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Phytochemistry 67 (2006) 1365–1371
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

A galloylated cyanogenic glycoside from the Australian endemic rainforest tree *Elaeocarpus sericopetalus* (Elaeocarpaceae)

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Received 31 January 2006; received in revised form 29 March 2006
Available online 22 May 2006

Abstract

A cyanogenic glycoside – 6′-*O*-galloylsambunigrin – has been isolated from the foliage of the Australian tropical rainforest tree species *Elaeocarpus sericopetalus* F. Muell. (Elaeocarpaceae). This is the first formal characterisation of a cyanogenic constituent in the Elaeocarpaceae family, and only the second in the order Malvales. 6′-*O*-galloylsambunigrin was identified as the principal glycoside, accounting for 91% of total cyanogen in a leaf methanol extract. Preliminary analyses indicated that the remaining cyanogen content may comprise small quantities of sambunigrin, as well as di- and tri-gallates of sambunigrin. *E. sericopetalus* was found to have foliar concentrations of cyanogenic glycosides among the highest reported for tree leaves, up to 5.2 mg CN g^{-1} dry wt.

Keywords: Australia; Chemotaxonomy; Cyanogenesis; Cyanogenic glycoside; Elaeocarpaceae; Gallic acid; Malvales; Sambunigrin; Tropical rainforest

1. Introduction

Elaeocarpus sericopetalus F. Muell. (“Northern Quandong”) is a small to medium sized tree, endemic to tropical rainforest in north eastern Queensland, Australia. The species belongs to the Elaeocarpaceae family, a small family of trees or shrubs (approx. 520 spp., 12 genera) distributed from temperate regions of South America, New Zealand and Japan to the tropics of Malaysia and the West Indies (Morley and Toelken, 1983; Mabberley, 1990). Within Australia the family is represented by 41 species in 6 genera (Hnatiuk, 1990), dominated by the largest genus *Elaeocarpus*, which occurs throughout Australia, East Asia, Malaysia and the Pacific Islands (Morley and Toelken, 1983). Many of the 27 species of *Elaeocarpus* found in Australia are endemic to northern Queensland rainforest (Coode, 1984), and several are important cultivated timber species (e.g. *Elaeocarpus angustifolius* Blume).

As part of a large study of cyanogenesis in Australian tropical rainforests, *E. sericopetalus* was the only species among eight *Elaeocarpus* spp. found to be cyanogenic (Miller et al., in press). Leaves of *E. sericopetalus* were found to be very highly cyanogenic; mature field-grown leaves had as much as 5.2 mg CN g^{-1} dry wt. The concentrations of cyanogenic glycosides in this species are among the highest reported for tree leaves.

Within the family Elaeocarpaceae and the order Malvales, alkaloids (e.g. indolizidine alkaloids) are considered common (Hegnauer, 1990; Mabberley, 1990; Gibbs, 1974). By contrast, cyanogenesis in the Elaeocarpaceae and Malvales is rare (Gibbs, 1974; Hegnauer, 1990; Lechtenberg and Nahrstedt, 1999). The list of cyanogenic species compiled by Tjon Sie Fat (1979) includes only four species from the Malvaceae, Tiliaceae and Sterculiaceae families of the Malvales. Several of these results were questioned by Hegnauer (1973, 1990). In addition, Gibbs (1974)

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doi:10.1016/j.phytochem.2006.03.022

identified only one cyanogenic species within the Malvales, and was highly skeptical about many of the positive results from other authors for about 15 genera across other families in the order. Hegnauer (1986) similarly, expressed doubt over positive results reported for species in Tiliaceae and Elaeocarpaceae families from the Philippines [Quisumbing (1951) cited in Hegnauer (1973, 1986)]. In the Elaeocarpaceae family, cyanogenesis has previously been reported in tissues from only two species: the leaves of *Vallea stipularis* var. *pyrifolia* F. Ballard (Gibbs, 1974), and the leaves [Greshoff (1898) cited in Hegnauer (1973)] and the bark (Pammel, 1911; Rosenthaler, 1919) of *Sloanea sigun* (Blume) K. Schum (syn. *Echinocarpus sigun*). Cyanogenic glycosides were recently characterised from a species in the Sterculiaceae, the first such report for the order Malvales (Seigler et al., 2005).

