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Generation of primary amide glucosides from cyanogenic glucosides

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ABSTRACT

The cyanogenic glucoside-related compound prunasinamide, (2R)-β-D-glucopyranosyloxyacetamide, has been detected in dried, but not in fresh leaves of the prunasin-containing species *Olinia ventosa*, *Prunus laurocerasus*, *Pteridium aquilinium* and *Holocalyx balansae*. Experiments with leaves of *O. ventosa* indicated a connection between amide generation and an excessive production of reactive oxygen species. *In vitro*, the Radziszewski reaction with H_2O_2 has been performed to yield high amounts of prunasinamide from prunasin. This reaction is suggested to produce primary amides from cyanogenic glycosides in drying and decaying leaves. Two different benzoic acid esters which may be connected to prunasin metabolism were isolated and identified as the main constituents of chlorotic leaves from *O. ventosa* and *P. laurocerasus*.

1. Introduction

For some cyanogenic plants primary amide glucosides have been detected whose structures correspond to the respective cyanogenic glycoside in that the nitrile moiety has been converted into a primary carboxamide group. So far, the amides were exclusively found in air-dried leaves (Nahrstedt and Rockenbach, 1993; Jaroszewski et al., 2002; Backheet et al., 2003), whereas fresh material of the same plants never yielded detectable amounts of amides (Olafsdottir et al., 1991; Adsersen et al., 1993). Nitriles may be converted into primary amides by a nitrile hydratase as stated by Chamberlain and Mackenzie (1981). Jaroszewski et al. (2002) suggested some kind of intramolecular catalysis to explain nitrile hydratation under the mild conditions of air-drying.

While *in vitro* nitrile hydratation under strong acidic or alkaline catalysis usually yields the corresponding carboxylic acid as main product (Schäfer, 1970), the Radziszweski reaction of nitriles with H_2O_2 (Schäfer, 1970) generates primary amides in high yields under mild conditions *via* a cyclic transition state that is formed by addition of a hydroperoxide anion being the actual nucleophilic agent in this pH-dependent reaction (Fig. 1). The peroxyimidic acid intermediate is highly reactive and can be isolated only with special precaution (Schäfer, 1970). The Radziszewski reaction has been used to detoxify jojoba meal by treatment with an alkaline solution of H_2O_2 thereby hydratating the toxic nitrile glucoside simmondsin into its corresponding amide (Verbiscar et al., 1980).

The aim of this study was to clarify whether prunasinamide (**2**) is generally present in air-dried leaves of prunasin (**1**) containing plants and to elucidate the conversion of cyanogenic glycosides to their corresponding amides during drying, assuming either specific enzymatic catalysis possibly contributing to a metabolic turnover of (**1**) without release of hydrogen cyanide (Jenrich et al., 2007; Sánchez-Pérez et al., 2008) or the Radziszewski reaction under the influence of H_2O_2 that evolves from chloroplasts and peroxisomes during drying (Smirnoff, 1993).

Dried leaves of the South African evergreen tree *Olinia ventosa* (L.) Cuf. (Oliniaceae) were reported to contain both the cyanogenic glucoside (**1**) and its corresponding amide (**2**) (Fig. 2, Nahrstedt and Rockenbach, 1993); leaves of *O. ventosa* as well as other plants containing (**1**) were used for the present investigations.

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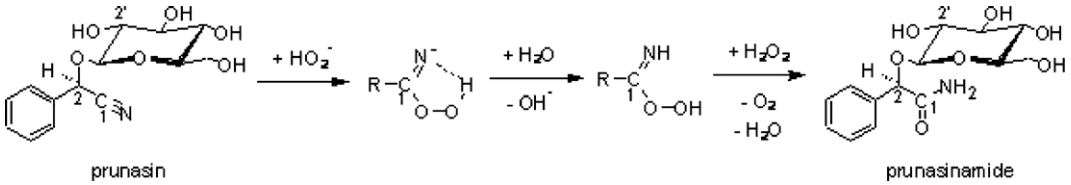

Fig. 1. Radziszewski reaction of prunasin with H_2O_2 yields the corresponding prunasinamide *via* a cyclic transition state.

