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OCCURRENCE OF THE CYANOGENIC GLUCOSIDE PRUNASIN AND ITS CORRESPONDING MANDELIC ACID AMIDE GLUCOSIDE IN *OLINIA* SPECIES (OLINIACEAE)*

ADOLF NAHRSTEDT and JÜRGEN ROCKENBACH

Institute of Pharmaceutical Biology and Phytochemistry, Westfälische Wilhelms-Universität, Hittorfstr. 56, D-48149 Münster, Germany

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Key Word Index—*Olinia emarginata*; *O. ventosa*; *O. radiata*; *O. rochetiana*; Oliniaceae; cyanogenesis; prunasin; mandelic acid amide glucoside; metabolism; nitrile hydratase.

Abstract—The cyanogenic glucoside prunasin (2-β-D-glucopyranosyloxy-2-phenylacetonitrile) was isolated from leaves and twigs of *Olinia emarginata*. It was also identified and quantified in leaves and stems of *O. ventosa*, *O. radiata* and *O. rochetiana*. The corresponding mandelic acid amide glucoside (2-β-D-glucopyranosyloxy-2-phenylacetic acid amide) was isolated from the leaves of *O. ventosa* and identified mainly by means of NMR spectral data and its degradation products; it also occurs in the leaves of *O. emarginata*. This is the first example of the co-occurrence of a cyanogenic glucoside and its corresponding amide indicating a possible hydration of the nitrile group of prunasin to a carboxy amide without affecting the glycosidic linkage; such reactions are catalysed by nitrile hydratases.

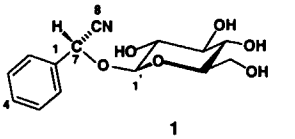
INTRODUCTION

The Oliniaceae of the Myrtales contain the single genus *Olinia* [1]. Of the eight species which are shrubs or small trees, four are found in South Africa: *Olinia ventosa* (L.) Cuf. (=*O. cymosa* Thunb.), *O. emarginata* Davy, *O. radiata* A. Juss and *O. rochetiana* A. Juss (=*O. usambarensis* Gilg and Engler). The leaves of *O. emarginata* and *O. ventosa* give off a benzaldehyde odour when crushed [2]. Recently, Fikenscher and Hegnauer [3] identified the cyanogenic glucoside prunasin (2-β-D-glucopyranosyloxy-2-phenylacetonitrile) from a blooming twig (herbarium specimen) of *O. cymosa* Thunb. (=*O. ventosa*) by paper chromatography and enzymatically catalysed degradation. During our study of cyanogenesis of South African plants, we were able to obtain air dried leaves and twigs of three of the above mentioned species, and a herbarium specimen in the case of *O. radiata*. We here describe the isolation and structure elucidation of the cyanogenic glucoside prunasin and its corresponding amide.

RESULTS AND DISCUSSION

The pulverized material was defatted with petrol followed by exhaustive extraction with acetone. The acetone extracts of all four plants showed a single cyanogenic zone on TLC plates in the range of prunasin when detected with the sandwich method [4] using an unspecific enzyme preparation for hydrolysis. GC of aliquots of the trimethylsilylated extracts also indicated prunasin for all four samples. Chromatography of the extract from *O. emarginata* on silica gel afforded a pure fraction which was identical with prunasin (2-β-D-glucopyranosyloxy-2-phenylacetonitrile, **1**) in all aspects (TLC, ^{1}H, ^{13}C NMR, isomerization in alkaline solution) [5, 6]. Methanolic extracts of the four plant samples were taken for quantitative determination of prunasin, via its enzymatically liberated hydrogen cyanide, using a Conway chamber [7] for HCN liberation and the ABS method [8] for determination of HCN (Table 1).