This study, therefore, constitutes the first report of cyanogenesis in the genus *Elaeocarpus*, and the first full characterisation of a cyanogen in the family Elaeocarpaceae, and only the second in the order Malvales. Given the paucity of knowledge of cyanogenesis within this taxonomic group, the aim of this work was to examine the distribution of cyanogenic glycosides in different plant tissues, and characterise the main cyanogenic glycosides present in the highly cyanogenic foliage of the Queensland endemic *E. sericopetalus*.

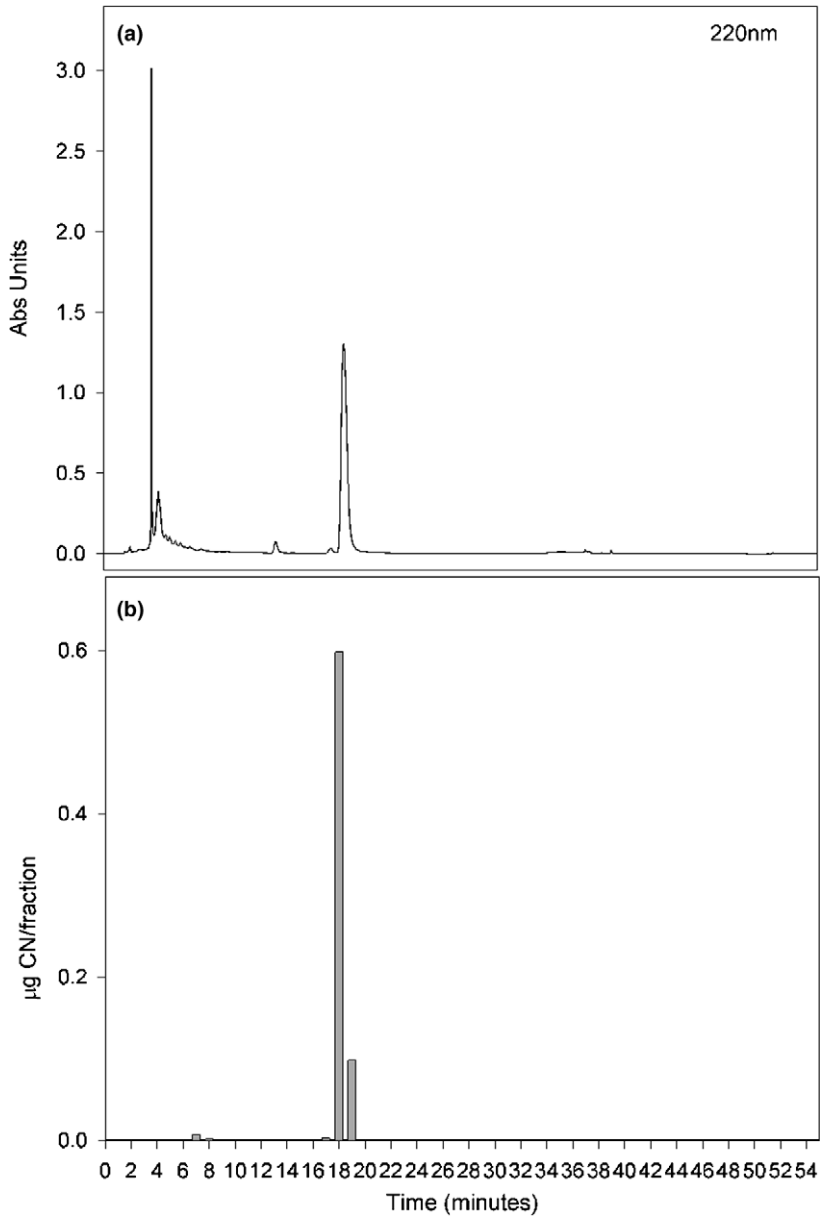


Fig. 1. (a) Chromatogram of *Elaeocarpus sericopetalus* leaf MeOH extract fractionated by RP-HPLC, and (b) cyanide content (µg) of 1 min fractions eluted from an analytical C_{18} column using 20% MeCN as the running solvent at 1 ml min^{-1}. The sample analysed was the 20% MeOH eluate from a C_{18} solid phase extraction cartridge. The majority of cyanogenic activity (99%) was associated with a single peak (**F2;** RT 17-18 min) identified as **1.** The remaining 1% of cyanide was detected at RT 7–8 min (**F1**).

