Phytochemistry 153 (2018) 79–93

Contents lists available at ScienceDirect

Phytochemistry

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

Glucosinolate turnover in Brassicales species to an oxazolidin-2-one, formed via the 2-thione and without formation of thioamide

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ARTICLE INFO

Article history:
Received 6 March 2018
Received in revised form
16 April 2018
Accepted 7 May 2018

Keywords:
Barbarea vulgaris R.Br. (Brassicaceae)
Glucosinolate hydrolysis
Turnover
1,3-Oxazolidine-2-thione
1,3-Oxazolidin-2-one
Nitrile
Thioamide
Reseda luteola
Nasturtium officinale

ABSTRACT

Glucosinolates are found in plants of the order Brassicales and hydrolyzed to different breakdown products, particularly after tissue damage. In *Barbarea vulgaris* R.Br. (Brassicaceae), the dominant glucosinolate in the investigated "G-type" is glucobarbarin, (*S*)-2-hydroxy-2-phenylethylglucosinolate. Formation of the nitrile from glucobarbarin was observed *in vitro*, while a previously suggested thioamide (synonym thionamide) was not confirmed. Resedine (5-phenyl-1,3-oxazolidin-2-one) was detected after glucobarbarin hydrolysis in crushed *B. vulgaris* leaves and siliques, but not in intact parts. The abundance increased for several hours after completion of hydrolysis. The corresponding 1,3-oxazolidine-2-thione (OAT), with the common name barbarin, was also formed, and appeared to be the precursor of resedine. Addition of each of two non-endogenous OATs, (*S*)-5-ethyl-5-methylOAT and (*R*)-5-vinylOAT (*R*-goitrin), to a leaf homogenate resulted in formation of the corresponding 1,3-oxazolidin-2-ones (OAOs), confirming the metabolic connection of OAT to OAO. Formation of OAOs was inhibited by prior brief heating of the homogenate, suggesting enzyme involvement. We suggest the conversion of OATs to OAOs to be catalyzed by an enzyme ("oxazolidinethionase") responsible for turnover of OAT formed in intact plants. Resedine had been reported as an alkaloid from another species - *Reseda luteola* L. (Resedaceae) - naturally containing the glucosinolate glucobarbarin. However, resedine was not detected in intact *R. luteola* plants, but formed after tissue damage. The formation of resedine in two families suggests a broad distribution of putative OATases in the Brassicales; potentially involved in glucosinolate turnover that needs myrosinase activity as the committed step. In agreement with the proposed function of OATase, several candidate genes for myrosinases in glucosinolate turnover in intact plants were discovered in the *B. vulgaris* genome. We also suggest that biotechnological conversion of OATs to OAOs might improve the nutritional value of Brassicales protein. HPLC-MS/MS methods for detection of these glucobarbarin products are described.

1. Introduction

1,3-Oxazolidin-2-ones (OAOs) are important as structural elements in modern antibiotics, the first of which was linezolid targeting bacterial protein synthesis (Diekema and Jones, 2001). Likewise, several OAOs are important as auxiliaries in chiral synthesis (Gnas and Glorius, 2006). They are also known as Evans auxiliaries and used for controlling aldol reactions (Evans et al., 1981). In this report, we argue that this class of compounds is also formed from enzymatic turnover of glucosinolates, a group of natural *S*-glucosides (Halkier and Gershenzon, 2006; Agerbirk and Olsen, 2012).

