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Cyanogenic glycosides from the rare Australian endemic rainforest tree *Clerodendrum grayi* (Lamiaceae)

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Abstract

The cyanogenic diglycoside lucumin ((*R*)-mandelonitrile-β-D-primeveroside) and monoglucoside prunasin ((*R*)-mandelonitrile-β-D-glucoside) were isolated from the foliage of the rare Australian rainforest tree species *Clerodendrum grayi* (Lamiaceae). This is the first reported isolation of the diglycoside lucumin from vegetative tissue (foliage), and the first reported co-occurrence of lucumin and prunasin. Furthermore, unusually, the diglycoside lucumin was the most abundant cyanogen accounting for approximately 60% of total cyanide in a leaf tissue.

Keywords: *Clerodendrum grayi*; Lamiaceae, Verbenaceae; Cyanogenesis; Cyanogenic glycoside; Lucumin, prunasin; Tropical rainforest; Chemotaxonomy

1. Introduction

Clerodendrum grayi Munir (Lamiaceae syn. Verbenaceae) is a subcanopy rainforest tree (3–5 m tall) endemic to the northern part of Queensland, Australia. It has a very limited distribution on the Atherton Tableland: it is only known south south east of Mareeba, between latitudes 17° and 18°S, and longitudes 145° and 146°E (Munir, 1989).

Recent revisions of the division between Lamiaceae and Verbenaceae families (Cantino, 1992; Wagstaff et al., 1997, 1998) have transferred the genus *Clerodendrum* to the Lamiaceae or "mint" family. The Lamiaceae is a large cosmopolitan family (>250 genera, 6700 spp. worldwide) and includes many economically important species such as medicinal and culinary herbs [e.g., *Lavandula* (lavender); *Mentha* (mint)], cultivated ornamentals [e.g., *Salvia* (sage)], and also tropical hardwood species (e.g., teak), formerly in the Verbenaceae. In terms of secondary metabolites, typical constituents are monoterpenoids, diterpenes or triterpenes [e.g., *Thymus* (thyme); *Ocimum* (basil)], as well as flavonoids and iridoid glycosides (Gibbs, 1974; Hegnauer, 1989; Taskova et al., 1997). By contrast, cyanogenesis in Lamiaceae, and also Verbenaceae, has rarely been reported. Even within the order Lamiales, cyanogenesis is considered rare, with only a few reports, mostly from early workers (e.g., Juliano, 1923; see also Gibbs, 1974).

As part of a large study of cyanogenesis in Australian tropical rainforests, *C. grayi* was found to be very highly cyanogenic, with foliar concentrations of cyanogenic compounds ranging from 1.8 to 4.8 mg CN g^{-1} dry wt in mature field-grown tree leaves (Miller, 2004). These concentrations are among the highest reported for tree leaves. Several species of *Clerodendrum* are known to be toxic (e.g., Pammel, 1911; Hurst, 1942; Webb, 1948; CFSAN, 2003) although the poisons have not always been detailed, and while a few species of *Clerodendrum* have been found to be cyanogenic – *C. intermedium* Berthold Thomas (Gibbs, 1974), *C. molle* var. *molle* (Adsersen et al., 1988)

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and *C.* sp (Tjon Sie Fat, 1979) – no cyanogenic constituents have previously been identified in the genus. Indeed, cyanogenic glycosides have only been identified in one species in the Lamiaceae family: prunasin and an isomer of amygdalin ((*R*)-mandelonitrile 2-*O*-β-D-glucoside-β-D-glucoside) were isolated from the leaves of the medicinal annual herb *Perilla frutescens* Britt. var. *acuta* Kudo (Aritomi et al., 1985). In addition, no cyanogenic glycosides have been identified from species formerly or currently in the Verbenaceae family. Therefore, the aim of this study was to identify the cyanogenic constituents in leaves of *C. grayi* Munir.

2. Results

2.1. Concentration of cyanogenic glycosides in C. grayi

Cyanogenic glycosides were distributed throughout all tissues sampled in *C. grayi*: leaves, woody stems, floral buds and flowers. Foliar concentrations of cyanogenic glycosides ranged from 1.1 to 4.9 mg CN g^{-1} dry wt. In floral buds and flowers, the concentrations of cyanogenic glycosides were 0.60–0.73 mg CN g^{-1} dry wt and 0.54–0.96 mg CN g^{-1} dry wt, respectively.