2. Results and discussion

HPLC analysis of air-dried leaves of *O. ventosa* showed large amounts of both (**1**) and (**2**), whereas (**2**) was not detected in fresh leaves or when leaves were dipped into boiling water for 10 s to inactivate enzymatic activity prior to drying. This strongly indicates that amide formation depends on intact enzymatic activity during the drying process.

Thus we argued that the conversion of (**1**) into (**2**) would be catalyzed by a nitrile hydratase such as the β-cyanoalanin hydrolase that has been purified from an acetone powder of *Lupinus angustifolius* (Castric et al., 1972). However, an acetone powder produced from the leaves of *O. ventosa* did not catalyze the formation of (**2**) from (**1**).

When leaves were analyzed in different stages of dehydration, a strong dependence of the content of (**2**) on the relative-water-content (RWC) of the leaves was observed; however (**2**) was not detectable in the tissue unless its RWC was below 35%. Desiccation-intolerant tissue such as most leaves are expected to suffer irreparable mechanical damage when dehydratized beyond this point (Smirnoff, 1993); in fact, (**2**) was not detected in control leaves which were kept humid in order to avoid tissue disruption for up to 72 h after detachment. At RWCs beyond 35% the content of (**2**) increased while the amount of (**1**) decreased by the same magnitude. Up to 50% of (**1**) was converted into (**2**) when the leaves had reached constant weight, yet containing 2–6% RWC of residual humidity (Fig. 3).

In order to examine the possible influence of H_2O_2 on the generation of (**2**), we analyzed leaves of *O. ventosa* which had become fully or partially chlorotic at the tree. Chlorosis is related to natural senescence or either pseudosenescence (Ougham et al., 2008), processes both known to evolve large amounts of ROS (del Río et al., 1998; Ougham et al., 2008). By analyzing green and chlorotic parts of those leaves, we found that the content of (**1**) was significantly lower in chlorotic leaf areas (Fig. 4). Interestingly, in chlorotic leaves which were firmly attached to the tree and showed low RWCs (between 7% and 22%) in their chlorotic parts (type A leaves), almost all (**1**) was converted to (**2**). However, there were also very easily detachable chlorotic leaves (type B) at the tree with only trace amounts of (**2**) in which (**1**) was obviously converted to a compound identified as *myo*-inositol-1-benzoate (**3**) by its NMR data (Chung and Chang, 1996; Fig. 4). To our knowledge, this is the first description of (**3**) as a natural compound. Notably, leaves of the evergreen shrub *Prunus laurocerasus* gave very similar results regarding the decrease of (**1**) and the appearance of (**2**) in chlorotic leaves of type A (Fig. 4), whereas *P. laurocerasus* type B leaves differed from *O. ventosa* type B leaves in that they contained β-D-glucose-1-benzoate (**4**) (identified by NMR data according to Horsley and Meinwald (1981)).

Some of the observations made here support the assumption that type B leaves of both evergreen species have bleached by undergoing a natural leaf senescence process: (i) the leaves drop off very easily at the base of their petioles due to an abscission process known to appear within senescence (Bleecker and Patterson, 1997); (ii) the benzoic acid esters (**3**) and (**4**) apparently arising from the degradation of (**1**) (the summarized molar contents of these substances are well negatively correlated to the content of prunasin; see Fig. 4) have lost the nitrile nitrogen which was present in (**1**). Though the fate of the nitrogen in (**1**) remains to be investigated, it seems reasonable that the benzoic acid esters result from the remobilization of nitrogen from senescing plant organs (Masclaux-Daubresse et al., 2008). Moreover, benzoic acid has been described as an allelopathic agent being released from naturally senescing leaves of the cyanogenic *Prunus serotina* (Horsley, 1979).

