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Structure and Biosynthesis of Benzoxazinoids: Plant Defence Metabolites with Potential as Antimicrobial Scaffolds

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Abstract

Benzoxazinoids, comprising the classes of benzoxazinones and benzoxazolinones, are a set of specialised metabolites produced by the plant family Poaceae (formerly Gramineae), and some dicots. The family Poaceae in particular contains several important crops like maize and wheat. Benzoxazinoids play a role in allelopathy and as defence compounds against (micro)biological threats. The effectivity of benzoxazinones in these functionalities is largely imposed by the subclasses (determined by *N* substituent). In this review, we provide an overview of all currently known natural benzoxazinoids and a summary of the current state of knowledge of their biosynthesis. We also evaluated their antimicrobial activity based on minimum inhibitory concentration (MIC) values reported in literature. Monomeric natural benzoxazinoids seem to lack potency as antimicrobial agents. The 1,4-benzoxazin-3-one backbone, however, has been shown to be a potential scaffold for designing new antimicrobial compounds. This has been demonstrated by a number of studies that report potent activity of synthetic derivatives of 1,4-benzoxazin-3-one, which possess MIC values down to 6.25 μg mL^{-1} against pathogenic fungi (e.g. *C. albicans*) and 16 μg mL^{-1} against bacteria (e.g. *S. aureus* and *E. coli*). Observations on the structural requirements for allelopathy, insecticidal, and antimicrobial activity suggest that they are not necessarily conferred by similar mechanisms.

Highlights

- Comprehensive overview of natural benzoxazinoids.
- Benzoxazinone subclass (*N* substituent) influences allelopathic and defence potency.
- Monomeric natural benzoxazinoids lack antimicrobial potency.
- Chemical modification of 1,4-benzoxazin-3-ones can enhance antimicrobial activity.
- Potential of 1,4-benzoxazin-3-one as a scaffold for design of antimicrobial drugs.

Keywords

Poaceae, cereal, plant defence, antibacterial, antifungal, classification, benzoxazinone, benzoxazolinone, synthetic, modification

Contents

1. Introduction

Benzoxazinoids are a set of specialised metabolites that are prominently described in species of the monocot plant family Poaceae. Benzoxazinoids have also been reported to be present in dicots of the families Acanthaceae, Calceolariaceae, Lamiaceae, Plantaginaceae, and Ranunculaceae (Adhikari et al., 2015; Frey et al., 2009; Niemeyer, 2009). Due to the agricultural importance of the crops in Poaceae such as wheat (*Triticum aestivum*) and maize (*Zea mays*), benzoxazinoids of this plant family will be our main focus. These specialised metabolites play a role in allelopathic plant-plant interactions, as defence compounds against (micro)biological threats, and as defence regulatory signals (Ahmad et al., 2011; Neal et al., 2012; Niemeyer, 2009; Sicker et al., 2000). Their allelopathic and defence functionalities, which are discussed in more detail in **Section 5**, suggest that benzoxazinoids could be potentially interesting leads for antimicrobial compounds. Promising lead structures might also be further modified to optimize their antimicrobial activity, as has been done for allelopathic activity (Macías et al., 2010; Macías et al., 2006b).

In this review we aim to provide a comprehensive overview of the structures of natural benzoxazinoids and to investigate their biosynthesis and biofunctionality with an emphasis on their antimicrobial potential.

2. Structure of natural benzoxazinoids

2.1. Classification and subclassification

Benzoxazinoids are divided into two classes: benzoxazinones (1,4-benzoxazin-3-one) and benzoxazolinones (1,3-benzoxazol-2-one) (**Figure 1**). Benzoxazolinones were first reported in rye in the 1950s (Virtanen and Hietala, 1955) and benzoxazinones shortly thereafter in the early 1960s, also in rye (Hietala and Virtanen, 1960; Honkanen and Virtanen, 1960). Within these classes, several subclasses are formed by compounds with various *N* substituents (Cambier et al., 1999; Niemeyer, 2009). The class of benzoxazinones is divided into three subclasses: lactams (*N*-hydro), hydroxamic acids (*N*-hydroxy), and methyl derivatives (*N*-methoxy) (**Table 1**). Within the class of benzoxazolinones, *N*-hydro derivatives are by far the most common due to loss of the *N* substituent in the transformation from benzoxazinone to benzoxazolinone (**Section 4.2**). Nevertheless, benzoxazolinones with other *N* substituents have been reported: *N*-hydroxy and *N*-methoxy derivatives were found in *Scoparia dulcis* (Wu et al., 2012). Possibly these compounds are formed enzymatically from *N*-hydro benzoxazolinones or by another yet to be discovered biosynthetic pathway. Additionally, *N*-glucosyl derivatives can be formed as detoxification products of benzoxazinoids (Hofmann et al., 2006; Maag et al., 2014; Sicker et al., 2001) (**Table 1**).

Various properties of benzoxazinoids, such as stability and reactivity (Grambow et al., 1986; Wouters et al., 2016), plant defence functionality (Niemeyer, 2009; Oikawa et al., 2004), effects on human health (Adhikari et al., 2015), and fragmentation in mass spectrometry (de Bruijn et al., 2016) were shown to be affected by *N* substitution.

2.2. Abbreviations and structural overview

The first benzoxazinoids were discovered over 50 years ago. Their systematic IUPAC names are long and inconvenient to use which led to the formulation of abbreviations based on the three letter code BOA. This code was complemented with prefixes of the letters H and M for hydroxy and methoxy substituents, respectively. BOA can refer to compounds with either a benzoxazinone or a benzoxazolinone backbone (**Figure 1**). Because this three letter code does not distinguish classes or subclasses, identification of compounds based on their abbreviation can be challenging, especially for newcomers in the benzoxazinoid field. To facilitate the correct use of these abbreviations and avoid inconsistency, this paper includes an overview of the presently known natural benzoxazinoids of different (sub)classes with their abbreviations (**Table 1)**. This overview does not include benzoxazinone *C*2 *O*-glycosides, which will be discussed in the following section. In addition to the benzoxazinoids in **Table 1**, zeaoxazolinone, a 7-methoxy-1,3-benzoxazol-2-one dimer from maize (Mohamed et al., 2014), is displayed in **Figure 2A**. Another dimeric benzoxazolinone, 5,5′-bis-benzoxazoline-2,2′-dione (1,3-benzoxazol-2-one dimer) was found in *Acanthus ilicifolius* (D'Souza et al., 1997) (**Figure 2B**).