2.2. Cyanogenic glycosides in C. grayi

Fractionation of a leaf MeOH extract of *C. grayi* by HPLC indicated the presence of two main cyanogenic compounds (Fig. 1). Similarly, only two cyanogenic compounds were detected following fractionation of non-polar cyanogens by HP-TLC (see Section 4). The two pairs of compounds proved to be the same (TLC, ESI-MS, GC–MS) and shall henceforth be referred to as compounds **1** and **2**. Based on HPLC analysis of the crude MeOH extract, the two main cyanogens accounted for 92% of total cyanide. The balance of cyanide (approximately 8%) indicated the presence of a third minor cyanogenic compound at HPLC RT 12–14 min, which was not detected in the extract fractionated by HP-TLC. The minor cyanogen was not purified in this study.

Compound **2** (HPLC RT 22 min; HPTLC $R_f = 0.4$ in solvent A) had a retention time coincident with authentic prunasin using HPLC and HP-TLC, under the same conditions. GC–MS compositional analysis following acid methanolysis and TMS-derivitisation identified glucose as the sole sugar. The molecular weight was determined by ESI-MS (positive: *m/z* 318 $[M+Na]^+$; negative: *m/z* 294 $[M-H]^-$) to be 295 amu, isobaric with prunasin/sambunigrin ($C_{14}H_{17}NO_6$). GC–MS analysis of TMS-derivatised

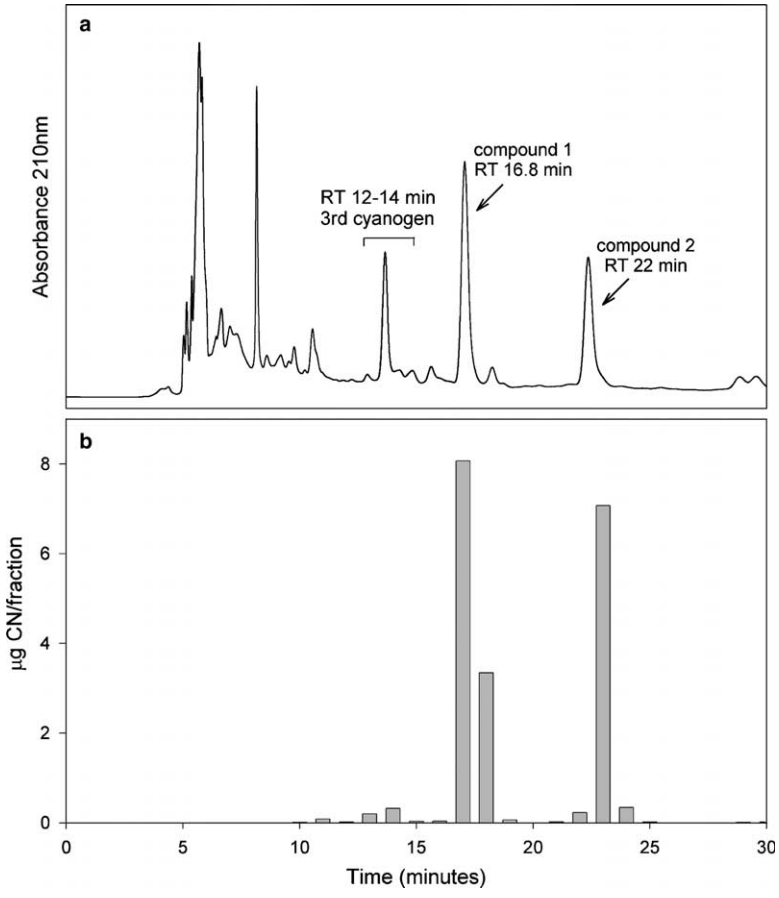

Fig. 1. (a) Fractionation of *Clerodendrum grayi* leaf MeOH extract by RP-HPLC (18% MeCN at 2 mL min^{-1}) analysed at λ 210 nm, and (b) cyanide content of fractions across the profile. The total CN detected is equivalent to that from approximately 4 mg dry wt leaf tissue.