Further examinations involved treatment of detached leaves in a senescence model used by Rontani et al. (2005) to enforce ROS generation in green leaves of *Petroselinum sativum*. Fresh green leaves of *O. ventosa* were kept under strong light irradiation (here 16 klx), while slowly drying out to a RWC of below 6% during the treatment. Leaves were thus being subjected to strong abiotic light and drought stress inducing a complete bleaching of the treated leaves and a complete disappearance of (**1**) (LOD was below 0.0004%), while concentrations of (**2**) matched that of (**1**) of fresh

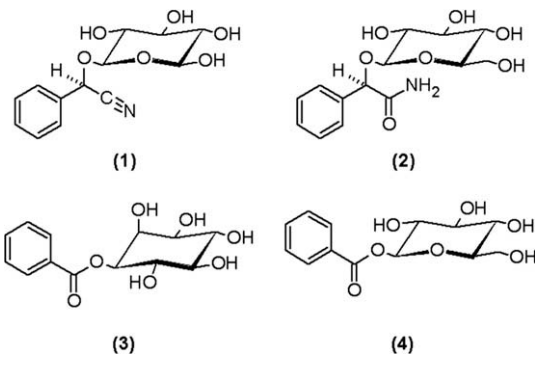

Fig. 2. Compounds isolated from *Olinia ventosa* (**1**–**3**), and *Prunus laurocerasus* (**1**, **2**, **4**), respectively.

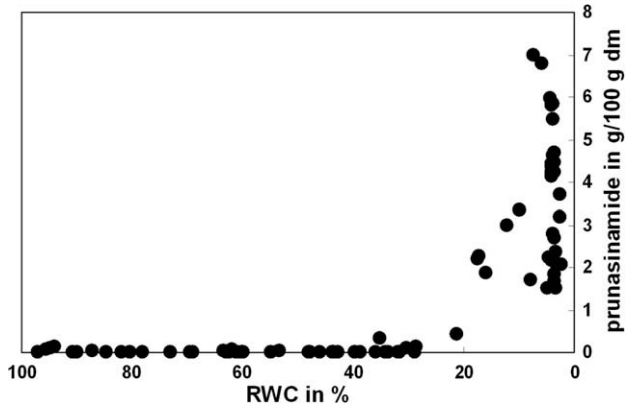

Fig. 3. Content of prunasinamide in drying *Olinia* leaves drastically increases when water content falls below 35% RWC.