It is well known that a related group of heterocycles, the substituted 1,3-oxazolidine-2-thiones (OATs), are found in many

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https://doi.org/10.1016/j.phytochem.2018.05.006
0031-9422/

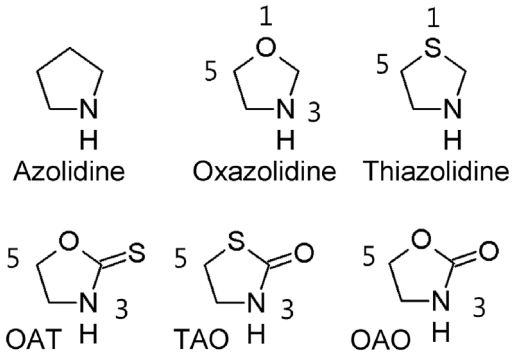

Fig. 1. Three heterocyclic ring systems found in Brassicales metabolites: 1,3-oxazolidine-2-thione (OAT), 1,3-thiazolidin-2-one (TAO), 1,3-oxazolidin-2-one (OAO), and their parent heterocyclic frames. Substituted OAT and TAO are known glucosinolate degradation products. In this paper, biochemical conversion of 5-substituted OATs into 5-substituted OAOs is suggested.

glucosinolate-containing plants. Both OAOs and OATs are named as deriving from the parent heterocycle azolidine (Fig. 1), explaining the middle 'A' in our abbreviations. Penicillin contains a third pattern, a thiazolidine ring (Fig. 1). Natural diversity of heterocyclic secondary metabolites was studied by several researchers, inspired by the remarkable progress in penicillin chemistry in the first half of the 20th century. Two reports independently showed that OATs were formed via cyclisation of β-hydroxyalkyl isothiocyanates produced from β-hydroxyalkyl glucosinolates (Greer, 1956; Kjær et al., 1956). For example, (*R*)-5-phenylOAT ((*R*)-barbarin, **2**) is formed via a never isolated β-hydroxy isothiocyanate derived from (*S*)-2-hydroxy-2-phenylethylglucosinolate (glucobarbarin, **1**) (Fig. 2) (Kjær and Gmelin, 1957; Agerbirk and Olsen, 2015). The enantiomer, (*S*)-barbarin, is formed from the epimeric glucosinolate epiglucobarbarin (Gmelin et al., 1970). Benzyl-activating substitution of glucobarbarins change the type of hydrolysis product to a 1,3-thiazolidin-2-one (TAO), an additional heterocycle in this plant group (Fig. 1) (Agerbirk and Olsen, 2015). Recently, 5-phenylTAO was also detected at low levels after fractionation of *B. vulgaris* extracts, but not in initial analysis of the extract (Pedras et al., 2015), perhaps indicating very low levels or formation as an artifact. Indeed, a thermally induced rearrangement of 5-phenylOAT to 5-phenylTAO is also well established (Lutfullin et al., 1976; Radulovic et al., 2017).

Glucosinolates are found throughout the order Brassicales (Blaževic et al., 2015), with around 144 documented structures reported by 2016 (Agerbirk and Olsen, 2012; Matich et al., 2012; Montaut et al., 2015; Agerbirk et al., 2015; Pedras et al., 2016; Olsen et al., 2016; Pfalz et al., 2016) and a further reported 3–5 structures from 2017, including (the isothiocyanate corresponding to) 5-phenylpentylglucosinolate (Dekic et al., 2017) and 4-hydroxy-3,5-dimethoxybenzylglucosinolate (Pagnotta et al., 2017). Although some structural features seem to be restricted to smaller taxonomic groups, there is also a great redundancy in structures found in widely separated taxonomic groups (Blaževic et al., 2015; Olsen et al., 2016). A widespread structural feature is β-hydroxylation of the glucosinolate side-chain catalyzed by GS-OH enzymes (Hansen et al., 2008; Liu et al., 2016; Olsen et al., 2016; Byrne et al., 2017). Indeed, the β-hydroxylated glucosinolate glucobarbarin (**1**) is found in Resedaceae (e.g. *Reseda luteola* L.) (Kjær and Gmelin, 1958; Radulovic et al., 2017) as well as in Brassicaceae (e.g. most or all *Barbarea* spp. and some accessions of watercress, *Nasturtium officinale* R.Br) (Kjær and Gmelin, 1957; Agerbirk and Olsen, 2011; Agerbirk et al., 2014).