2.3. Glycosylation of benzoxazinones

Benzoxazinones are glycosylated in an early step of the biosynthesis (**Section 3.1**). Glycosylation is used as a mechanism to store benzoxazinones inside plant cells and to prevent self-toxicity (Cambier et al., 1999; von Rad et al., 2001). Glycosides are unable to undergo ring-opening, limiting their reactivity (**Figure 3** and **Section 4**). So far, glycosides reported in Poaceae are mainly glucosides, whereas in the family Plantaginaceae galactosides were also detected (Wu et al., 2012). Recent studies also indicate the presence of dihexosides in wheat and rye (*Secale cereale*) (de Bruijn et al., 2016; Hanhineva et al., 2011), tri- and tetrahexosides in wheat and rye beers (Pihlava and Kurtelius, 2016), and acetylhexosides in wheat (de Bruijn et al., 2016). Upon metabolism by mammals, benzoxazinoids may also be conjugated with uronic acids (Adhikari et al., 2012). In addition, reglucosylation of aglycones, with inversion of stereochemistry (from 2*R* to 2*S*), is a mechanism of benzoxazinoid detoxification found in some insects (Wouters et al., 2014).

2.4. Substituents on the benzene moiety (R_2 to R_5)

The most common natural substituents at positions R_2 to R_5 are hydroxyl or methylated hydroxyl (methoxyl) groups (Niemeyer, 2009). Other substituents at these positions occurring more rarely in nature are chloro-, acetyl- or glucopyranosyloxy-groups (**Table 1**). Chloro-derivatives, for example, can be found in maize (Anai et al., 1996) and *A. ilicifolius* (Acanthaceae) (Kanchanapoom et al., 2001a).

3. Benzoxazinone biosynthesis and distribution in Poaceae

3.1. General biosynthesis of benzoxazinones

The basic biosynthetic pathway of benzoxazinones (up to DIBOA-Glc) was first studied and elucidated in maize (Frey et al., 1997), and later also in wheat (Nomura et al., 2002; Nomura et al., 2003) and rye (Bakera et al., 2015; Rakoczy-Trojanowska et al., 2017). The biosynthesis starts from indole-3-glycerolphosphate (IGP), which is consecutively converted to HBOA, the first benzoxazinoid, in 4 steps by the enzymes BX1 to BX4 (**Figure 3**). A subsequent glucosylation and hydroxylation leads to DIBOA-Glc, which serves as the starting point for hydroxamic acid biosynthesis. All further downstream benzoxazinoids are synthesized as glucosides and most of the enzymes involved are unable to use the aglycones as substrates (Jonczyk et al., 2008; Oikawa et al., 2002). The formation of these downstream metabolites has mainly been studied in maize and to a lesser extent in wheat and rye (Dutartre et al., 2012; Handrick et al., 2016; Jonczyk et al., 2008; Makowska et al., 2015; Meihls et al., 2013; Tanwir et al., 2017). A summary of the current state of knowledge on the biosynthetic pathways of benzoxazinones is shown in **Figure 3**. As shown on the right-hand side of this figure, benzoxazinones can be transformed to benzoxazolinones, which is further discussed in **Section 4.2**. Next to the Poaceae, benzoxazinoids have been reported in dicots of the families Acanthaceae, Calceolariaceae, Lamiaceae, Plantaginaceae, and Ranunculaceae (**Table 1**). The biosynthesis in these families might be similar to what has been reported in Poaceae, as indicated by analogues of the benzoxazinoid biosynthetic enzymes that have been found in dicots (Dick et al., 2012; Schullehner et al., 2008). The overview in **Figure 3** indicates that several benzoxazinoid biosynthetic pathways require further investigation (dashed arrows). These aspects are discussed in the next section.

3.2. Tentative pathways in benzoxazinone biosynthesis

According to the generally accepted pathway, as shown in **Figure 3**, HBOA is converted to DIBOA by BX5, which is then glucosylated by BX8 or BX9. Whereas HBOA aglycons are relatively stable, DIBOA aglycons are reactive (**Section 4**) and phytotoxic (**Section 5**). Therefore, the formation of free DIBOA within the plant cell is likely omitted. One possibility could be the stabilisation and rapid glycosylation of DIBOA within a metabolon (i.e. a complex of sequential metabolic enzymes) by metabolic channelling. This would be similar to what has been demonstrated for toxic or labile intermediates in other secondary metabolic pathways (e.g. dhurrin biosynthesis in sorghum) (Jørgensen et al., 2005). As an alternative, we propose a pathway in which HBOA is glycosylated prior to oxidation to form DIBOA-Glc. It was previously proposed that HBOA-Glc and DIBOA-Glc are in fact a redox pair (Hofman and Hofmanová, 1969), which might present a mechanism for the possible interconversion of these compounds. The existence of an enzyme which catalyses the oxidation of HBOA-Glc to DIBOA-Glc has not been thoroughly explored in literature.

Maize BX8 and BX9 were both shown to be able to glycosylate HBOA albeit at a much lower conversion rate than DIBOA and DIMBOA (von Rad et al., 2001). The glycosylation of DIMBOA, however, does not seem to serve a function within the biosynthetic pathway, as it is already formed as a glycoside. The formation of HBOA-Glc would be a logical starting point for the biosynthesis of lactams. Further hydroxylation and methylation to produce DHBOA-Glc, HMBOA-Glc, and HM$_2$BOA-Glc might be performed by the same or similar enzymes as those involved in hydroxamic acid biosynthesis (BX6, BX7, and BX13) or by a yet to be discovered part of the BX enzyme-cluster. The compounds TRIBOA-Glc and TRIMBOA-Glc are intermediates of the biosynthesis and are not typically accumulated and detected in maize tissues (Cambier et al., 1999; Handrick et al., 2016). Possibly, DHBOA-Glc serves a similar role as an intermediate in HMBOA-Glc synthesis. An analogue of TRIMBOA-Glc as an intermediate for the biosynthesis of HM$_2$BOA-Glc is not yet known. The biosynthetic pathway of benzoxazinone lactams presents a gap in our current knowledge. Lactams are the least prevalent subclass of benzoxazinones in maize explaining the lack of research on their biosynthesis, however, lactams are more prominent in other species, such as rye (Tanwir et al., 2013).

The methyl derivative equivalent of DIBOA, 4-*O*-Me-DIBOA-Glc, was annotated in wheat seedlings exposed to fungal stress based on LC-MS analysis (de Bruijn et al., 2016). Thus far, its biosynthesis has not yet been fully elucidated. It was shown that a DIMBOA-Glc 4-*O*-methyltransferase from wheat was also able to convert DIBOA-Glc to 4-*O*-Me-DIBOA-Glc *in vitro*, but the latter compound was not detected *in planta* in that study (Oikawa et al., 2002).