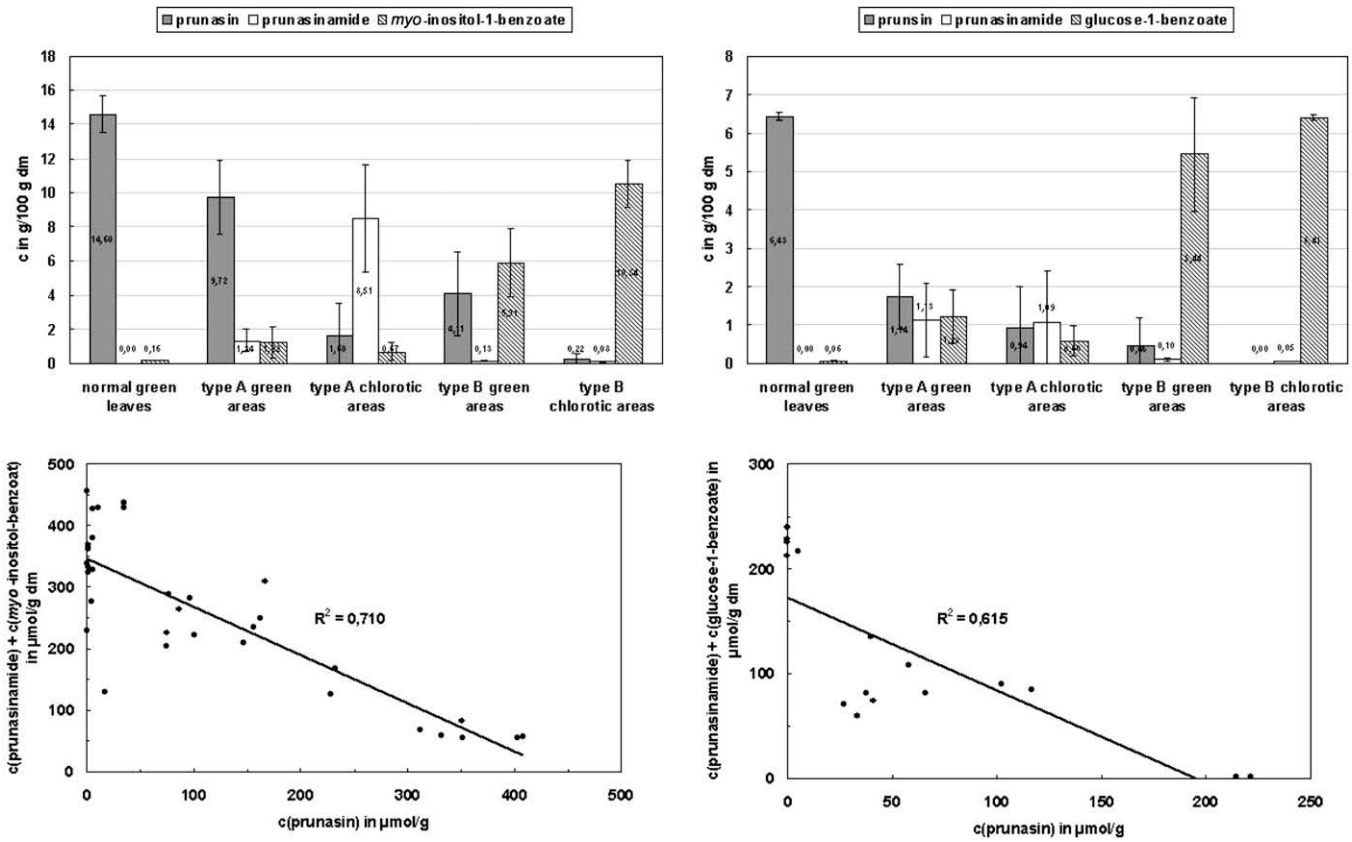

Fig. 4. HPLC analysis of chlorotic leaves of two distinct kinds, both to be found on *O. ventosa* (left panels) and *P. laurocerasus* (right panels) show remarkable losses in their contents of prunasin especially in their chlorotic parts. Prunasin seems to be converted in either prunasinamide (type A leaves) or a species-specific benzoic acid ester (type B leaves), as the summarized molar contents of these substances are well negatively correlated to the content of prunasin (lower panels).

green leaves (14.5%). Control leaves which were protected from light during parallel treatment, stayed green and showed contents of (**1**) and (**2**) comparable to that of normally air-dried leaves (8.7% for (**1**), 4.7% for (**2**); Fig. 5). Thus photochemical damage of the phototrophic leaf tissue caused the entire conversion of (**1**) into (**2**). Notably, leaves treated here correspond to the chlorotic leaves of type A described above with respect to their RWC and contents of (**1**) and (**2**). As normal green leaves were subjected to abiotic stress in this model, the process leading to bleaching should rather be classified as pseudosenescence (Ougham et al., 2008).

To further examine the influence of H_2O_2 on the generation of (**2**), leaves were treated with xenobiotics known to increase the production of ROS. The herbicide paraquat acts as a redox-cycler in the plant's chloroplasts and thus generates large amounts of superoxide-radicals, which are in turn quickly metabolized to H_2O_2 by the enzyme superoxide dismutase (Halliwell and Gutteridge, 1989). On the other hand, aminotriazole increases the amount of H_2O_2 in the tissue through the inhibition of the H_2O_2 decomposing enzyme catalase, which is located in peroxisomes (Smirnoff, 1993).

When whole, healthy leaves of *O. ventosa* were allowed to float in solutions of each xenobiotic for 24 h, the ratio of (**1**):(**2**)

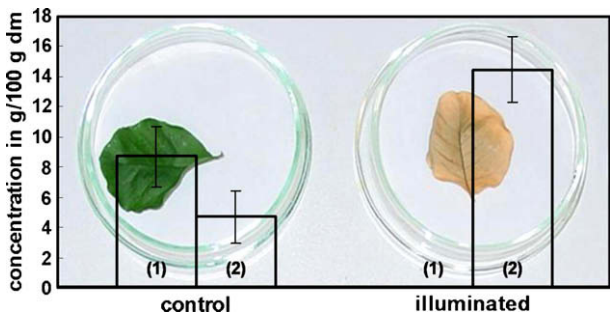

Fig. 5. In leaves of *O. ventosa* which were subjected to strong light and drought stress, prunasin was completely converted into prunasinamide.