The results confirm the previously observed occurrence of prunasin in *O. ventosa* (=*O. cymosa*) [3] and show that it is also common in the vegetative parts of other South African *Olinia* species as the only cyanogenic glycoside. As is shown in Table 1, the glucoside is present in the leaves and twigs with higher amounts in the leaves; *O. rochetiana*, however, is an exception in that the twigs accumulate considerably more prunasin than the leaves. According to Cronquist [9] the Oliniaceae belong to the order Myrtales of which members of the genus *Eucalyptus* (Myrtaceae), also known to be cyanogenic, were shown to contain prunasin [10].



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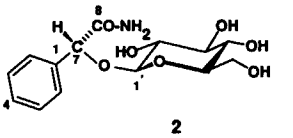

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*Part of the Ph.D. Thesis of J. Rockenbach, University of Münster, 1991.

Table 1. Prunasin content in *Olinia* leaves

Plant species	μmol g^{-1} HCN* leaves	twigs
O. emarginata	3.4	1.1
O. ventosa	2.4	0.9
O. rochetiana	0.05	1.6
O. radiata	0.1	n.d.

*Corresponding to μmol g^{-1} prunasin in air-dried tissues.

The TLC of the extracts obtained from *O. ventosa* and *O. emarginata* showed a greenish zone after detection with anisaldehyde–H_2SO_4 slightly above amygdalin (2*R*-gentiobiosyl-2-phenylacetonitrile) and below prunasin, both of which were used as reference substances. The corresponding compound was purified from the extract of *O. ventosa* by DCCC and centrifugal thin-layer chromatography (CTLC) [11] on silica gel to give **2** in high yield as a single compound as indicated by TLC and GLC. The DCI-MS showed signals at *m/z* 314 $[M+H]^+$ and 331 $[M+NH_4]^+$ corresponding to a relative molecular mass of 313 and indicating a nitrogen containing compound. The IR spectrum of **2** showed a strong amide absorption at 1660 cm^{-1}. The ^1H NMR spectrum of **2** in methanol-d_4 (Table 2) was similar to that of prunasin; it showed signals between 7.1 and 7.4 ppm corresponding to a monosubstituted aromatic ring system, a singlet at 5.16 ppm indicating an isolated methine proton, the doublet of an anomeric proton with a coupling constant $J_{1'-2'}$ of 7.3 Hz and the residual sugar protons between 3 and 3.9 ppm integrating to 6 protons. The proton decoupled ^{13}CNMR spectrum of **2** (Table 2) again was very similar to that of prunasin but contained a carbonyl resonance at 176.0 ppm.

Enzymatic hydrolysis of **2** yielded glucose (TLC, GLC) and an aglycone after extraction with ethyl acetate. The latter exhibited the same ^1H NMR spectrum as mandelic acid amide that was synthesized from L-(−)-mandelic acid with retention of configuration at C-2 (see Experimental). The optical rotation of the isolate obtained after hydrolysis of **2** was negative as was the optical rotation of the synthesized mandelic acid amide indicating the *R*-configuration according to the literature [27]. Thus, **2** is *R*-mandelic acid amide glucoside (2-β-D-glucopyranosyloxy-2-phenylacetic acid amide).

To our knowledge this is the first report of the co-occurrence of a cyanogenic glycoside and its corresponding amide in the same plant. Compound **2** can be considered as the product of hydration of the nitrile group of **1**. Such reactions are catalysed by nitrile hydratases and have been observed for aliphatic nitriles in micro-organisms, fungi and bacteria [12–17]; the resulting amide can be further hydrolysed by amidases to give the corresponding free acid and ammonia [12, 14–16]. Aromatic and heterocyclic nitriles, however, are often directly converted by micro-organisms [14, 18], also under anaerobic conditions [19], to the corresponding acids and ammonia by nitrilases with little or no formation of the free amide. Only a few examples are known of similar metabolic steps in higher plants [12]. A β-cyanolalanine hydratase from blue lupins [20] and from *Asparagus officinalis* [21] catalyses the hydrolysis of β-cyanoalanine to asparagine. For cyanogenic glycosides corresponding metabolic steps have never been observed in intact higher plants. Non-glycosylated amides have been described as degradation products of cyanogenic glycosides in processed plant material. *R*-Mandelic acid amide was detected in aqueous extracts of apricot kernels [22] as a product of the degradation of amygdalin. Mandelic acid was found in extracts of the leaves, twigs and seeds of *Prunus* species; it is thought to be a degradation product of prunasin [10]. So-called amygdalin amide (mandelic acid amide gentiobioside) was found as a

