MeOH–$CHCl_3$–H_2O–HCOOH (12:5:2:1), 87% aq. ethanol, or water, and the composition of each extract was determined by g.l.c. of the trimethylsilylated derivatives. The g.l.c. profiles were similar to that in Fig. 1 and indicated that **1** was not an artifact of the extraction procedure.

DISCUSSION

The name cardiomanol is proposed for (*E*)-4-hydroxy-2-(hydroxymethyl)but-2-enyl β-D-allopyranoside (**1**).

Allose is rare in Nature. It is *O*-glycosylically linked to a sesquiterpenoid in *Cibotium barometz* (family Dicksoniaceae)[18] and to phenols in *Osmunda asiatica* (Osmundaceae)[19] and *Arachniodes standishii* (Dryopteridaceae)[20], and is *C*-linked in the xanthone, dilatatin, from *Hymenophyllum dilatatum* (Hymenophyllaceae)[21].

The generic classification of the Hymenophyllaceae is contentious. The conservative approach recognises 2 genera, *Hymenophyllum* and *Trichomanes*, but more radical approaches recognise up to 42 genera[22]. Irrespective of this, *C. reniforme*, the only species in *Cardiomanes*, is considered to be isolated phylogenetically from the other species in the family and, in a preliminary examination,

cardiomanol was not detected in *Trichomanes (Selenodesmium) dentatum* or *Hymenophyllum (Mecodium) demissum.*

Metabolically, cardiomanol is relatively inactive, since the radioactivity incorporated was dramatically less than that into the other sugars (Table I). The radioactivity of the products of hydrolysis of cardiomanol indicated a non-specific incorporation of ^{14}C into the allose and aglycon.

Despite its low rate of biosynthesis, cardiomanol is the major soluble carbohydrate in all parts of the plant. On a weight basis, it was 20-fold more abundant than sucrose in old leaves, and 3-fold more abundant in the rhizome.

The physiological bases of tolerance to desiccation are not well understood[23], but the high content of cardiomanol in *C. reniforme* (>10% of the dry weight of the lamina, Table I) suggests that it may be involved. Monosaccharides and oligosaccharides have been linked with the tolerance to desiccation of a wide range of organisms, through their ability to preserve the integrity of membranes at low water potentials[24]. Morphologically, the filmy ferns differ from other vascular plants in that the leaf lamina is only one, or in some species including *C. reniforme,* a few cells thick[25]. Stomata are absent, the epidermal cells lack a well developed cuticle, and it is presumed that exchange of water and gases occurs directly through the leaf surface. These ferns conform to the definition of poikilohydrous plants, that is those plants which lack the ability to modulate water exchange significantly[26]. The frond of *C. reniforme* is remarkable amongst the filmy ferns in that the lamina is kidney shaped, up to about 100 mm across, and is undissected[25]. Ecologically, the filmy ferns are restricted to sites of frequent high humidity, mainly in the tropics and southern temperate zones. However, many are epiphytes and can tolerate considerable desiccation[27]. *C. reniforme,* typically on the forest floor and a low-to-mid epiphyte in lowland and lower mountain forests throughout New Zealand, is also abundant on the scoria field of the recent volcanic island Rangitoto. Although experimental evidence is lacking, this pattern of distribution and field observations indicate it to possess considerable tolerance to desiccation, partly manifest by the in-rolling of the leaves under dry conditions[25,28].

Secondary metabolites are widely implicated in protection against pathogens and predation. Tests on agar medium indicated that cardiomanol does not possess antifungal or antibacterial activity.

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