From *R. luteola*, two alkaloids were described in an early report (Lutfullin et al., 1976). One was a substituted OAT, while the other was a substituted OAO, a further heterocyclic ring in the order Brassicales (Fig. 1). The OAT was named resedinine, but was identical to barbarin. Hence the older name has priority, and the name resedinine should be abandoned. The OAO was named resedine (**3**) and identified as 5-phenylOAO (Fig. 2). Relations to plant biochemistry or glucosinolate turnover were not investigated.

Of the many known OATs in the Brassicales, one has received particular attention: (*S*)-5-vinylOAT ((*S*)-goitrin) formed from the glucosinolate progoitrin ((*R*)-2-hydroxybut-3-enylglucosinolate) in oilseed rape (Greer, 1956). Despite decades of breeding efforts to lower levels of progoitrin and goitrin (Nour-Eldin et al., 2017), these metabolites remain a limiting factor for the use of protein from oilseed rape in fodder. Some of the antinutritional effects of goitrin and other OATs seem to be related to disturbance of iodine metabolism and catalysis of nitrite reactions in mammals (reviewed in Agerbirk et al., 2014; Felker et al., 2016). A recent investigation has broadened the range of known effects of OATs (Radulovic et al., 2017).

Glucosinolate hydrolysis is catalyzed by isoenzymes of thioglucoside glucohydrolase (E.C. 3.2.1.147) commonly known as myrosinases. Myrosinase-catalyzed complete turnover of glucosinolates happens after tissue disruption, and is caused by the mixing of enzyme and substrate that were spatially separated in intact plants (Wittstock et al., 2016a; b). Classical myrosinases of the "TGG" group were for many years regarded as the sole enzymes catalyzing this reaction. However, other myrosinases responsible for turnover of glucosinolates in intact plants have been identified in recent years (Pastorczyk and Bednarek, 2016; Wittstock et al., 2016a; b). Some unexpected, non-conventional degradation products have been reported, suggesting a varied and complex metabolism of glucosinolates in intact plants (Montaut and Bleeker, 2010; Agerbirk and Olsen, 2012; Frisch et al., 2015; Pedras et al., 2016; Klein and Sattely, 2017). To our knowledge, nothing is known of metabolism or detoxification of OATs in intact plants.

From non-enzymatic breakdown of glucobarbarin and progoitrin catalyzed by high concentrations of ferrous salts, thioamides were reported in high yields, on the base of limited analytical evidence (Austin et al., 1968a; b). A later report provided circumstantial evidence (UV-spectrum of a minor chromatographic peak) for the formation of low levels of the thioamide from epiglucobarbarin at similar high ferrous ion conditions, and discussed the possibility of ferrous ion catalyzed turnover of β-hydroxyalkyl glucosinolates in biological material (Bellostas et al., 2008).

In order to better understand ecological functions of glucobarbarin in our main experimental plant *B. vulgaris*, we set out to investigate its breakdown products *in vitro* as well as *in vivo*. Our initial objective was to determine the biochemical hydrolysis products from glucobarbarin, expected to be barbarin, 3-hydroxy-3-phenylpropanenitrile (**4**) and possibly the related thioamide (hypothetic "**5**"). However, an unexpected product with the same nominal mass as a potential thioamide fragment appeared, so an additional goal was to identify this OAO product and test for an enzymatic formation from the corresponding OAT. We here report lack of formation of a thioamide, scarce formation of the nitrile, and unexpected formation of OAOs via OATs catalyzed by a heat sensitive factor in glucobarbarin-containing plants. The formation of the OAO, resedine (**3**), is characterized and the connection to glucosinolate turnover investigated.

2. Results and discussion

2.1. Identification of the nitrile from glucobarbarin hydrolysis

One glucobarbarin product, barbarin (**2**), was already at hand, and ion trap HPLC-MS was optimized for detection in the relevant