compound **1** (purified by either HPLC or HPTLC), indicated the presence of a single major species with the same the same retention time and mass spectrum as authentic (*R*)-prunasin (Fig. 2). In the case of the HPLC-purified sample, (*R*)-prunasin represented 95% of the total sample, with 5% attributable to (*S*)-sambunigrin (Fig. 2), similar to the proportion of (*S*)-sambunigrin detected in the authentic prunasin standard (Sigma M-0636). No (*S*)-sambunigrin was detected in the sample purified by HP-TLC, indicating that some racemisation may occur during HPLC purification, under the conditions used. Based on these analyses, compound **2** was concluded to be (*R*)-prunasin [(*R*)-mandelonitrile β-D-glucoside] (Fig. 4).

Compound **1** (HPLC RT 16.8 min; $R_f = 0.22$ in solvent A was determined to be a disaccharide glycoside. Following solvolysis and TMS-derivitisation, derivatives of Glc and Xyl in the molar ratio of 1:1 were detected by GC–MS. Methylation linkage analysis afforded 1,5-*O*-diacetyl-2,3,4-*O*-tri-methyl xylitol and 1,5,6-*O*-tri-acetyl-2,3,4-*O*-tri-methyl glucitol. These derivatives were identified by GC–MS, based on their relative elution times and mass spectra compared with authentic standards. The presence of these per-methylated alditol acetates unambiguously defines the sequence β-D-xylopyranosyl 1–6 glucose.

The molecular weight of compound **1** was determined by ESI-MS (positive: *m/z* 450 $[M+Na]^+$; negative *m/z* 426.4 $[M-H]^-$) to be 427 amu. Fragmentation (MS/MS) of the parent ion was consistent with the structure of the cyanogenic glycosides (*R*)-lucumin/(*S*)-epilucumin (MW 427; $C_{19}H_{25}NO_{10}$; Fig. 3). In positive mode, fragmentation (MS/MS) of the parent ion yielded fragment ions at *m/z* 317.2 $[MW294 + Na]^+$ and *m/z* 318.2 $[MW295 + Na]^+$ corresponding to mass losses of 133 and 132, consistent with the loss of the xylose and cyanohydrin moieties, respectively. Fragmentation of the parent ion in negative mode yielded the fragment ions *m/z* 295.0 corresponding to the molecular weight of the disaccharide moiety, and *m/z* 294.0, consistent with loss of the xylose residue, as well as smaller ions at *m/z* 160.9, 148.8, 133.0, and 131.9 (Fig. 3). The *R* configuration at the chiral carbon of the aglycone was determined by ^{1}H NMR. Comparison of ^{1}H NMR (pyridine-d_5) with the literature values for lucumin (Takeda et al., 1997) and epilucumin (Nahrstedt et al., 1983), in particular the cyanohydrin proton (H-7) resonance, verified that compound **1** was (*R*)-lucumin ((*R*)-mandelonitrile 6-*O*-β-D-glucoside-β-D-xyloside) (Fig. 4).

3. Discussion

Two cyanogenic glycosides – (*R*)-prunasin and (*R*)-lucumin – were purified from the highly cyanogenic leaf tissue of *C. grayi*. This constitutes only the second report of cyanogenic glycosides in the family Lamiaceae, and the first report for the genus *Clerodendrum*.

This is the first reported co-occurrence of prunasin and its corresponding primeveroside. The majority of cyanogenic species contain a single cyanogenic glycoside, with the exception of species containing linamarin and lotaustralin. These glycosides typically, if not always, co-occur (Seigler, 1991). In instances where glycosides do co-occur in the same plant tissues, these are most commonly mixtures of enantiomeric glycosides. This is particularly common within the cyclopentanoid series of glycosides found in the Achariaceae, Flacourtiaceae (Jaroszewski and Olafsdottir, 1987), Malesherbiaceae, Passifloraceae (Olafsdottir et al., 1989), and Turneraceae (Olafsdottir et al., 1990) families, but mixtures also occur of phenylalanine-derived glycosides [e.g., co-occurrence of (*R*)-prunasin and (*S*)-sambunigrin in *Prunus turneriana* (Miller et al., 2004), and *Acacia* spp. (Conn et al., 1985; Maslin et al., 1988)].