3.3. Distribution of benzoxazinones in Poaceae

Several recent reviews have addressed the genetic background of benzoxazinoid production between the different species within the Poaceae family (Dutartre et al., 2012; Makowska et al., 2015). Three agriculturally important crops that produce benzoxazinoids are maize, wheat, and rye. The profile of benzoxazinones produced between these species varies (**Figure 4**). Overall, maize produces the most diverse profile of benzoxazinones, whereas rye possesses the lowest diversity. Based on the biosynthetic pathways involved (**Figure 3**), it seems like wheat does not possess an active BX14-like enzyme to perform the conversion of DIM$_2$BOA-Glc into HDM$_2$BOA-Glc. It is, however, able to produce HDMBOA-Glc which indicates the presence of an active BX10-like enzyme. Rye has not been shown to produce HDMBOA-Glc and other methyl derivatives.

Several other well-known species from the Poaceae family, such as rice (*Oryza sativa*), oat (*Avena sativa*), and barley (*Hordeum vulgare*) do not produce benzoxazinoids. Interestingly, some other members of the genus *Hordeum*, e.g. *Hordeum lecheri*, have been found to produce benzoxazinoids (Grün et al., 2005). There have also been some reports of benzoxazinoid production in sorghum (Malan et al., 1984; Niemeyer, 1988). Several other less well-known species in the Poaceae (e.g. *Aegilops speltoides*) have been reported to produce benzoxazinoids (Dutartre et al., 2012). As shown by the phylogenetic trees presented by Dutartre and co-workers, the development of the benzoxazinoid biosynthetic cluster does not necessarily follow the general phylogenetic relationships between the different species, as is exemplified by the genus *Hordeum* (Dutartre et al., 2012).

For a more in-depth perspective of benzoxazinoid phylogenomics, we would like to refer readers to the aforementioned reviews (Dutartre et al., 2012; Makowska et al., 2015) (focussed on Poaceae). In addition, there are several studies that provide more information about benzoxazinoids in dicots (Dick et al., 2012; Frey et al., 2009; Schullehner et al., 2008). The main benzoxazinoids produced by dicots are similar to those produced by Poaceae (**Table 1**). In **Section 2** and **Table 1**, several notable molecules unique to dicots are shown such as chloro-derivatives of HBOA and DIBOA, and the production of benzoxazinone galactosides rather than glucosides.

3.4. Benzoxazinoid content and induction *in planta*

The main benzoxazinoid in maize and wheat is DIMBOA (and its glycosides) (Köhler et al., 2015; Villagrasa et al., 2006), whereas in rye this is DIBOA (and its glycosides) (Oikawa et al., 2002; Rakoczy-Trojanowska et al., 2017). Reported values for the total quantities of benzoxazinoids in tissues of different species are highly variable. They range from 4.8 and 95 µg g^{-1} dry weight in wheat and rye grains (Tanwir et al., 2013), respectively, to over 1900 µg g^{-1} dry weight in rye shoots (Schulz et al., 2013), and to several mg g^{-1} fresh weight in maize (Köhler et al., 2015; Meihls et al., 2013). Comparing individual or total benzoxazinoid content from different literature sources is not straightforward. One of the underlying causes is that many papers focus solely on the analysis of a select number of benzoxazinones (usually DIMBOA and DIBOA) whereas in other works total benzoxazinoid content might include glycosides, other benzoxazinones, and benzoxazolinones. Secondly, the quantitative analysis of these molecules is challenging and has been addressed in multiple publications (Bonnington et al., 2003; Eljarrat and Barceló, 2001; Pedersen et al., 2017). This is mainly due to the activity of glycosidases during sample treatment and the reactivity of the resulting benzoxazinone aglycons (**Section 4**) (Cambier et al., 1999; Grambow et al., 1986). Furthermore, there are several factors which can have a large effect on the total content and composition of benzoxazinoids reported in plants: (i) plant species and cultivar (Copaja et al., 2006; Eljarrat and Barceló, 2001; Schulz et al., 2013; Zasada et al., 2007), (ii) plant age (Cambier et al., 1999; Köhler et al., 2015; Tanwir et al., 2017), (iii) tissue type (e.g. root, shoot) (Cambier et al., 1999; Copaja et al., 2006; Villagrasa et al., 2006), and (iv) growth conditions (e.g. fertilization) (Walker et al., 2012).

Several induction methods have been described to elicit *in planta* production or diversification of benzoxazinoids. Abiotic elicitation methods include treatment with jasmonic acid (Oikawa et al., 2002), *cis*-jasmone (Moraes et al., 2008), chitin and chitosan oligomers, or copper chloride (Oikawa et al., 2001). Biotic elicitation methods include exposure to fungi (Ahmad et al., 2011; Oikawa et al., 2004), bacteria (Walker et al., 2012), insects, or insect larvae (Ahmad et al., 2011; Oikawa et al., 2004).

4. Reactivity of benzoxazinones and benzoxazolinones

As previously described, benzoxazinones are stored as stable glycosides in the plant (**Sections 2.3** and **3.1**, and **Figure 3**). Upon cell damage or when exuded from roots, the glycosides come into contact with glycosidases which leads to the release of the less stable and more reactive benzoxazinone aglycones. The storage of defence compounds as glycosidic precursors which are released under stress conditions by glycosidase action is also observed in plants of other families. In Brassicaceae, for example, glucosinolates (precursors) are deglycosylated leading to the formation of isothiocyanates, which can react with thiols or amines contributing to antimicrobial functionality and other bioactivities (Dufour et al., 2015). Benzoxazinone aglycones can undergo a variety of reactions, as described in the following sections.

4.1. Oxo-cyclo tautomerism of benzoxazinone aglycons

Several mechanisms for the reactivity of ring-closed benzoxazinone aglycones have been suggested, such as metal-complexation by hydroxamic acids (Tipton and Buell, 1970). Another proposed mechanism is based on the electrophilicity of the nitrenium (N^+) ion which is formed when the *N* substituent acts as a leaving group (Hashimoto and Shudo, 1996; Wouters et al., 2016). Additionally, benzoxazinone aglycones can undergo oxo-cyclo tautomerism in which the heterocycle assumes its ring-opened configuration. In the ring-opened configuration, benzoxazinones possess an α-dicarbonyl moiety which can react with thiols and amines (Wouters et al., 2016). In addition, ring-opening can lead to transformation of benzoxazinones to benzoxazolinones, which is discussed in the next section.

4.2. Transformation of benzoxazinones to benzoxazolinones

One of the possible follow-up reactions of ring-opening of benzoxazinone aglycones is the spontaneous transformation into the corresponding benzoxazolinone (**Figure 3**) (Wouters et al., 2016). Several different multi-step mechanisms have been proposed for this transformation, all of which involve a deprotonation step and eventually lead to loss of the *N* substituent (R_1) (Wouters et al., 2016). The reactivity of aglycones towards this transformation is mainly determined by their *N* substituent which acts as the leaving group. Methyl derivatives (*N*-methoxy) are more reactive than hydroxamic acids (*N*-hydroxy), whereas lactams (*N*-hydro) are practically unreactive because H is not a suitable leaving group (Grambow et al., 1986; Wouters et al., 2016). Due to the deprotonation step, the reaction is pH dependent. It proceeds rapidly around neutral pH and even more quickly in alkaline conditions. Below pH 7, the degradation rate decreases, with a rate constant at pH 4 which is approximately 150-fold lower than that at pH 7, as demonstrated for DIBOA (Bredenberg et al., 1962).