2. Results and discussion

2.1. Concentrations of cyanogenic glycosides in *E. sericopetalus*

Cyanogenic glycosides were distributed throughout all tissues sampled in *E. sericopetalus*: leaves, woody stems and fruit. The concentration of cyanogenic glycosides in the freeze dried, ground leaf tissue from six *E. sericopetalus* saplings (>3 m tall) and one tree ranged from 3.5 to 5.2 mg CN g^{-1} dry wt; concentration was unrelated to plant size. In woody stem and immature fruit (incl. fruit flesh and seed) samples, the concentrations of cyanogenic glycosides were 0.11 mg CN g^{-1} dry wt. and 1.08 mg CN g^{-1} dry wt, respectively. In the foliage, there was sufficient endogenous β-glycosidase for complete hydrolysis of cyanogenic glycosides. By contrast, in stem and fruit, hydrolysis of cyanogenic glycosides was incomplete after 24 h without the addition of enzyme partially purified from *E. sericopetalus* foliage. In addition, in each case, β-glucosidase from almonds (emulsin from *Prunus amygdalus* Batsch added at 1.04 units ml^{-1}) was not as effective as the extracted *E. sericopetalus* enzyme in fully catabolising the cyanogens. This is not surprising given that the *E. sericopetalus* leaf MeOH extract was found to be dominated by galloylated compounds, and emulsin, like other β-glucosidases, has been reported only to cleave non-modified terminal glucose residues (Nahrstedt et al., 1983; Hseih and Graham, 2001).

2.2. Identification of cyanogenic glycosides in *E. sericopetalus*

Fractionation of leaf MeOH extracts of *E. sericopetalus* by RP-HPLC yielded four cyanogenic regions. The most abundant cyanogenic fraction **F2** (RT 17–18 min; analytical HPLC; λ_{max} 220 nm) accounted for 91% of total cyanide in the crude MeOH extract and was the only cyanogenic constituent purified for characterisation (Fig. 1).

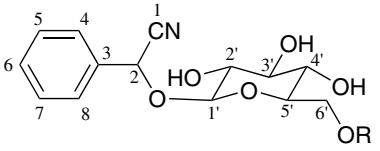
Fraction **F2** was purified by preparative C_{18} HPLC, affording 6′-*O*-galloylsambunigrin (**1**). High resolution ESI(+)MS analysis of **1** revealed a pseudo molecular ion (M+Na) consistent with a molecular formula ($C_{21}H_{21}NO_{10}$, Δmmu = 0.1) requiring 12 double bond equivalents. The ^{1}H NMR spectrum (CD_3OD, 400 MHz) of **1** was close to sambunigrin (**2**) (Seigler et al., 2002) and prunasin (**3**) (Seigler et al., 2002), but with the addition of a singlet at δ_H 7.10 (*s*, 2H). By consideration of the molecular formula of **1** the extra resonance could be explained by a galloyl moiety.

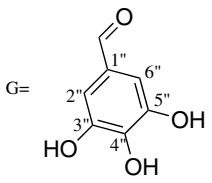
6′-*O*-galloylprunasin (**4**) has been reported twice before in plants (Isaza et al., 2001; Ling et al., 2002). Isaza et al. (2001) reported their NMR data in an unspecified mixture of d_6-acetone and D_2O, so no direct comparison with data for **1** could be made. By contrast, Ling et al. (2002) reported the NMR data in CD_3OD, enabling comparison with the ^{1}H NMR and ^{13}C NMR spectra of **1**. The ^{13}C NMR data for **1** was in close agreement with **4**, with the largest difference being only 1.0 ppm. However, the ^{1}H NMR data of **1** differed significantly, with downfield chemical shifts of δ_H 0.40 (H-1′), 0.23 (H-5′), and 0.09 (H-2) and an upfield chemical shift of δ_H 0.14 (H-2′) when compared with **4** (Ling et al., 2002). These differences suggested that **1** was indeed different to **4**.

Further NMR experiments (Table 1) were used to complete the gross structure of **1**. In particular, an HMBC correlation from the glucose methylene (δ_H 4.58 and 4.45) to the galloyl carbonyl (δ_C 168.3) placed the galloyl group at the 6′ position. These data confirmed **1** had an identical gross structure to **4**.