The co-occurrence of prunasin and lucumin here is noteworthy, not only because it was hitherto unreported, but

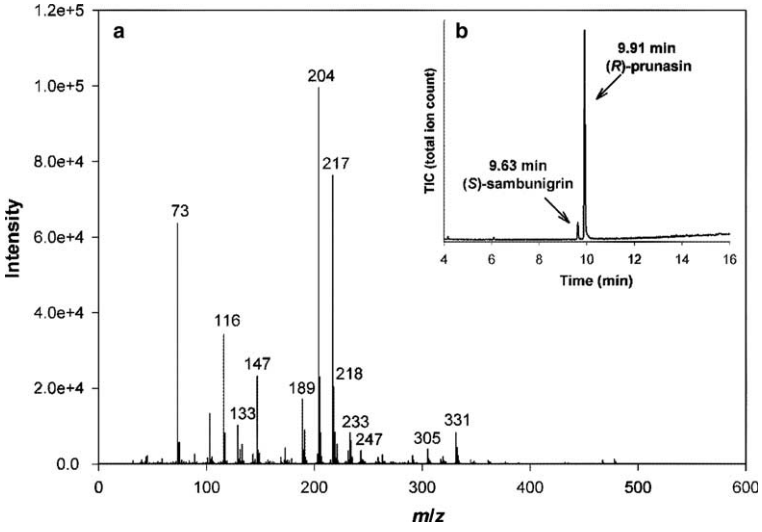

Fig. 2. (a) GC mass spectrum of the TMS derivative of compound **2** [(*R*)-prunasin RT 9.91 min] purified by HPLC, and (b) the corresponding GC chromatogram illustrating the presence of 5% (*S*)-sambunigrin in the HPLC-purified sample.

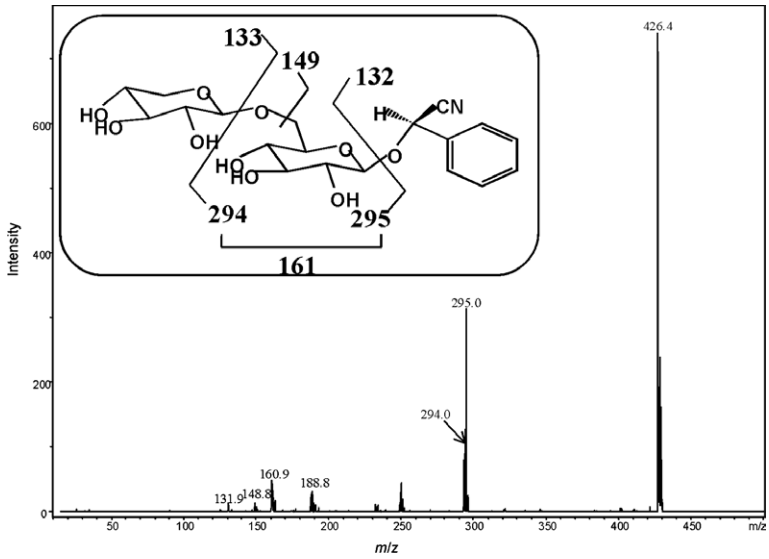

Fig. 3. ESI mass spectra of compound **1** isolated from *Clerodendrum grayi* foliage. In negative mode, the dominant ion *m*/*z* 426.4 $[M-H]^-$ was consistent with a molecular weight of 427 amu and the cyanogenic glycoside lucumin/epilucumin. Fragmentation (MS/MS) of the parent ion yielded ions at *m*/*z* 295.0, 294.0, 249.9, 188.8, 160.9, 148.8 and 131.9, 133.0, consistent with predicted fragmentation pattern.

