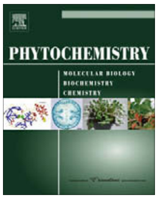
Phytochemistry 70 (2009) 388–393

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem



Generation of primary amide glucosides from cyanogenic glucosides

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ARTICLE INFO

Article history:
Received 23 May 2008
Received in revised form 2 November 2008
Available online 3 February 2009

Keywords:
Olinia ventosa
Oliniaceae
Prunus laurocerasus
Rosaceae
Pteridium aquilinium
Pteridaceae
Holocalyx balansae
Caesalpiniaceae
Cyanogenic glycosides
Primary amides
Prunasin
Prunasinamide
Senescence
Pseudosenescence
Radziszewski reaction

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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doi:10.1016/j.phytochem.2008.11.008

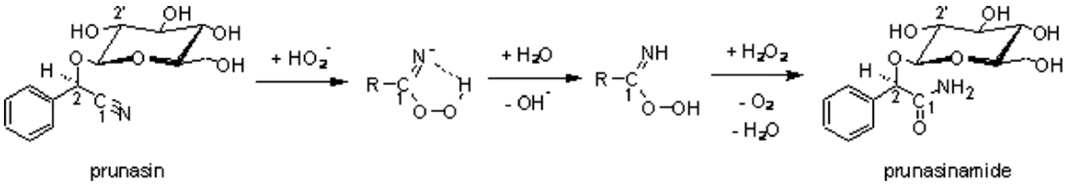

Fig. 1. Radziszewski reaction of prunasin with H_2O_2 yields the corresponding prunasinamide *via* a cyclic transition state.

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

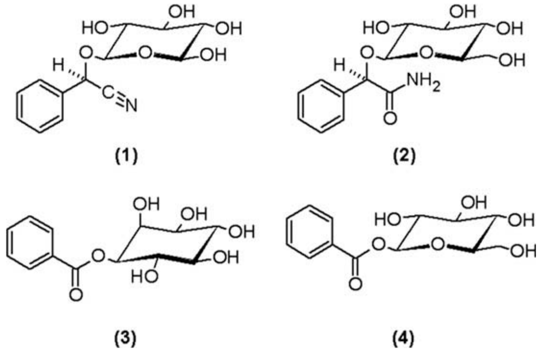
(1) (2) (3) (4)

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

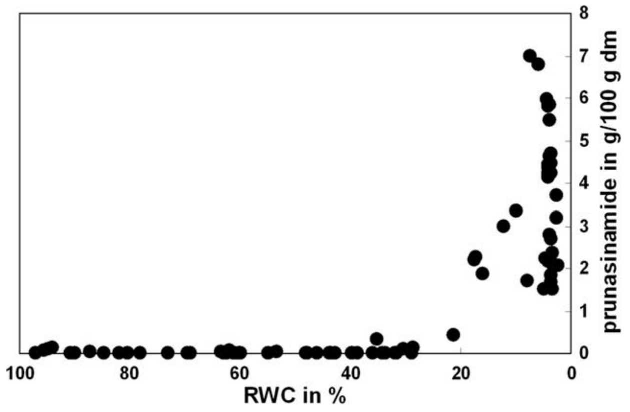

Fig. 3. Content of prunasinamide in drying *Olinia* leaves drastically increases when water content falls below 35% RWC.