Given the challenges of resolving differences in the sugar moieties of **1** and **4** by NMR, the alditol acetates of **1** were analysed by GC–MS. GC–MS analysis of the TMS derivative following acid methanolysis of **1** had previously indicated glucose. The alditol acetates of **1** ran as a single peak with identical retention times relative to the SI standard (RRT 0.972), and the same ion spectrum, corresponding with that for the glucitol standard. In addition, the alditol acetate of **1** was added to a mix of hexitol standards (alditol acetates of mannose, galactose and glucose); the uniform amplification of the glucose signal confirmed 1-6 glucose as the sugar.

The chirality of the cyanogenic centre (C-2) was suggested to be *S* due to characteristic resonances in the ^{1}H NMR spectrum of **1**. The glucose anomeric resonance (H-1′) of **1** (δ_H 4.66, *d* 7.9 Hz) was much closer to that published for sambunigrin (**2**) (cf. δ_H 4.672, *d* 7.8) than prunasin (**3**) (cf. δ_H 4.253, *d* 7.8) (Seigler et al., 2002). In addition, the ^{1}H NMR spectrum of **1** showed a downfield shift of 0.09 ppm for H-2, compared with that published for **4** (Ling et al., 2002), which was also consistent with a sambunigrin moiety and hence *S* configuration at C-2.





1 2*S* R = G
2 2*S* R = H
3 2*R* R = H
4 2*R* R = G

Enzymatic hydrolysis of **1** with tannase was carried out following the procedure of Isaza et al. (2001). The hydrolysis released two products: gallic acid was detected by LC/

Table 1
NMR data for **1** (CD_3OD, 400 MHz)

No.	δ_C	δ_H [*m*, J (Hz)]	gHMBC	COSY
1	118.3			
2	69.0	5.79 (*s*)	C-1, C-3, C-4/C-8, C-1′	
3	134.9			
4/8[a]	128.8	7.49 (*m*)	C-2, C-5/C-7, C-6	H-5/H-7
5/7[a]	129.9	7.37 (*m*)	C-3, C-4/C-8	H-4/H-8
6	130.7	7.37 (*m*)	C-4/C-8	
1′	102.4	4.66 (*d*, 7.9)	C-2, C-2′, C-3′	H-2′
2′	74.7	3.26 (*m*)	C-1′, C-3′, C-4′	H-1′, H-3′
3′	77.7	3.45 (*m*)		
4′	71.5	3.45 (*m*)		
5′	75.9	3.63 (*m*)	C-3′, C-4′, C-6′	H-4′
6′a	64.3	4.58 (*dd*, 1.9, 12.0)	C-4′, C-5′, C-7″	H-5′, H-6′b
6′b		4.45 (*dd*, 5.3, 12.0)	C-4′, C-5′, C-7″	H-5′, H-6′a
1″	121.3			
2″/6″[a]	110.2	7.10 (*s*)	C-1″, C-2″/C-6″[b], C-3″/C-5″, C-4″, C-7″	
3″/5″[a]	146.6			
4″	140.0			
7″	168.3			

[a] Carbon atoms grouped due to symmetry.
[b] HMBC correlation from H-2″ to C-6″ and H-6″ to C-2″.

MS with another compound with MW 295 consistent with either sambunigrin or prunasin. Subsequent ^{1}H NMR (600 MHz, CD_3OD) revealed this compound was sambunigrin (**2**), supporting the assignment of **1** as 6′-*O*-galloylsambunigrin (**1**).

Elaeocarpus sericopetalus is among a small number of reported species that contain cyanogenic glycosides with organic acid residues, in this case, gallic acid. The only previous reports of cyanogenic glycosides with a galloyl moiety are those by Isaza et al. (2001) and Ling et al. (2002) of 6′-*O*-galloylprunasin from two species in the Melastomataceae family. Isaza et al. (2001) isolated 6′-*O*-galloylprunasin (**4**) from leaves of the Colombian medicinal plant *Monochaetum multiflorum* (Bompl.) Naudin, while in the Malaysian medicinal plant *Phyllagathis rotundifolia* (Jack) Bl., **4** was the most abundant of seven galloylated cyanogenic glycosides in leaves, accounting for around 50% by mass of total cyanogen (Ling et al., 2002). The galloylated cyanogenic glycosides were restricted to the leaves where prunasin was a minor constituent (11% of cyanogen); however, in the combined root/stem fraction, prunasin was the only cyanogenic glycoside (Ling et al., 2002).