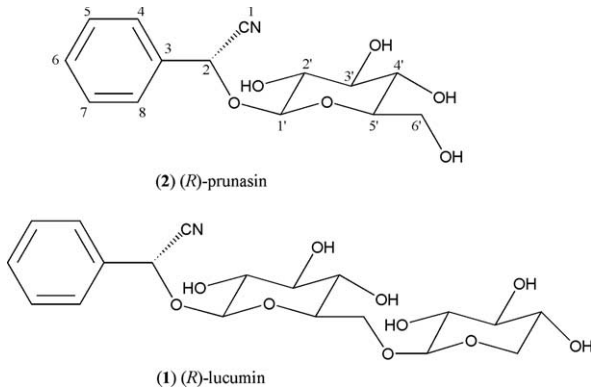

Fig. 4. Chemical structures of the cyanogenic glycosides (**1**) (*R*)-lucumin ((*R*)-mandelonitrile 6-*O*-β-D-glucoside-β-D-xyloside), and (**2**) (*R*)-prunasin ((*R*)-mandelonitrile β-D-glucoside) isolated from *Clerodendrum grayi* leaf tissue. **1** and **2** were present in the ratio 1.58:1 (mol:mol).

also because cyanogenic glycosides with a disaccharide moiety are typically restricted to generative plant parts. This is the first report of lucumin in vegetative tissue. Furthermore, the diglycoside is the major cyanogenic glycoside in *C. grayi*, lucumin constituting almost 60% of total leaf cyanide. In contrast to prunasin, which is widely distributed within different plant tissues and among diverse taxonomic groups, lucumin has only been isolated from the seeds of two species in the Sapotaceae family, *Lucuma mammosa* Gaertn. (Eyjólfsson, 1971) and *Calocarpum sapota* (Jacq.) Merr. (Takeda et al., 1997), where it was the sole cyanogen, and from seeds of *Xeranthemum cylindraceum*, where it occurred with zierin and zierinxyloside (Hübel et al., 1982). However, lucumin may also be present in leaves of *Pouteria* spp. Preliminary investigations of leaf extracts of *Pouteria subrotata* Cronquist (one of five cyanogenic *Pouteria* spp.), also in the Sapotaceae, indicated a constituent with chromatographic properties similar to lucumin/epilucumin (Thomsen and Brimer, 1997), although this is yet to be confirmed. Epilucumin has only been reported from seeds of *X. cylindraceum* (Asteraceae; Schwind et al., 1990).

Lucumin (and epilucumin) are part of a small group of cyanogenic diglycosides found in plants. This group includes amygdalin, vicianin, linustatin and neolinustatin (Smith et al., 1980), proacaciberin (Nartey et al., 1981) and zierinxyloside (Hübel et al., 1982), the sugar moieties of which are gentiobiose, vicianose or primeverose. More recently, several more complex diglycosides containing organic acid residues have been isolated [e.g., amygdalin and 2-acyl derivatives in seeds of *Merremia dissecta* (Jacq.) Hallier (Convolvulaceae; Nahrstedt et al., 1990); *Anthemis* glycosides A and B in seeds of *Anthemis cairica* Vis. (Asteraceae; Nahrstedt et al., 1983)]. With rare exception (e.g., vicianin in aerial parts of the fern *Davallia*; Kofod and Eyjólfsson, 1969), cyanogenic diglycosides are restricted to generative parts of the plants, the fruits and seeds. Amygdalin is characteristic of the generative parts of the Prunoideae and Maloideae (Rosaceae family), and is also found in the underground parts of *Gerbera jamesonii hybrida* (Asteraceae; Nagumo et al., 1985) along with prunasin, and vicianin. Vicianin is also found in seeds of *Vicia sativa* L. (Fabaceae; Ressler and Tatake, 2001), proacaciberin in pods of *Acacia sieberiana* DC var. *woodii* (Burtt Davy) Keay and Brenan (Nartey et al., 1981) and linustatin/neolinustatin in linseed (Smith et al., 1980).

While co-occurring monosaccharide and disaccharide glycosides are common in seed tissue [e.g., prunasin and amygdalin in seeds of rosaceous species (Nahrstedt, 1972, 1973; Frehner et al., 1990; Ohtsubo and Ikeda, 1994; Dicenta et al., 2002; Zhou et al., 2002; Kumarasamy

et al., 2003)], they are rare in leaf material. In the rare reports of co-occurrence, when quantified, the diglycoside typically only constitutes a small proportion of total cyanogen. The di-glucoside amygdalin has once been reported in the leaves of *Prunus* spp. (along with prunasin), but it only accounted for 1% of total cyanide (Santamour, 1998). Low concentrations of dhurrin-6′-glucoside, in addition to major constituent dhurrin, were detected in leaves of *Sorghum bicolor* (Selmar et al., 1996). In the only previous report from Lamiaceae, prunasin and a novel isomer of amygdalin were isolated from the leaves of the medicinal herb *Perilla frutescens* var. *acuta* (Aritomi et al., 1985), and prunasin and oxyanthin (prunasin-6′-apioside) were found in seeds and leaves of *Oxyanthus pyriformis* ssp. *pyriformis* (Hochst.) (Rubiaceae; Rockenbach and Nahrstedt, 1990). The relative amounts of the glycosides were, however, not reported in these studies.