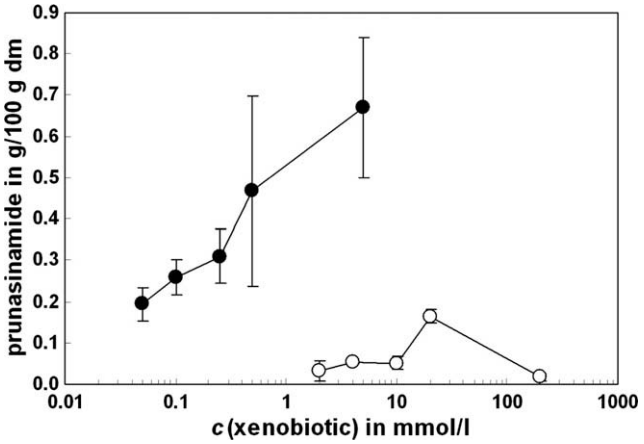

Fig. 6. A 24 h treatment of *O. ventosa* leaves with the ROS inducing xenobiotics paraquat (●) or aminotriazole (○) resulted in a dose dependent increase of prunasinamide concentration. Untreated leaves contained less than 0.05% prunasinamide (data not shown).

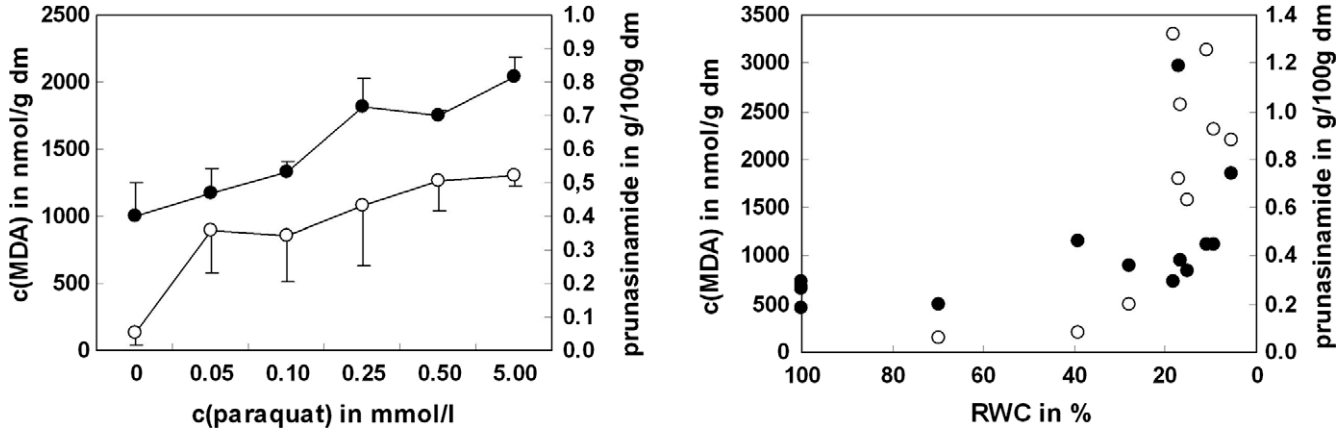

Fig. 7. Both treatment with paraquat (left panel) and drying (right panel) of *O. ventosa* leaves resulted in an increase of both prunasinamide concentrations (○) and the concentrations of malondialdehyde (MDA) (●), a marker substance for oxidative stress.

decreased while the sum of (**1**) + (**2**) remained constant. While a clear dose-dependence was observed with paraquat, the effect of aminotriazole declined after having reached a maximum at 20 mM concentration (Fig. 6). These data strongly suggest that endogeneous H_2O_2 is involved in the conversion of (**1**) to (**2**).

In order to confirm that the increased production of ROS and thereby H_2O_2 causes the increase of (**2**), malondialdehyde (MDA), a product of lipid peroxidation by active oxygen species, was quantified during drying experiments and paraquat treatments with whole green leaves of *O. ventosa*. MDA concentrations were determined using derivative spectroscopy of the conjugate formed upon reaction of MDA with thiobarbituric acid as described by Merzylak et al. (1992). As shown in Fig. 7, increasing amounts of MDA paralleled increasing amounts of (**2**) indicating the dependence of (**2**)-formation from H_2O_2.