As found in *P. rotundifolia,* where multiple galloylated cyanogens were identified (Ling et al., 2002), there was some evidence for a number of galloylated cyanogens in the *E. sericopetalus* foliar extract. Preliminary LC–MS analysis of the 10% and 40% MeOH eluates from solid phase extraction cartridges, which included minor cyanogenic fractions **F3** and **F4**, indicated molecular weights consistent with di- and tri-gallates of sambunigrin; however, these were not present in sufficient quantity to purify for full characterisation.

Cyanogenic glycosides esterified with phenolic acids other than gallic acid have also been reported. Examples of prunasin derivatives are grayanin, a caffeoyl ester of prunasin from *Prunus grayana* Maxim. bark (Shimomura et al., 1987), and 6′-*O*-malonylprunasin isolated from leaves of *Merremia dissecta* (Convolvulaceae; Nahrstedt et al., 1989). In both instances, organic acid residues are attached at the C-6 of the glucose. Interestingly, while 6′-*O*-malonylprunasin (5% total cyanogen) was isolated along with prunasin (95% total cyanogen) from leaves of *M. dissecta* (Convolvulaceae; Nahrstedt et al., 1989), the seeds contained three disaccharide cyanogenic glycosides: amygdalin, and two derivatives esterified with phenolic acids [the 6″-(4-hydroxy)benzoate and 6″-(4-hydroxy)-*E*-cinnamate of amygdalin; Nahrstedt et al., 1990]. In addition, the cyanogenic glycosides, 4′-*O*-*p*-coumarate and 4′-*O*-caffeate of prunasin, where the organic acid residues are substituted at C-4, were isolated from the fern *Microlepia strigose* (Thunb.) C.Presl. (Dennstaedtiaceae; Wada et al., 1997). Several more complex cyanogenic glycosides with phenolic acid residues have also been reported: the complex di-glycosides with *p*-coumaric acid residue from *Anthemis* spp. (Asteraceae; Nahrstedt et al., 1983), and xeranthin, a complex tri-glycoside with caffeic acid residue from *Xeranthemum cylindraceum* (Asteraceae; Schwind et al., 1990).

In the majority of these studies, the phenolic acid derivatives co-occur with the cyanogenic glycoside precursor [e.g. with prunasin (Nagumo et al., 1985; Nahrstedt et al., 1989), epilucumin (Nahrstedt et al., 1983) or amygdalin (Nahrstedt et al., 1990)]. However, this is not always the case; prunasin was not reported with its organic acid esters by Isaza et al. (2001) or Shimomura et al., (1987; from the bark of *P. grayana*). In this study, the presence of sambunigrin could not ultimately be confirmed. While both HPLC RT and the MW (295 amu) of the minor cyanogenic fraction **F1** in the 10% MeOH SPE eluate, suggested trace amounts of sambunigrin (**2**) in the *E. sericopetalus* extract, insufficient material was available for confirmation by ^{1}H NMR.

This is the first published characterisation of a cyanogenic glycoside from the family Elaeocarpaceae, and only the second from the order Malvales. Given the structure of the aglycone (non-hydroxylated phenyl ring), and cooccurrence with sambunigrin, albeit in small quantities, it seems likely that **1** is biosynthetically derived from the amino acid phenylalanine. The findings here for *E. sericopetalus* are consistent with a reference to an unpublished report of phenylalanine-derived sambunigrin in the leaves of *Sloanea sigun* (Blume) K. Schum (syn. *Echinocarpus sigun*) [Elaeocarpaceae; Hegnauer and Fikenscher unpub data (1983) cited in Hegnauer (1990)], the only other reference to a cyanogenic constituent in Elaeocarpaceae. Interestingly, tyrosine-derived dhurrin and taxiphyllin were recently isolated from leaves of *Guazuma ulmifolia* Lam.

in the Sterculiaceae family, the only previous report for the Malvales (Seigler et al., 2005).