The disaccharide residue primeverose occurs frequently in other plant glycosides (Bailey, 1965) [e.g., β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides) are the most abundant aroma compounds in tea leaves; Mizutani et al., 2002]. By contrast, xylose (primeverose) is rare in cyanogenic glycosides. In addition to lucumin and its epimer (*S*)-epilucumin, the primeverose moiety is restricted to a rare group of cyanogenic glycosides, the complex *Anthemis* glycosides A and B (*A. cairica*; Nahrstedt et al., 1983), and the primeverosides of benzaldehyde cyanohydrin and *m*-hydroxybenzaldehyde cyanohydrin (zierin and zierinxyloside) in the fruits of *X. cylindraceum* (Asteraceae; Schwind et al., 1990).

Interestingly, lucumin has been reported to be resistant to hydrolysis by most β-glycosidases (see Brimer et al., 1995). In this study, lucumin was more effectively hydrolysed by enzyme partially purified from *C. grayi* leaf tissue, than by either emulsin (β-glucosidase from almonds) or pectinase from *Rhizopus* sp. In fact, addition of these enzymes at levels of over 1 U mL^{-1} resulted in the hydrolysis of only one quarter of the total cyanogenic glycoside content over a 24 h period. This finding is consistent with a previous report for a compound predicted, but not confirmed, to be lucumin/epilucumin in *Pouteria* spp. (Thomsen and Brimer, 1997). The catabolism of lucumin merits further investigation.

Given the relative rarity of cyanogenic glycoside reports from the Lamiaceae, and even within the order Lamiales, it is difficult to draw any conclusions about the biogenetic origins of glycosides within these taxonomic groups. However, at the subclass level (Asteridae sensu Cronquist, 1981), the isolation of prunasin and lucumin from *C. grayi*, in addition to prunasin and an isomer of amygdalin from *Perilla frutescens* var. *acuta* (Lamiaceae; Aritomi et al., 1985), underlines the tendency of cyanogenic members of this subclass to have cyanogens biosynthetically derived from the amino acid phenylalanine. Within Asteridae, phenylalanine-derived glycosides have been isolated in the following orders: Scrophulariales [family Scrophulariaceae; Hegnauer, 1973 and Myoporaceae; Finnemore and Cox, 1929], Rubiales [Rubiaceae; Rockenbach and Nahrstedt, 1990; Rockenbach et al., 1992], Dipsacales [Caprifoliaceae; Jensen and Nielsen, 1973; see also Buhrmester et al., 2000], and in the Solanales [Convolvulaceae; Nahrstedt et al., 1989, 1990]. Within the order Asterales, cyanogenic glycosides are also typically derived from phenylalanine, with prunasin, sambunigrin, and holocalin and its epimer zierin present in most subfamilies (Fikenscher et al., 1980). Furthermore, prunasin is common in *Gerbera* spp. (Fikenscher et al., 1980), and amygdalin, vicianin and prunasin were found in the underground parts of *Gerbera jamesonii hybrida* (Nagumo et al., 1985). The only apparent exceptions within Asteridae are firstly, within the subtribe Calendulae (Asterales), where linamarin and lotaustralin have been isolated (Butler, 1965), and secondly, within the suborder Boraginales (Lamiales) where the tyrosine-derived dhurrin was isolated from *Borago officinalis* L. (Boragnaceae) (Van Valen, 1979).

4. Experimental

4.1. Plant material

Leaf samples were obtained from five trees growing in upland rainforest on the Atherton Tableland, Queensland, Australia. Leaves were snap frozen in liquid nitrogen, freeze-dried and ground using an analytical mill (IKA® Labortechnik, Janke & Kunkel, Germany). The mean concentration of cyanogenic glycosides in the ground tissue homogenate was 4.6 mg CN g^{-1} dry wt. Voucher specimens have been lodged at the University of Melbourne (specimens MELU 102115, 102116, 102117) and Queensland herbaria (specimen BRI AQ. 578815).