The conversion of (**1**) to (**2**) was also shown by an *in vitro* experiment when (**1**) was treated with an excess of H_2O_2 (40 °C, pH 6.8: Radziszewski reaction) and was almost completely converted into (**2**). This experiment confirms that the generation of (**2**) in drying leaves occurs most likely *via* the Radziszewski reaction (Fig. 8). In contrast to the non-enzymatically catalyzed *in vitro* nitrile hydratation with high concentration of ammonia as reported by Turczan et al. (1978), neither the corresponding acid was observed nor epimerization took place at the methine carbon (C-2).

Because the generation of H_2O_2 is a general feature attributed to photosynthetic tissue and senescence, the production of (**2**) from (**1**) should occur in other cyanogenic plants containing (**1**) when the rate of H_2O_2 production exceeds the quenching capacity of the antioxidative protection system of the cells. In leaves of *Pteridium aquilinum* (L.) Kuhn and *Holocalyx balansae* Mich., known to contain (**1**) (Nahrstedt, 1976; Seigler, 1977), (**2**) was easily detectable after air drying, but not after prior heat-treatment.

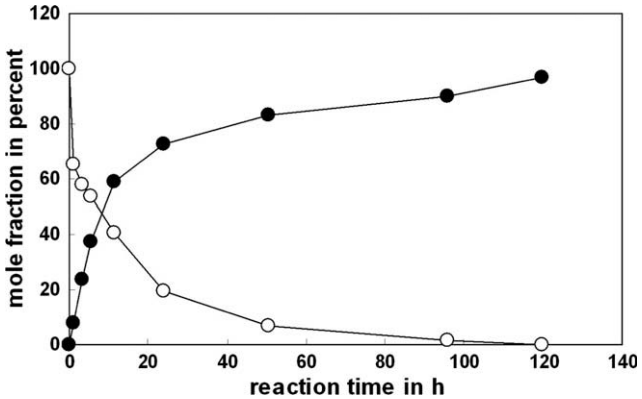

Fig. 8. *In vitro* reaction of prunasin (○) with H_2O_2 (40 °C, pH 6.8) resulted in a complete conversion into prunasinamide (●).

3. Conclusions

In plants, H_2O_2 and other ROS are continuously evolved from different sites, in particular in photosynthetic tissue, by enzymatically catalyzed reactions which can be inhibited by heat-pretreatment. H_2O_2 generated under normal physiological conditions is quickly detoxified by different defense systems (Halliwell and Gutteridge, 1989). Our data show that in *O. ventosa* (**2**) is produced when increased H_2O_2 generation occurs as during drought and light stress, further when catalase is inhibited or superoxide radical generation is increased. Finally, conversion of (**1**) to (**2**) proceeds most likely *via* the Radziszewski reaction which resembles the *in vivo* conversion in leaves.

Thus the generation of (**2**) can be attributed to enzymatic activities that finally generate H_2O_2, although the contribution of a nitrile hydratase as previously suggested by Lechtenberg and Nahrstedt (1999) cannot be ruled out completely. Production of (**2**) was demonstrated for drying leaves of *O. ventosa*, *P. laurocerasus*, *P. aquilinum* and *H. balansae*, which are phylogenetically not closely related to each other. Its conversion from (**1**) can thus be designated as a pre-mortality reaction, as it was only observed under conditions ultimately leading to the death of the tissue; future research should clarify whether in senescent leaves (**2**) is an intermediate to finally form (**3**) or (**4**) depending on the plant species. The conversion of cyanogenic glycosides to their corresponding primary amides has consequences for the analysis of cyanogenic material when being air-dried at normal temperatures which usually causes loss of cyanogenic glycosides. This is probably not only due to active β-glucosidases as hitherto assumed, but also to pre-mortality reactions in favor of the corresponding amides. *In vivo* formation of corresponding amides is also of interest for the detoxification of cyanogenic foodplants, e.g. when the leaves of cassava are used for nutrition (Padmaja, 1995) or jojoba (Verbiscar et al., 1980). However, the overall toxicity of the corresponding amide glucosides is yet not known.