3. Experimental

3.1. Plant material

Leaf samples were obtained from six saplings (all >3 m tall) and one large tree growing in upland rainforest at two sites on the Atherton Tablelands (17°13.3′S, 145°40.4′E, 900 m a.s.l. and 17°27.3′S, 145°28.6′E 1100 m a.s.l.), Qld, Australia and pooled for analysis. Leaves were collected and stored in air-tight plastic bags on ice for up to 2 h, and were then frozen in liquid nitrogen, freeze-dried and ground using an analytical mill (IKA® Labortechnik, Janke and Kunkel, Germany). The concentration of cyanogenic glycosides in the freeze-dried, ground tissue homogenate was 4.8 mg CN g^{-1} dry wt. Woody stem and immature fruit (seed and fruit flesh) samples were also obtained from the large tree and were assayed for cyanide. Due to limited tissue samples, only cyanogenic glycosides in leaf tissue were purified. Voucher specimens have been lodged at the University of Melbourne (specimens MELU 102135, 102304) and Queensland herbaria (specimen BRI AQ. 578817).

3.2. Detection and quantification of cyanogenic glycosides

Cyanogenic glycoside concentration in plant material and in leaf tissue extracts was measured by hydrolysing the glycoside and trapping the evolved cyanide in a 1 M NaOH well (Gleadow et al., 1998; Brinker and Seigler, 1989). Freeze-dried, ground plant tissue (20 mg) was incubated for 20 h at 37 °C with 1 ml of 0.1 M citrate buffer–HCl (pH 5.5), conditions which allowed for complete conversion of the cyanogenic glycoside to cyanide (data not shown). Previous experiments had shown that tissue contained sufficient endogenous β-glucosidase for complete hydrolysis of the cyanogenic glycosides; however, in stem and fruit, the complete hydrolysis of cyanogenic glycosides required the addition of enzyme partially purified from *E. sericopetalus* foliage.

To detect and quantify cyanogenic compounds in extracts and fractions during purification, glycosides were hydrolysed using β-glucosidase enzyme partially purified from the same leaf tissue used for cyanogenic glycoside purification (see below). Cyanide in the NaOH well was determined using the method of Gleadow and Woodrow (2002) adapted from Brinker and Seigler (1989) for use with a photometric microplate reader with incubator (Labsystems Multiskan® Ascent, Helsinki, Finland).

3.3. Cyanogenic β-glycosidase purification

Cyanogenic β-glycosidase was partially purified from the same *E. sericopetalus* leaf tissue used for the identification of the cyanogenic constituents. Freeze-dried tissue (5 g) was extracted at 4 °C in a protein extraction buffer (Gleadow et al., 1998), filtered, and centrifuged (20 min at 27,000*g*) to remove remaining tissue. The supernatant was fractionated by adding solid $(NH_4)_2SO_4$, and proteins precipitating between 35% and 90% $(NH_4)_2SO_4$ saturation were collected following centrifugation (20 min at 27,000*g*), resuspended in a minimum amount of buffer (0.1 M citrate buffer–HCl, pH 5.5) and desalted using a dialysis cassette (Slide-A-Lyzer® 3.5 K, MWCO 3500, Pierce, Rockford, IL, USA) in 0.1 M citrate buffer–HCl (pH 5.5). Aliquots of the crude enzyme preparation were incubated and tested for CN to verify that no cyanogenic glycoside had been extracted in the protein preparation. The desalted enzyme preparation was tested for activity against the crude MeOH extract over a 24 h period at 37 °C to determine the minimum quantity of crude enzyme needed for complete hydrolysis of cyanogenic glycosides (data not shown). In subsequent experiments, several times the required minimum enzyme was added. Both β-glucosidase from almonds (emulsin from *Prunus amygdalus* Batsch; E.C. 3.2.1.21, Sigma G-0395 added at 1.04 units ml^{-1}), and pectinase from *Rhizopus* sp. (Macerase® Pectinase, E.C. 3.2.1.15; 441201 Calbiochem®, Calbiochem-Novabiochem Corp., CA, USA added at 1.22 units ml^{-1}) were also tested for activity but failed to fully hydrolyse the cyanogenic glycosides. The extracted *E. sericopetalus* enzyme was therefore used for all subsequent assays.