4.2. 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 (15–20 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. 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. 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). The cyanide detected by this method is directly proportional to

the concentration of cyanogenic glycoside, with, for example, 1 mg CN equivalent to 11.35 mg prunasin.

4.3. Cyanogenic β-glucosidase purification

Cyanogenic β-glucosidase was partially purified from the same 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® 3.5K, 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 amygdalis* Batsch; E.C. 3.2.1.21, Sigma G-0395 added at 1.04 U 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 U mL^{-1}) were also tested for activity but failed to fully hydrolyse the cyanogenic glycosides. The extracted *C. grayi* enzyme was therefore used for all subsequent assays.

4.4. Purification of cyanogenic compounds

4.4.1. Extraction and fractionation by HPLC

Tissue (8 g) was extracted using the similar procedure as Miller et al. (2004). Homogenised, freeze-dried tissue was de-fatted by three extractions with petroleum ether (solvent:tissue, 10:1 v/w), filtered (Whatman® 541 filter paper, Whatman Asia Pacific, San Centre, Singapore), and then twice extracted with cold MeOH, and filtered. 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 phase was 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%, 40%, 60%, 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.

Cyanogenic glycosides eluted in H_2O, 10% and 20% MeOH. The greatest proportion of cyanide (82%) was detected in the 10% MeOH fraction, only 3% in H_2O, and 12% in 20% MeOH. The highest yielding fraction, the 10% MeOH eluate, was concentrated in vacuo and fractionated by RP-HPLC using 18% MeCN–H_2O (2 mL min^{-1}) run isocratically for 25 min, and then increasing to 95% MeCN–H_2O over 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.

The majority of cyanogenic activity in the 10% MeOH eluate (approximately 95%) was associated with two peaks, compound **1** (RT 16.8 min, λ_{max} 210 nm in 18% MeCN) and compound **2** (RT 22 min, λ_{max} 208 nm in 18% MeCN), which accounted for 58% and 37% of the total CN content, respectively. Compound **2** co-eluted with prunasin under equivalent HPLC conditions. Compounds**1** and **2** were present in the ratio 1.56:1 based on the CN yield per peak. An unresolved third peak (approximately RT 12–14 min), accounting for approximately 3% total CN in the 10% MeOH eluate (Fig. 1) and for 8% total CN in the crude MeOH extract (data not shown), was not purified for characterisation in this study.

4.4.2. Non-polar fraction analysis and extraction

No assumption was made about the solubility and polarity of the cyanogenic constituents. Therefore, $CHCl_3$ and petroleum ether fractions were assayed for cyanide using alkaline saponification (Selmar et al., 1990). Due to significant CN detected in both the petroleum ether and $CHCl_3$ fractions, *C. grayi* leaf tissue (3 g) was extracted for lipids according to Christie (1993). The $CHCl_3$:MeOH (2:1, v/v, 60 mL) extract was partitioned with 0.9% NaCl (w/v, 15 mL), and the lower phase fractionated on a silica column by elution with $CHCl_3$, acetone:MeOH (9:1, v/v), and MeOH (Leray, 2001). These solvents fractionate the sample into the broad classes of neutral lipids, glycolipids and phospholipids, respectively (Leray, 2001). Eluates were concentrated in vacuo and aliquots assayed for cyanogenic compounds, with the addition of enzyme partially purified from the same *C. grayi* leaf tissue. The majority of cyanogenic activity (90%) was in the acetone:MeOH (9:1. v/v) eluate which was fractionated using HP-TLC.