Table 2. NMR data for prunasin and the corresponding acid amide

	^1H NMR		^{13}C NMR	
	1	**2**	**1**	**2**
1	—	—	134.9	137.2
2,6	7.55–7.61 (*m*)	7.29–7.34 (*m*)	130.2	129.6
3,5	7.44–7.50 (*m*)	7.14–7.27 (*m*)	129.0	129.3
4			131.0	129.9
7	5.91 (*s*)	5.16 (*s*)	68.5	79.4
8	—	—	119.7	176.0
1′	4.26 (*d*, 7.7)	3.89 (*d*, 7.3)	102.1	100.2
2′			74.8	75.0
3′	3.34	2.88	77.9[a]	77.5[a]
4′	−3.20	−3.21	71.5	71.5
5′			78.4[a]	78.1[a]
6′	A 3.93 (*dd*, 2.1, 12.1)	A 3.65 (*dd*, 2.3, 12.2)	62.9	62.6
	B 3.70 (*dd*, 5.5, 11.9)	B 3.48 (*dd*, 5.7, 12.0)		

In MeOH-d_4; δ-scale in ppm; for numbering see formulae.
[a]Signals interchangeable in vertical columns.

by-product after heat sterilization of aqueous amygdalin solutions formerly used as anticancer drugs [23]. Recently two non-glucosylated amides corresponding to cyclopentenoid cyanogenic glucosides have been isolated from the leaves and stems of *Passiflora suberosa* L.; they are considered to be artifacts produced from the accompanying cyanogenic glucosides suberin A and epivolkenin [24].

The high amount of **2** found in *O. ventosa*, the restriction of the chirality at C_2 of **2** when compared to **1** and the occurrence of **2** in the crude MeOH extract indicate that **2** is not an artificial product of the isolation procedure. Thus, a nitrile hydratase activity capable of converting **1** into **2** should be present in the leaves of *O. ventosa*; this activity is under investigation. In conclusion, our results indicate a new route of metabolism of cyanogenic glycosides in the intact higher plant.

EXPERIMENTAL

General. NMR spectra were recorded at 199.9 MHz (^{1}H) and 50.29 MHz (^{13}C). Chemical shifts are given in ppm relative to TMS, coupling constants in Hz. IR spectra were recorded with KBr pellets (2 mg:200 mg). The elemental analysis was performed with 10 mg of **2**. Reference compounds are from the collection of A.N.

Plant material. Leaves and twigs of *O. emarginata* and *O. ventosa* were collected in 1990 in the area of Kirstenbosch (South Africa); the leaves of *O. radiata* (5 g) were from the Herbarium in Durban, South Africa; the material of *O. rochetiana* (*ca* 15 g) was obtained from the collection of J. E. Burrows through E. van Jaarsveld (Natl. Botanic Garden, Kirstenbosch). Vouchers are deposited in the Institute in Münster under PBMS 67 (*O. ventosa*), PBMS 68 a-c (*O. emarginata*) and PBMS 69 (*O. rochetiana*); a voucher of *O. radiata* is deposited in the Herbarium in Durban.