Whether alternative pathways exist to produce benzoxazolinones *in planta* is unknown. The detection of benzoxazolinones, especially those with *N*-hydroxy and *N*-methoxy substituents (Wu et al., 2012) (**Section 2.1**, **Table 1**), in some plants might indicate the existence of pathways dedicated to the production of specific benzoxazolinones.

4.3. Transformation products of benzoxazolinones

As demonstrated by the discovery of zeaoxazolinone in maize (**Figure 2A**) (Mohamed et al., 2014) and 5,5′-bis-benzoxazoline-2,2′-dione in *A. ilicifolius* (**Figure 2B**) (D'Souza et al., 1997), benzoxazolinones can form dimers *in planta*. Surprisingly, zeaoxazolinone consists of two 7-methoxy-1,3-benzoxazol-2-one subunits, although the monomeric 7-methoxy derivative has not yet been found (**Table 1**). The exact mechanism of dimer formation and the enzymes involved needs to be further elucidated.

Benzoxazolinones can be detoxified by plants or insects using various mechanisms, e.g. hydroxylation, and *O* or *N* glycosylation (**Table 1**) (Hofmann et al., 2006; Maag et al., 2014; Schulz et al., 2016; Wieland et al., 1998). Furthermore, in soil, benzoxazolinones can undergo microbial transformations (Fomsgaard et al., 2004; Schulz et al., 2013; Wouters et al., 2016). In the presence of bacteria or fungi, benzoxazolinones are converted to aminophenol intermediates (e.g. 2-aminophenol), which can subsequently react to aminophenoxazinones (e.g. 2-amino-phenoxazin-3-one) (Fomsgaard et al., 2004; Macías et al., 2009). Besides aminophenoxazinones, other transformation products formed via aminophenol intermediates are acetamides and malonamic acids (Fomsgaard et al., 2004; Schulz et al., 2013). These transformation products, however, have lost their benzoxazinoid structural motif and are therefore not considered in more detail in this work.

5. *In planta* functionality of benzoxazinoids

Allelopathic functionality of benzoxazinoids has been well-established and is one of the main functions of these compounds. Benzoxazinoid levels are especially high in roots and root exudates of young maize, wheat, and rye, as they compete with other plants during early growth (Niemeyer, 2009; Schulz et al., 2013). General structure-activity relationships observed for allelopathic or phytotoxic activity are that hydroxamic acids (usually represented by DIBOA and DIMBOA) are more potent than lactams (usually represented by HBOA and HMBOA). The benzoxazolinones BOA and MBOA are in between hydroxamic acids and lactams in terms of allelopathic potency. DIBOA and DIBOA-Glc typically seem to be the most potent allelopathic natural benzoxazinoids, exhibiting significant root length inhibition at concentrations between 10^{-3} to 10^{-4} M (depending on target species) (Macías et al., 2005, 2006a). Interestingly, glycosylation of DIBOA does not seem to affect its activity (Macías et al., 2005, 2006a; Wouters et al., 2016). It was also shown to be possible to enhance selectivity and potency of the phytotoxic activity by chemical modification of 4-hydroxy-1,4-benzoxazin-3-one (Macías et al., 2010; Macías et al., 2006b).

Besides allelopathy, benzoxazinoids also play a role as defence compounds against insects and microorganisms. In general, accumulation of benzoxazinoids is positively correlated with resistance against disease and insects in maize, wheat, and rye (Ahmad et al., 2011; Copaja et al., 2006; Meihls et al., 2013; Niemeyer, 2009; Søltoft et al., 2008). As with allelopathy, hydroxamic acids seem to be more potent than lactams (Wouters et al., 2016). Moreover, benzoxazinones with increased methylation (i.e. *N*-OCH_3 or *C*7-OCH_3) were found to be correlated with disease resistance (Søltoft et al., 2008). In another study, it was suggested that methylation of DIMBOA-Glc to HDMBOA-Glc might be a mechanism by which MBOA production is accelerated, as HDMBOA will more quickly transform to MBOA than DIMBOA (Oikawa et al., 2004). In that study, they observed increased concentrations of HDMBOA-Glc in maize upon fungal infection and larval feeding. Additionally, MBOA was shown to be more effective at inhibiting fungal germination than its precursors (Oikawa et al., 2004).

Wouters and co-workers postulated that the capacity to undergo ring-opening increased activity of benzoxazinones against insects. Consequently, glycosides were found to be less active than aglycons (Wouters et al., 2016). The toxicity of MBOA to insects was lower than that of DIMBOA (Wouters et al., 2016), suggesting that insecticidal activity is probably conferred by benzoxazinones via another mechanism (**Section 4**) than antifungal activity.

These defence functionalities associated with benzoxazinoids indicate that they might be potentially interesting leads for antimicrobial compounds. As an example, the antibiotic ofloxacin shares structural features (1,4-benzoxazin ring system) with natural benzoxazinoids (Hayakawa et al., 1986).

6. Antimicrobial potential of natural benzoxazinoids

One of the first reports on the antimicrobial potential of benzoxazinoids described the inhibition of the gram-negative bacterium *Xanthomonas stewartii* (i.e. *Pantoea stewartii*), the causal agent of Stewart's wilt, by MBOA from maize (Whitney and Mortimore, 1961). A more recent study indicated that DIMBOA also possesses antibacterial properties against the pathogenic bacterium *Staphylococcus aureus*, albeit at high concentrations (Gleńsk et al., 2016). The novel benzoxazinoid zeaoxazolinone (**Figure 2A**) showed fungal inhibition comparable to clotriamazole against amongst others *Aspergillus flavus* and *Candida albicans* (Mohamed et al., 2014). Unfortunately, no MIC values were reported for this dimeric benzoxazinoid. The antifungal and possibly antibacterial potential of this promising compound should be further investigated. The antimicrobial activity of 5,5′-bis-benzoxazoline-2,2′-dione (**Figure 2B**) has not yet been evaluated.