4. Experimental

4.1. General experimental procedures

NMR spectra were recorded on a Varian Mercury 400 spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C). ESI-mass data were

obtained on a Micromass Quattro LC-Z. IR-data were recorded on a Nicolet 4700 ATR-FTIR-spectrometer, optical rotation data on an Autopol® Automatic Polarimeter. (**1**) used for the Radziszewski reaction has been isolated from *O. ventosa* in high purity. Other chemicals were purchased from Lancaster synthesis (aminotriazole), Sigma–Aldrich (paraquat, 4-hydroxybenzoic acid) and VWR International (H_2O_2, solvents, and other chemicals).

4.2. Plant material

Leaves and fronds of the examined plants were collected from plants grown in the greenhouse (*O. ventosa*) and in the botanical garden (*P. laurocerasus, P. aquilinum*) of the Institute of Pharmaceutical Biology and Phytochemistry (Münster) or were kindly provided from the botanical Garden Berlin-Dahlem (*H. balansae*). Vouchers are deposited in the Institute in Münster under PBMS 193 (*H. balansae*), PBMS 219 (*O. ventosa*), PBMS 220 (*P. laurocerasus*) and PBMS 221 (*P. aquilinum*). Chlorotic leaves (type A and type B) were collected from the same plants.

4.3. Proceeding and characterization of plant material

Leaves were oven-dried at 30 °C without air-circulation and heat-treated at different stages of dehydration in order to stop amide generation at that point. RWC was determined gravimetrically. With the knowledge of each leaf's dry mass, which was determined from an aliquot after 2 h of drying at 120 °C, each leaf's RWC was calculated in any particular situation by weighing the leaf and relating its current absolute water content to its initial absolute water content (right after detachment).

For treatment in the senescence model (Rontani et al., 2005), fresh leaves were stored in petri dishes together with a dry piece of filter paper. The petri dishes were then illuminated in a phytotron using a mercury-vapour-lamp at 25 °C for 7 days; illumination was damped to 16 klx (16 h/d) using a net. Control samples were handled in the same way but protected from illumination.

4.4. Treatment with xenobiotics

Whole fresh and healthy leaves were used for treatment. Test solutions were prepared by dissolving paraquat and aminotriazole each in 25 mM phosphate-buffer, pH 6.8, containing 0.05% Tween 20. Samples were allowed to float in 40 ml of the respective test solutions for 24 h under illumination (Phytotron, 16 klx (16 h/d), 25 °C).

4.5. In vitro conversion of (*1*)

A mixture of 34 mM (**1**) and 100 mM H_2O_2 in 50 mM phosphate-buffer, pH 6.8, was shaken at 40 °C in a thermomixer. Samples of 100 µl were taken over a period of 120 h and mixed with 1.00 ml of a 10% aq. soln. of $Na_2S_2O_3$ right after sampling in order to stop the reaction.

4.6. Quantification of (*1*), (*2*), (*3*) and (*4*)

Fifty milligrams of dry plant material were homogenized in 1.00 ml of MeOH together with 0.20 mg of 4-hydroxybenzoic acid (internal standard). 400 µl of the supernatant were mixed with 1.6 ml H_2O and applied to a SPE-Catrige (Merck LiChrolut®, 500 mg RP-18 40–63 µm) which was eluted with 10 ml of MeOH–H_2O (2:8). Ten microliters of the eluate was used for HPLC analysis (stationary phase: ProSep C18, 5 µm, 150 × 4 mm; mobile phase: MeOH–H_2O–TFA (100:891:9), isocratic at 1 ml/min). Quantification was achieved using the internal-standard-method at 202 nm (Waters 996 PDA-detector). The low wavelength provided an improved selectivity while increased baseline noise caused by the eluent's absorption at 202 nm did not reduce *s/n* when compared to detection at 254 nm. All concentrations were calculated in percentage related to dry mass.

4.7. Isolation

Powdered oven-dried leaves (670 g) of *O. ventosa* were defatted in a Soxhlet-extractor with CH_2Cl_2 and subsequently extracted with EtOH to give 127 g of dry matter. A 57 g aliqoute was chromatographed over 0.7 kg of Sephadex LH20 using EtOH–H_2O (75:25) as mobile phase. Fractions were tested for (**1**) and (**2**) using TLC (Nahrstedt and Rockenbach, 1993). Positive fractions were combined and freeze-dried (51 g). A 10 g aliquot was suspended in 20 ml H_2O and applied to 16 g of Extrelut®, which was successively extracted with 200 ml each of CH_2Cl_2, EtOAc, EtOAc-*n*-BuOH (3:1), and *n*-BuOH (each satd. with H_2O). The residue of the EtOAc-fraction (4.8 g) was recrystallized from $CHCl_3$/MeOH to give 2 g of (**1**). The residue of the EtOAc–*n*-BuOH-fraction (2.2 g) was further separated by MPLC (stationary phase: Europrep C-18, 18…32 µm, 36 × 500 mm; mobile phase: MeOH–H_2O-gradient) to give 0.6 g of (**2**) and 90 mg of (**3**).