Isolation. Air dried material (*ca* 100 g, *O. ventosa* and *O. emarginata*) was defatted with petrol in a Soxhlet extractor and subsequently exhaustively extracted with acetone. The residue of the acetone extract was suspended in H_2O, filtered and extracted once with petrol to remove the chlorophyll. For isolation of **1** the aq. phase was then dried and chromatographed on a silica gel column (60 × 5 cm) using EtOAc–H_2O–MeOH 79:10:11 as mobile phase. Compound **1** eluted between 870 and 1000 ml. For isolation of **2** the dry residue of the aq. phase was subjected to droplet countercurrent chromatography (DCCC) using a Büchi 670 (296 columns, 2.7 mm i.d.; Büchi, Flawil, Switzerland); the lower phase of $CHCl_3$–MeOH–H_2O–PrOH 5:6:4:1 was used as the stationary phase, the upper as the mobile phase in the ascending mode (0.2 ml min^{-1}). The frs between 265 and 315 ml containing **2** were concd and purified by centrifugally accelerated thin layer chromatography (CTLC) with the Chromatotron (Harrison, Palo Alto, Ca) on silica gel (2 mm). Eluents were *n*-hexane–EtOAc 20:80 followed by EtOAc–CH_2Cl_2–Me_2CO 4:2:3; the eluates were fractionated and checked for **2** by TLC. A final purification was achieved by repeating the above described DCCC with the positive frs obtained from the CTLC with a final yield of 300 mg; $[\alpha]_D^{20}$ −135° (MeOH; *c* 0.36).

Hydrolysis. β-Glucosidase from bitter almonds (Serva, Heidelberg, F.R.G.) and an unspecific enzyme preparation with β-glucosidase, β-glucuronidase and esterase activity (Röhm EL 1-77) were used in citrate–phosphate buffer at pH 6.

Measurement of hydrogen cyanide. Plant material, extracts and frs were tested for cyanogenesis by means of the Feigl/Anger test [25, 26] using the unspecific enzyme preparation for hydrolysis. Extracts and some frs were monitored for cyanogenic compounds by TLC using the sandwich-picrate method [4]. For quantitative determination of HCN, Conway chambers [7] and the Röhm enzyme were used; HCN was determined photometrically using the ABS method [8].

TLC system. Precoated TLC plates (Merck Darmstadt F.R.G., 5735); EtOAc–Me_2CO–CH_2Cl_2–H_2O–MeOH 40:30:12:8:10; detection anisaldehyde–H_2SO_4 and the sandwich method according to ref. [4]. R_f **1**: 0.69, **2**: 0.44, amygdalin: 0.37.

GC system. DB5 capillary column 30 m × 0.25 mm; N_2, 1.25 ml min^{-1}; 200–320°, 5° min^{-1}, then isothermally; injector: 250°, FID 320°; R_t TMS–sambunigrin: 11.07 min, TMS–**1**: 11.44, TMS–**2**: 14.74. Compounds (*ca* 1 mg) were dissolved in 2–3 drops pyridine, silylated with 0.1 ml BSTFA and 3 drops TMCS and used for GC after 4 hr at ambient temp. For isomerization *ca* 1 mg of **1** was dissolved in 0.5 ml 0.01 M NH_3 and freeze dried; the resulting mixt. of **1** and 2*S*-sambunigrin (*ca* 60:40) was silylated and chromatographed in the GC system.

HPLC system. RP 18 Hypersil ODS, 5 μm, 250 × 4 mm; MeOH–H_2O–MeCN 14:80:6, 1 ml min^{-1}; UV detection at 264 and 278 nm (Waters PDA detector 990); R_t mandelic acid: 4.3 min, **2**: 4.4, mandelic acid amide: 5.7, **1**: 11.5.

Synthesis of R*-mandelic acid amide.* *R*-Mandelic acid (350 mg) was heated in a surplus of thionylchloride for 15 min; the cooled mixt. was poured into ice-cold conc. NH_3 and the ppt. collected by filtration, dried and purified by TLC using the above described system. The ^{1}H NMR in $CDCl_3$ of mandelic acid amide thus produced showed a doublet at δ3.53 (OH-proton), a doublet at 5.09 (methine proton), two broad singlets at 5.6 and 6.0 (amide protons) and the aromatic proton resonances at 7.35–7.5 ppm. $[\alpha]_D^{20}$ (EtOH; *c* 1.75) was −42.9°; $[\alpha]_D^{20}$ of the isolate obtained after hydrolysis of **2** was −82.4°, (EtOH; *c* 3.2) lit. −66.6° (acetone; *c* 2.5) [27].

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