Despite the extensive research on benzoxazinoids, only a limited number of papers evaluated activity of these compounds against human pathogens, commonly represented by *S. aureus* (gram-positive bacterium), *Escherichia coli* (gram-negative bacterium), and *C. albicans* (yeast, fungus). Three papers by Bravo, Lazo and co-workers (Bravo et al., 1997; Bravo and Lazo, 1993, 1996) and one other paper (Gleńsk et al., 2016) reported minimum inhibitory concentration (MIC) values of benzoxazinoids against these three pathogens (**Table 2**). The antifungal activity of these compounds has been studied more extensively than their antibacterial activity. MBOA and DIMBOA (MIC 450 and 500 µg mL^{-1}, respectively) are most effective against fungi. As expected, the antifungal activity of benzoxazinoid glucosides (i.e. detoxified derivatives) is very limited (MIC >1000 µg mL^{-1}) (Bravo and Lazo, 1996). MIC values against bacteria have only been reported for the most common natural benzoxazinoids, namely the benzoxazinone aglycons DIMBOA and DIBOA, and the benzoxazolinones BOA and MBOA. Overall, DIMBOA is the natural benzoxazinoid with the lowest reported MIC values (**Table 2**). Our general observations regarding antimicrobial activity of monomeric natural benzoxazinoids are that: (i) hydroxamic acids are more active than lactams, (ii) hydroxylation at *C*7 increases activity, and (iii) glycosylation decreases activity. This is in accordance with a previous review (Wouters et al., 2016). Interestingly, the second and third observation are in contrast with findings for allelopathy (phytotoxicity) where these factors do not seem to affect activity (**Section 5**). This suggests that allelopathy, antimicrobial, and insecticidal activity are not necessarily conferred by the same structural motifs. Nevertheless, it seems that hydroxamic acids are more active than lactams with regard to all three of these functionalities.

Even DIMBOA, the most antimicrobial monomeric natural benzoxazinoid, has relatively poor activity (lowest reported MIC 250 µg mL^{-1} against *S. aureus*) (Gleńsk et al., 2016) compared to other natural compounds, such as flavonoids (e.g. MIC 3.9-15.6 µg mL^{-1} of apigenin against *S. aureus*) (Gibbons, 2004) and prenylated isoflavonoids (e.g. MIC 3.13 µg mL^{-1} of licoricidin against *S. aureus*) (Gibbons, 2004).

Interestingly, the benzoxazolinone transformation product 2-amino-phenoxazin-3-one was selectively very potent as an antimicrobial compound against the species *Helicobacter pylori* (MIC 1 µg mL^{-1}) (Hanawa et al., 2010), some *Mycobacterium* spp. (MIC 2.8 – 11.3 µg mL^{-1}) (Shimizu et al., 2004), and *Chlamydia pneumoniae* (MIC 2.1 µg mL^{-1}) (Uruma et al., 2005). Activity of this compound against many other bacteria (e.g. *E. coli* and *S. aureus*) was limited (Atwal et al., 1992; Shimizu et al., 2004).

Overall, the antimicrobial potential of monomeric natural benzoxazinoids seems to be limited, yet several known antifungals such as carbendazim and boscalid contain the NHCO structure (in open chain configuration) coupled to an aromatic system (Śmist et al., 2016). This suggests that structural modification of benzoxazinoids might enhance their antimicrobial activity.

7. Potential of the 1,4-benzoxazin-3-one scaffold

In addition to their work on natural benzoxazinoids, Bravo and Lazo also evaluated some synthetic derivatives of these compounds. They showed that some synthetic 1,4-benzoxazin-3-one hydroxamic acids lacking the *C*2 hydroxyl group possessed more antifungal potential against *C. albicans* than DIMBOA (Bravo and Lazo, 1993, 1996). These studies first demonstrated the potential of benzoxazinoids as scaffolds for the design of antimicrobial compounds. The 1,4-benzoxazin-3-one scaffold in particular, typically without the hydroxamic acid motif conferred by the *N*-OH group, was further utilized in several other studies in which more extensive modifications were applied.

One study showed that three synthetic benzoxazinones, with a combination of a *C*8 chloro and a *C*2 alkane (*n*-propyl, *n*-butyl, *n*-pentyl) substituent, had antifungal activity comparable to fluconazole (Özden et al., 2000). Another study found two other benzoxazinone derivatives, namely a *C*2 ethyl and *N*-acetyl benzoxazinone, with promising antifungal activity against a variety of phytopathogenic fungi (Śmist et al., 2016). Unfortunately, no MIC values were determined in these studies.

Several other studies do, however, report MIC values for synthetic 1,4-benzoxazin-3-ones and these values are up to 40-fold lower than those reported for DIMBOA (**Figure 5**). For example, a number of compounds with *N*-alkyl and *C*7 halogen substituents were found to possess good activity against several pathogenic bacteria but limited antifungal activity (Fang et al., 2011). In addition, two other studies describe multiple compounds with good activity against pathogenic bacteria as well as fungi (Alper-Hayta et al., 2006; Yalcin et al., 2003). An example of an all-round active molecule is 2-(2-ethyloxy-2-oxo-ethyl)-6-chloro-7-nitro-1,4-benzoxazin-3-one with MIC 12.5 µg mL^{-1} against *C. albicans*, and MIC 25 µg mL^{-1} against *S. aureus* and *E. coli* (**Figure 5**) (Alper-Hayta et al., 2006). Most of the antimicrobial synthetic 1,4-benzoxazin-3-one derivatives do not possess a hydroxyl-moiety at *C*2 and can therefore not undergo ring-opening. Neither is the formation of a nitrenium ion or metal complexation very likely, considering their structure. Establishing structure-activity relationships for these antimicrobial compounds might help in elucidating their antimicrobial mode-of-action.

Overall, synthetic benzoxazinoids based on a 1,4-benzoxazin-3-one scaffold show promising antimicrobial activity which might be further enhanced by targeted modifications based on previously reported findings. Considering the general observation that hydroxamic acids are more reactive and bioactive than lactams, perhaps its 4-hydroxy derivative would be an even more potent scaffold which was already used to produce synthetic allelopathic compounds (Macías et al., 2010).

8. Conclusions

In this review, we provided an overview of all 32 currently known natural benzoxazinoids (excluding *C*2 glycosides), which includes one dimeric benzoxazolinone. The current state of knowledge on the biosynthesis of these compounds in Poaceae was summarised and a gap in the knowledge on the pathways responsible for lactam biosynthesis was identified. Within the family Poaceae, maize, wheat, and rye are the major benzoxazinoid producers, differences in the benzoxazinoid diversity between these species suggests a more advanced development of the biosynthesis in maize than in the other two species. The subclass (*N* substituent) of benzoxazinones amongst others affects their allelopathy and defence functionality.

We evaluated the antimicrobial activity of natural benzoxazinoids based on MIC values reported in literature. Even though these compounds play an important role in plant defence and allelopathy, monomeric natural benzoxazinoids seem to lack potency as antimicrobial agents. Observations on the structural requirements for allelopathy, insecticidal, and antimicrobial activity suggest that they are not necessarily conferred by similar mechanisms.