3.4. Purification of cyanogenic compounds

Homogenised, freeze-dried tissue (12 g) was extracted using the similar procedure as Miller et al. (2004). Following extraction with petroleum ether (solvent:tissue, 10:1 v/w), tissue was twice extracted with cold MeOH, and filtered (Whatman® 541 filter paper, Whatman Asia Pacific, San Centre, Singapore). The filtrate volume was reduced by rotary evaporation (40 °C), and an equivalent volume of $CHCl_3$ was added, with sufficient H_2O to facilitate phase separation. The MeOH/$CHCl_3$ phase was collected, concentrated in vacuo, resuspended in H_2O and fractionated by elution through a solid-phase extraction cartridge (Maxi-Clean™ C_{18}, 900 mg cartridge, Alltech Associates, Baulkham Hills, Australia) at 1 ml min^{-1}, using a step MeOH gradient (0%, 10%, 20%, 30%, 40%, 100% MeOH in H_2O). Fractions (10 ml) were collected, concentrated in vacuo, and tested quantitatively for cyanogenic glycosides by the addition of β-glucosidase enzyme partially purified from the same leaf tissue.

The majority of cyanogenic activity was retained in the 20% and 30% MeOH eluates. All cyanogenic eluates were concentrated in vacuo and fractionated by analytical RP-HPLC using 20% MeCN–H_2O (1 ml min^{-1}) for 30 min, increased to 95% MeCN–H_2O over 20 min and held for 5 min, through a Phenomenex Luna C_{18} column (250 mm × 10 mm × 5 μm particle size; Phenomenex,

Pennant Hills, Australia). Fractions (1 min) were concentrated in vacuo and tested for the presence of cyanogenic glycoside using enzyme partially purified from the same leaf tissue.

Across all eluates, there were four regions of cyanogenic activity – fractions **F1** (RT 7–8 min), **F2** (RT 17–18 min), **F3** (RT 28–30 min) and **F4** (RT 38–40 min). The 20% and 30% MeOH eluates were the highest yielding fractions, and in each case, a dominant peak (**F2**; RT 17–18 min; λ_{max} 220 nm in 20% MeCN) accounted for 99% and 97% of total cyanide, respectively. The most abundant cyanogenic compound (**F2**; RT 17–18 min, λ_{max} 220 nm, analytical HPLC) accounted for 91% of total cyanide in the crude MeOH extract and was the only cyanogenic fraction purified for characterisation (Fig. 1). In the 20% MeOH eluate, the remaining 1% of cyanide was associated with **F1** (RT 7–8 min); in the 30% MeOH eluate, 0.6% of total cyanide was associated with **F1** (RT 7–8 min) and 1.5% with **F3** (RT 28–29 min). The lower yielding 10% and 40% eluates afforded multiple fractions with cyanogenic activity. The 10% MeOH eluate, indicated two cyanogenic fractions, **F1** (RT 7–8 min; 64% of CN), and **F2** (RT 17–18 min; 27% of CN). The 40% MeOH eluate yielded three regions of cyanogenic activity, **F2** (5.5% of CN), **F3** (RT 28–30 min; 48% of CN), and a fourth fraction (**F4**; RT 38–40 min) where 34% of CN was detected. Due to the low yields of 10% and 40% MeOH fractions and the poor baseline resolution fractions **F1**, **F3**, and **F4**, these were not purified.

Subsequent purification of **1**(**F2**) was carried out by preparative RP-HPLC, using 23% MeCN–H_2O at 2 ml min^{-1} for 20 min, through a Phenomenex Luna C_{18} preparative column (250 mm × 10 mm × 5 μm particle size; Phenomenex, Pennant Hills, Australia), the MeCN–H_2O percentage then increased to 95% over 15 min, affording 5 mg of pure **1**. Under these conditions, **1** eluted at RT 24–25 min (λ_{max} 220 nm).

4. Structure elucidation

4.1. GC–MS analyses

4.1.1. Monosaccharide composition analysis

The monosaccharide composition of **1** was determined by GC–MS, after acid methanolysis and TMSi (trimethylsilyl) derivatisation according to Ralton and McConville (1998) and McConville et al. (1990). Following derivatisation, samples containing 0.1 mM *scyllo*-inositol (SI) as an internal standard were dried under N_2, resuspended in hexane and analysed by GC–MS (HP6890-MSD, Agilent Technologies, Forest Hill, Vic., Australia). Derivatised samples (1 μl) were injected at 250 °C onto a column (HP-1MS; 30 m × 250 μm × 0.25 μm) and analysed with a temperature program increasing from 140 to 250 °C at 5 °C min^{-1}, for 10 min, and then increasing to 265 °C at 15 °C min^{-1} for 5 min. The flow rate of He carrier gas was 1.0 ml min^{-1}.