From 47 g of powdered senescent leaves of *P. laurocerasus*, 0.14 g of (**4**) was isolated by the same procedure as used for the isolation of (**1**) from green leaves of *O. ventosa*.

4.8. Nitrile hydratase assay

Leaves of *O. ventosa* were grinded under liquid nitrogen and further comminuted by an ultra turrax T25 at 6000 rpm under continuous extraction with cold acetone ($T = -20$ °C) until an almost colourless powder was obtained. This dry acetone powder (2.5 g) was extracted with 20 ml of 50 mM citrate buffer, pH 5.8, and centrifuged for 30 min at 6000 rpm and 4 °C. Supernatant (2.5 ml) was desalted on a Sephadex G25 column equilibrated with the same buffer. Two hundred and fifty microliters of the enzyme solution was used in a 1000 µl-assay with either (**1**) or (**2**) (final concentration 8 mM) and variable amounts of gluconolactone (final concentration 0, 50, 100, 250 mM) to inhibit possible glucosidases. Samples were screened for (**2**) after 12 h of incubation at 30 °C using HPLC. Additionally, 100 µl of each sample were monitored for HCN release using Feigl–Anger paper (Brinker and Seigler, 1989).

4.9. Thiobarbituric acid-reactive substances assay

A sample of plant material representing 50 mg of dry matter was ground with liquid nitrogen. One thousand microliters of 2.5% thiobarbituric acid dissolved in 20% trichloroacetic acid was added. The samples were then heated to 95 °C in a thermo mixer for 20 min, cooled and centrifuged at 14000 rpm for 10 min. The second derivatives of the absorption spectra were recorded ($d\lambda = 2$ nm) using a Shimadzu UV–Visible Recording Spectrophotometer UV-260. The amplitude between the second derivative minimum at 532 nm and its satellite maximum at 552 nm was proportional to the concentration of MDA and used for quantification. 1,1,3,3-Tetraethoxypropane treated under the same conditions was used for calibration.

4.10. (2R)-2-β-D-Glucopyranosyloxymandelonitrile (prunasin) (*1*)

Colourless crystals. *mp* 146.8 °C. $[\alpha]_D^{20}$ – 66.4 (MeOH, $c = 0.10$). Spectral data were identical to those published by Nahrstedt and Rockenbach (1993) (NMR) and Aritomi et al. (1985) ([α]).

4.11. (2R)-2-β-D-Glucopyranosyloxymandelamide (prunasinamide) (**2**)

White powder. $[\alpha]_D^{20}$ – 141.0 (MeOH, $c = 0.17$). Spectral data were identical to those published by Backheet et al. (2003) (IR, NMR) and Nahrstedt and Rockenbach (1993) ([α]).

4.12. myo-Inositol-1-benzoate (**3**)

White powder. $[\alpha]_D^{20}$ – 19.0 (H_2O, $c = 0.10$). IR spectral data, ν_{max} cm^{-1}: 3428 *s* (O–H) , 2932 *w* (C–H), 1704 *s* (>C=O), 1277 *s* (C–O–C), 1071 *vs* (C–O). NMR spectral data were identical to those published by Chung and Chang (1996) and Zapata et al. (1991).

4.13. β-D-Glucose-1-benzoate (**4**)

Colourless crystals. $[\alpha]_D^{20}$ – 18.1 (H_2O, $c = 0.10$). Spectral data were identical to those published by Horsley and Meinwald (1981) (IR, [α], HNMR, CNMR (DMSO-d_6), Heimhuber et al. (1990) (HNMR (CH_3OD)) and Ushiyama et al. (1989) (CNMR (CH_3OD)).

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