The 1,4-benzoxazin-3-one backbone has been shown to be a potential scaffold for designing new antimicrobial compounds with activity against pathogenic bacteria and fungi. This has been demonstrated by a number of studies that report potent activity (MIC down to 6.25-16 μg mL^{-1} against *C. albicans, E.coli,* and *S. aureus*) of synthetic derivatives of 1,4-benzoxazin-3-one.

Conflict of interest

The authors declare no conflict of interest regarding this research.

References

Adhikari, K. B., Lærke, H. N., Mortensen, A. G., Fomsgaard, I. S., 2012. Plasma and urine concentrations of bioactive dietary benzoxazinoids and their glucuronidated conjugates in rats fed a rye bread-based diet. J. Agric. Food. Chem. 60, 11518-11524.

Adhikari, K. B., Tanwir, F., Gregersen, P. L., Steffensen, S. K., Jensen, B. M., Poulsen, L. K., Nielsen, C. H., Høyer, S., Borre, M., Fomsgaard, I. S., 2015. Benzoxazinoids: Cereal phytochemicals with putative therapeutic and health-protecting properties. Mol. Nutr. & Food Res. 59, 1324-1338.

Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y. H., Martin, J., Smart, L., Glauser, G., Erb, M., Flors, V., Frey, M., Ton, J., 2011. Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. Plant Physiol. 157, 317-327.

Alipieva, K. I., Taskova, R. M., Evstatieva, L. N., Handjieva, N. V., Popov, S. S., 2003. Benzoxazinoids and iridoid glucosides from four *Lamium* species. Phytochem. 64, 1413-1417.

Alper-Hayta, S., Akı-Sener, E., Tekiner-Gulbas, B., Yıldız, I., Temiz-Arpacı, O., Yalcın, I., Altanlar, N., 2006. Synthesis, antimicrobial activity and QSARs of new benzoxazine-3-ones. Eur. J. Med. Chem. 41, 1398-1404.

Anai, T., Aizawa, H., Ohtake, N., Kosemura, S., Yamamura, S., Hasegawa, K., 1996. A new auxin-inhibiting substance, 4-Cl-6,7-dimethoxy-2-benzoxazolinone, from light-grown maize shoots. Phytochem. 42, 273-275.

Bakera, B., Makowska, B., Groszyk, J., Niziołek, M., Orczyk, W., Bolibok-Bragoszewska, H., Hromada-Judycka, A., Rakoczy-Trojanowska, M., 2015. Structural characteristics of *ScBx* genes controlling the biosynthesis of hydroxamic acids in rye (*Secale cereale* L.). J. Appl. Genet. 56, 287-298.

Baumeler, A., Hesse, M., Werner, C., 2000. Benzoxazinoids-cyclic hydroxamic acids, lactams and their corresponding glucosides in the genus *Aphelandra* (Acanthaceae). Phytochem. 53, 213-222.

Bonnington, L., Eljarrat, E., Guillamón, M., Eichhorn, P., Taberner, A., Barceló, D., 2003. Development of a liquid chromatography-electrospray-tandem mass spectrometry method for the quantitative determination of benzoxazinone derivatives in plants. Anal. Chem. 75, 3128-3136.

Bravo, H. R., Copaja, S. V., Figueroa-Duarte, S., Lamborot, M., Martín, J. S., 2005. 1,4-benzoxazin-3-one, 2-benzoxazolinone and gallic acid from *Calceolaria thyrsilora* Graham and their antibacterial activity. Z. Naturforsch., C: Biosci. 60, 389-393.

Bravo, H. R., Copaja, S. V., Lazo, W., 1997. Antimicrobial activity of natural 2-benzoxazolinones and related derivatives. J. Agric. Food. Chem. 45, 3255-3257.

Bravo, H. R., Copaja, S. V., Martín, J. S., 2004. Contents of 1,4-benzoxazin-3-ones and 2-benzoxazolinone from *Stenandrium dulce* (Nees). Z. Naturforsch., C: Biosci. 59, 177-180.

Bravo, H. R., Lazo, W., 1993. Antimicrobial activity of cereal hydroxamic acids and related compounds. Phytochem. 33, 569-571.

Bravo, H. R., Lazo, W., 1996. Antialgal and antifungal activity of natural hydroxamic acids and related compounds. J. Agric. Food. Chem. 44, 1569-1571.

Bredenberg, J. B., Honkanen, E., Virtanen, A. I., 1962. Kinetics and mechanism of decomposition of 2,4-dihydroxy-1,4-benzoxazin-3-one. Acta Chem. Scand. 16, 135-&.

Cambier, V., Hance, T., de Hoffmann, E., 1999. Non-injured maize contains several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. Phytochem. Anal. 10, 119-126.

Copaja, S. V., Villarroel, E., Bravo, H. R., Pizarro, L., Argandona, V. H., 2006. Hydroxamic acids in *Secale cereale* L. and the relationship with their antifeedant and allelopathic properties. Z. Naturforsch., C: Biosci. 61, 670-676.

D'Souza, L., Wahidulla, S., Mishra, P. D., 1997. Bisoxazolinone from the mangrove *Acanthus illicifolius*. Indian J. Chem. Sect. B - Org. Chem. Incl. Med. Chem. 36, 1079-1081.

de Bruijn, W. J. C., Vincken, J.-P., Duran, K., Gruppen, H., 2016. Mass spectrometric characterization of benzoxazinoid glycosides from *Rhizopus*-elicited wheat (*Triticum aestivum*) seedlings. J. Agric. Food. Chem. 64, 6267-6276.

Dick, R., Rattei, T., Haslbeck, M., Schwab, W., Gierl, A., Frey, M., 2012. Comparative analysis of benzoxazinoid biosynthesis in monocots and dicots: Independent recruitment of stabilization and activation functions. Plant Cell 24, 915-928.

Dufour, V., Stahl, M., Baysse, C., 2015. The antibacterial properties of isothiocyanates. Microbiol. 161, 229-243.

Dutartre, L., Hilliou, F., Feyereisen, R., 2012. Phylogenomics of the benzoxazinoid biosynthetic pathway of Poaceae: Gene duplications and origin of the *Bx* cluster. BMC Evol. Biol. 12.

Eljarrat, E., Barceló, D., 2001. Sample handling and analysis of allelochemical compounds in plants. Trac-Trend Anal Chem 20, 584-590.

Fang, L., Zuo, H., Li, Z. B., He, X. Y., Wang, L. Y., Tian, X., Zhao, B. X., Miao, J. Y., Shin, D. S., 2011. Synthesis of benzo[b][1,4]oxazin-3(4*H*)-ones via smiles rearrangement for antimicrobial activity. Med. Chem. Res. 20, 670-677.