4.1.2. Methylation linkage analysis

To confirm the monosaccharide composition of **1**(**F2**), the GC–MS of the alditol acetate was analysed. The *E. sericopetalus* glycoside (**1**) and hexose sugar standards were analysed by methylation linkage analysis according to the method of Ciucanu and Kerek (1984), modified by McConville et al. (1990). The partially methylated alditol acetates (PMAAs) were resuspended in DCM (50 μl) and analysed by GC–MS. Samples were injected at 250 °C onto the column (HP-1MS; 30 m × 250 μm × 0.25 μm) and separated using a temperature program increasing from 80 to 140 °C at 30 °C min^{-1}, and from 140 to 250 °C at 5 °C min^{-1}, and held at that temperature for 20 min. The flow rate was 1.0 ml min^{-1}. The retention time of PMAAs relative to *scyllo*-inositol (SI) hexaacetate internal standard, and the mass spectra were compared with authentic hexose sugar standards.

5. General experimental procedures

NMR experiments were performed on either Varian Inova 400, Varian Unity 400 plus or Bruker Avance 600 spectrometers. Chiroptical measurements ($[\alpha]_D$) were obtained on a Jasco Dip-1000 digital polarimeter in a 100 mm by 2 mm cell. Ultraviolet (UV) absorption spectra were obtained using a Shimadzu UV-1650PC Spectrophotometer. ESI(±)MS were acquired using a Micromass ZMD mass detector or an Agilent 1100 series LC/MSD. High resolution (HR) ESI-MS measurements were obtained on a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III source.

5.1. Enzymatic hydrolysis of *1*

To confirm the chirality of the C-2, **1** was hydrolysed following the method of Isaza et al. (2001). A sample of **1** (0.5 mg) was dissolved in H_2O (1 ml) and 500 μl of 1 mg ml^{-1} aqueous solution of tannase (Sigma #42395) added. After overnight incubation at 37 °C, the reaction was checked by LC/MS (Zorbax Stablebond C_8, 150 × 4.6 mm, 5 μm, 90% H_2O/MeCN(0.05% HCO_2H) to 50% H_2O/MeCN(0.05% HCO_2H) over 15 min at 1 ml min^{-1}). Gallic acid eluted at RT 2.6 min (ESI(±)MS *m/z* 171 $(M+H)^+$, 193 $(M+Na)^+$ and 169 $(M-H)^-$) and sambunigrin at RT 6.6 min (ESI(±)MS *m/z* 318 $(M + Na)^+$ and 340 $(M + HCO_2)^-$). ^1H NMR (600 MHz, CD_3OD) δ 7.58 (2 H, *m*, ArH) 7.43 (3H, *m*, ArH), 6.04 (1H, *s*, ArH), 4.67 (1H, *d*, 8.0, H-1′), 3.93 (1H, *dd*, 2.3, 12.0, H-6′a), 3.67 (*overlapping*, H-6′b), 3.40 (2H, *overlapping*, H-3′/H-5′), 3.27 (1H, *m*, H-2′) for sambunigrin and δ 7.01 (*s*) for gallic acid.

5.2. 6′-*O*-galloylsambunigrin (*1*)

Colourless oil; $[\alpha]_D^{21} -41^\circ$ (MeOH; *c* 0.4); UV (MeOH) λ_{max} nm (log ε): 216 (9.1), 278 (10.2); ESI(±)MS *m*/*z* 470 $(M+Na)^+$, 446 $(M-H)^-$; ^1H and ^{13}C NMR (see Table 1); HRESI(+)MS *m*/*z* 470.1062 ($C_{21}H_{21}NO_{10}Na$ calc. 470.1063).

Acknowledgements

We thank Rigel Jensen for assistance with plant identification in the field, and Assoc. Prof. Malcolm McConville, Dept. of Biochemistry and Molecular Biology, The University of Melbourne, for assistance with GC–MS analyses and useful discussions. Thanks also goes to Isao Kouno, Nagasaki University and Takashi Yoshida, Okayama University, for sending authentic samples of 6′-*O*-galloylprunasin for structure comparison. Field work was supported by an Individual Award from the Queen's Trust for Young Australians to R.E. Miller.

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