4.4.3. Thin layer chromatography (HP-TLC)

The acetone:MeOH (9:1, v/v) eluate from the silica column was concentrated under N_2, resuspended in $CHCl_3$:MeOH (2:1, v/v), and fractionated on aluminium-backed Silica gel 60 HP-TLC plates (Merck, Damstadt, Germany) developed in solvent A ($CHCl_3$:MeOH:H_2O, 65:35:4, v/v). Cyanogenic glycosides were visualised with orcinol spray reagent (20 mg mL^{-1} orcinol monohydrate in EtOH: conc. H_2SO_4:H_2O; 75:10:5, v/v) and development for 5 min at 110 °C. Orcinol detects carbohydrate. Bands

were purified by preparative HP-TLC, and silica scrapings twice extracted with 200 μL $CHCl_3$:MeOH (2:1; v/v). Aliquots of each fraction were assayed for CN, and the purity of fractions was assessed by analytical HP-TLC. Two cyanogenic fractions were detected, **T1** ($R_f = 0.22$) and **T2** ($R_f = 0.4$). Band **T1** required no further separation, while band **T2** was further purified by HP-TLC developed in solvent B (methyl acetate:$CHCl_3$:PrOH:MeOH:0.2% aq. KCl, 25:25:25:10:9, v/v).

Purified **T1** and **T2** were partitioned between water and *n*-BuOH (1:2 v/v) and both upper and lower phases assayed for cyanide, and analysed by HP-TLC using solvent system A. Material in the upper BuOH phase was used for both ESI-MS and GC–MS analyses.

4.5. GC–MS analyses

4.5.1. Monosaccharide composition analysis

The monosaccharide composition of HP-TLC purified cyanogenic compounds (**T1** and **T2**) 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 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}.

4.5.2. Methylation linkage analysis

To determine the linkage composition of the sugar moieties, HP-TLC purified cyanogens and controls (an oligosaccharide of known composition, and a silica blank) 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 hexaacetate internal standard, and the mass spectra were compared with authentic pentose and hexose sugar standards.

4.6. ESI-MS

HPLC and HP-TLC purified samples were injected by direct infusion in 20% MeCN at a flow rate of 180 μL h^{-1} into an esquire 3000 (Bruker Daltronics, Bremen, Germany) and analysed using Bruker Daltronics Data Analysis 3.0 in both positive and negative modes. Parent ions were further fragmented (MS/MS). An authentic standard of prunasin (Sigma M-0636) was also analysed.

4.7. Nuclear magnetic resonance

^{1}H NMR was recorded in pyridine-d_5 on a Bruker DRX 600 MHz spectrometer at 25 °C. Chemical shifts are given in ppm (δ) and were referenced to the middle pyridine solvent signal at 7.19 ppm. Data were compared to that in the literature for lucumin (Takeda et al., 1997) and epilucumin (Nahrstedt et al., 1983).

^{1}H NMR spectral data: δ 3.73–3.78 (1H, *m*, Xyl H-5b), 4.05–4.11 (5H, *m*, Glc H-2, 3,4,5 and Xyl H-3), 4.24–4.27 (2H, *m*, Xyl H-2,4), 4.30–4.36 (2H, *m*, Xyl H-5a, Glc H-6b), 4.84 (1H, *dd*, $J = 2.0$, 10.3, Glc H-6a), 4.90 (1H, *d*, $J = 7.3$, Glc H-1), 5.17 (1H, *d*, $J = 7.5$, Xyl H-1), 6.55 (1H, *s*, H-7), 7.25–7.31 (3H, *m*, H-2,4,6), 7.83–7.85 (2H, *m*, H-3,5).

4.8. GC–MS analysis of TMS-derivatives

In order to determine the orientation of the chiral carbon in compound **2**/**T2** (MW 295 amu) the TMS-ether was analysed by GC–MS. The mass spectra of TMS-ethers resolve epimers (*R*)-prunasin and (*S*)-sambunigrin (Nahrstedt, 1981; Seigler, 1991). Compound **2**/**T2** was derivatised using Tri-Sil® Reagent (HMDA:TMCS:Pyridine, 2:1:10) (Pierce, Rockford, IL, USA) and analysed by GC–MS. Aliquots (1 μL) were injected at 315 °C onto a column (HP5MS Hewlett–Packard; 30 m × 0.25 mm × 0.25 μm, Agilent Technologies, Palo Alto, CA, USA) and separated using a temperature program increasing from 200 to 315 °C, with a gradient of 5 °C min^{-1} and a flow rate of 1.3 mL min^{-1} (see Buhrmester et al., 2000; Gleadow et al., 2003). The spectrum was analysed and compared with authentic prunasin (Sigma M-0636). Under these conditions, the retention times of the TMS-ethers of prunasin and sambunigrin were 9.91 and 9.63 min, respectively.

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