Fielder, D. A., Collins, F. W., Blackwell, B. A., Bensimon, C., Apsimon, J. W., 1994. Isolation and characterization of 4-acetyl-benzoxazolin-2-one (4-ABOA), a new benzoxazolinone from *Zea mays*. Tetrahedron Lett. 35, 521-524.

Fomsgaard, I. S., Mortensen, A. G., Carlsen, S. C. K., 2004. Microbial transformation products of benzoxazolinone and benzoxazinone allelochemicals - a review. Chemosphere 54, 1025-1038.

Frey, M., Chomet, P., Glawischnig, E., Stettner, C., Grün, S., Winklmair, A., Eisenreich, W., Bacher, A., Meeley, R. B., Briggs, S. P., Simcox, K., Gierl, A., 1997. Analysis of a chemical plant defense mechanism in grasses. Science 277, 696-699.

Frey, M., Schullehner, K., Dick, R., Fiesselmann, A., Gierl, A., 2009. Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. Phytochem. 70, 1645-1651.

Gibbons, S., 2004. Anti-staphylococcal plant natural products. Nat. Prod. Rep. 21, 263-277.

Gleńsk, M., Gajda, B., Franiczek, R., Krzyżanowska, B., Biskup, I., Włodarczyk, M., 2016. In vitro evaluation of the antioxidant and antimicrobial activity of DIMBOA [2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one]. Nat. Prod. Res. 30, 1305-1308.

Grambow, H. J., Lückge, J., Klausener, A., Müller, E., 1986. Occurrence of 2-(2-hydroxy-4,7-dimethoxy-2*H*-1,4-benzoxazin-3-one)-*β*-D-glucopyranoside in *Triticum aestivum* leaves and its conversion into 6-methoxy-benzoxazolinone. Z. Naturforsch., C: Biosci. 41, 684-690.

Grün, S., Frey, M., Gierl, A., 2005. Evolution of the indole alkaloid biosynthesis in the genus Hordeum: Distribution of gramine and DIBOA and isolation of the benzoxazinoid biosynthesis genes from *Hordeum lechleri*. Phytochem. 66, 1264-1272.

Handrick, V., Robert, C. A. M., Ahern, K. R., Zhou, S. Q., Machado, R. A. R., Maag, D., Glauser, G., Fernandez-Penny, F. E., Chandran, J. N., Rodgers-Melnik, E., Schneider, B., Buckler, E. S., Boland, W., Gershenzon, J., Jander, G., Erb, M., Köllner, T. G., 2016. Biosynthesis of 8-O-methylated benzoxazinoid defense compounds in maize. Plant Cell 28, 1682-1700.

Hanhineva, K., Rogachev, I., Aura, A. M., Aharoni, A., Poutanen, K., Mykkänen, H., 2011. Qualitative characterization of benzoxazinoid derivatives in whole grain rye and wheat by LC-MS metabolite profiling. J. Agric. Food. Chem. 59, 921-927.

Hashimoto, Y., Shudo, K., 1996. Chemistry of biologically active benzoxazinoids. Phytochem. 43, 551-559.

Hayakawa, I., Atarashi, S., Yokohama, S., Imamura, M., Sakano, K. I., Furukawa, M., 1986. Synthesis and antibacterial activities of optically-active ofloxacin. Antimicrob. Agents Chemother. 29, 163-164.

Hietala, P. K., Virtanen, A. I., 1960. Precursors of benzoxazolinone in rye plants II. Precursor I, the glucoside. Acta Chem. Scand. 14, 502-504.

Hofman, J., Hofmanová, O., 1969. 1,4-Benzoxazine derivatives in plants - Sephadex fractionation and identification of a new glucoside. Eur. J. Biochem. 8, 109-112.

Hofman, J., Masojídková, M., 1973. 1,4-Benzoxazine glucosides from *Zea mays*. Phytochem. 12, 207-208.

Hofmann, D., Knop, M., Hao, H., Hennig, L., Sicker, D., Schulz, M., 2006. Glucosides from MBOA and BOA detoxification by *Zea mays* and *Portulaca oleracea*. J. Nat. Prod. 69, 34-37.

Honkanen, E., Virtanen, A. I., 1960. The synthesis of precursor II of benzoxazolinone formed in rye plants, and the enzymic hydrolysis of precursor I, the glucoside. Acta Chem. Scand. 14, 504-507.

Huo, C. H., An, D. G., Wang, B., Zhao, Y. Y., Lin, W. H., 2005. Structure elucidation and complete NMR spectral assignments of a new benzoxazolinone glucoside from *Acanthus ilicifolius*. Magn. Reson. Chem. 43, 343-345.

Jonczyk, R., Schmidt, H., Osterrieder, A., Fiesselmann, A., Schullehner, K., Haslbeck, M., Sicker, D., Hofmann, D., Yalpani, N., Simmons, C., Frey, M., Gierl, A., 2008. Elucidation of the final reactions of DIMBOA-glucoside biosynthesis in maize: Characterization of *Bx6* and *Bx7*. Plant Physiol. 146, 1053-1063.

Jørgensen, K., Rasmussen, A. V., Morant, M., Nielsen, A. H., Bjarnholt, N., Zagrobelny, M., Bak, S., Møller, B. L., 2005. Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. Curr. Opin. Plant Biol. 8, 280-291.

Kanchanapoom, T., Kamel, M. S., Kasai, R., Picheansoonthon, C., Hiraga, Y., Yamasaki, K., 2001a. Benzoxazinoid glucosides from *Acanthus ilicifolius*. Phytochem. 58, 637-640.

Kanchanapoom, T., Kasai, R., Picheansoonthon, C., Yamasaki, K., 2001b. Megastigmane, aliphatic alcohol and benzoxazinoid glycosides from *Acanthus ebracteatus*. Phytochem. 58, 811-817.

Kato-Noguchi, H., Kosemura, S., Yamamura, S., 1998. Allelopathic potential of 5-chloro-6-methoxy-2-benzoxazolinone. Phytochem. 48, 433-435.

Köhler, A., Maag, D., Veyrat, N., Glauser, G., Wolfender, J. L., Turlings, T. C. J., Erb, M., 2015. Within-plant distribution of 1,4-benzoxazin-3-ones contributes to herbivore niche differentiation in maize. Plant Cell Environ. 38, 1081-1093.

Le-Van, N., Wratten, S. J., 1984. Compound 30.4, an unusual chlorinated 1,4-benzoxazin-3-one derivative from corn (*Zea mays*). Tetrahedron Lett. 25, 145-148.

Maag, D., Dalvit, C., Thevenet, D., Köhler, A., Wouters, F. C., Vassão, D. G., Gershenzon, J., Wolfender, J. L., Turlings, T. C. J., Erb, M., Glauser, G., 2014. 3-*β*-D-Glucopyranosyl-6-methoxy-2-benzoxazolinone (MBOA-*N*-Glc) is an insect detoxification product of maize 1,4-benzoxazin-3-ones. Phytochem. 102, 97-105.

Macías, F. A., Chinchilla, N., Arroyo, E., Molinillo, J. M. G., Marín, D., Varela, R. M., 2010. Combined strategy for phytotoxicity enhancement of benzoxazinones. J. Agric. Food. Chem. 58, 2047-2053.

Macías, F. A., Marín, D., Oliveros-Bastidas, A., Castellano, D., Simonet, A. M., Molinillo, J. M. G., 2005. Structure-activity relationships (SAR) studies of benzoxazinones, their degradation products and analogues. Phytotoxicity on standard target species (STS). J. Agric. Food. Chem. 53, 538-548.

Macías, F. A., Marín, D., Oliveros-Bastidas, A., Castellano, D., Simonet, A. M., Molinillo, J. M. G., 2006a. Structure-activity relationship (SAR) studies of benzoxazinones, their degradation products, and analogues. Phytotoxicity on problematic weeds *Avena fatua* L. and *Lolium rigidum* Gaud. J. Agric. Food. Chem. 54, 1040-1048.

Macías, F. A., Marín, D., Oliveros-Bastidas, A., Molinillo, J. M. G., 2006b. Optimization of benzoxazinones as natural herbicide models by lipophilicity enhancement. J. Agric. Food. Chem. 54, 9357-9365.

Macías, F. A., Marín, D., Oliveros-Bastidas, A., Molinillo, J. M. G., 2009. Rediscovering the bioactivity and ecological role of 1,4-benzoxazinones. Nat. Prod. Rep. 26, 478-489.

Makowska, B., Bakera, B., Rakoczy-Trojanowska, M., 2015. The genetic background of benzoxazinoid biosynthesis in cereals. Acta Physiol. Plant. 37.

Malan, C., Visser, J. H., van de Venter, H. A., 1984. Screening for DIMBOA (benzoxazinone) concentration among South African inbred maize lines and sorghum cultivars. S. Afr. J. Plant Soil 1, 99-102.

Meihls, L. N., Handrick, V., Glauser, G., Barbier, H., Kaur, H., Haribal, M. M., Lipka, A. E., Gershenzon, J., Buckler, E. S., Erb, M., Köllner, T. G., Jander, G., 2013. Natural variation in maize aphid resistance is associated with 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside methyltransferase activity. Plant Cell 25, 2341-2355.

Mohamed, G. A., Ibrahim, S. R. M., Abdelkader, M. S. A., Al-Musayeib, N. M., Ghoneim, M., Ross, S. A., 2014. Zeaoxazolinone, a new antifungal agent from *Zea mays* roots. Med. Chem. Res. 23, 4627-4630.

Moraes, M. C. B., Birkett, M. A., Gordon-Weeks, R., Smart, L. E., Martin, J. L., Pye, B. J., Bromilow, R., Pickett, J. A., 2008. *cis*-Jasmone induces accumulation of defence compounds in wheat, *Triticum aestivum*. Phytochem. 69, 9-17.

Neal, A. L., Ahmad, S., Gordon-Weeks, R., Ton, J., 2012. Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. Plos One 7.

Niemeyer, H. M., 1988. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defense chemicals in the Gramineae. Phytochem. 27, 3349-3358.

Niemeyer, H. M., 2009. Hydroxamic acids derived from 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one: Key defense chemicals of cereals. J. Agric. Food. Chem. 57, 1677-1696.

Nomura, T., Ishihara, A., Imaishi, H., Endo, T. R., Ohkawa, H., Iwamura, H., 2002. Molecular characterization and chromosomal localization of cytochrome P450 genes involved in the biosynthesis of cyclic hydroxamic acids in hexaploid wheat. Mol. Genet. Genomics 267, 210-217.

Nomura, T., Ishihara, A., Imaishi, H., Ohkawa, H., Endo, T. R., Iwamura, H., 2003. Rearrangement of the genes for the biosynthesis of benzoxazinones in the evolution of Triticeae species. Planta 217, 776-782.

Oikawa, A., Ishihara, A., Hasegawa, M., Kodama, O., Iwamura, H., 2001. Induced accumulation of 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) in maize leaves. Phytochem. 56, 669-675.

Oikawa, A., Ishihara, A., Iwamura, H., 2002. Induction of HDMBOA-Glc accumulation and DIMBOA-Glc 4-*O*-methyltransferase by jasmonic acid in poaceous plants. Phytochem. 61, 331-337.

Oikawa, A., Ishihara, A., Tanaka, C., Mori, N., Tsuda, M., Iwamura, H., 2004. Accumulation of HDMBOA-Glc is induced by biotic stresses prior to the release of MBOA in maize leaves. Phytochem. 65, 2995-3001.

Özden, S., Özden, T., Attila, I., Küçükislamoglu, M., Okatan, A., 1992. Isolation and identification via high-performance liquid chromatography and thin-layer chromatography of benzoxazolinone precursors from *Consolida orientalis* flowers. J. Chromatogr. 609, 402-406.

Özden, S., Özturk, A. M., Göker, H., Altanlar, N., 2000. Synthesis and antimicrobial activity of some new 4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-ones. Farm. 55, 715-718.

Pedersen, H. A., Heinrichson, K., Fomsgaard, I. S., 2017. Alterations of the benzoxazinoid profiles of uninjured maize seedlings during freezing, storage, and lyophilization. J. Agric. Food. Chem. 65, 4103-4110.

Pihlava, J. M., Kurtelius, T., 2016. Determination of benzoxazinoids in wheat and rye beers by HPLC-DAD and UPLC-QTOF MS. Food Chem. 204, 400-408.

Pratt, K., Kumar, P., Chilton, W. S., 1995. Cyclic hydroxamic acids in dicotyledonous plants. Biochem. Syst. Ecol. 23, 781-785.

Rakoczy-Trojanowska, M., Orczyk, W., Krajewski, P., Bocianowski, J., Stochmal, A., Kowalczyk, M., 2017. *ScBx* gene based association analysis of hydroxamate content in rye (*Secale cereale* L.). J. Appl. Genet. 58, 1-9.

Schullehner, K., Dick, R., Vitzthum, F., Schwab, W., Brandt, W., Frey, M., Gierl, A., 2008. Benzoxazinoid biosynthesis in dicot plants. Phytochem. 69, 2668-2677.

Schulz, M., Filary, B., Kühn, S., Colby, T., Harzen, A., Schmidt, J., Sicker, D., Hennig, L., Hofmann, D., Disko, U., Anders, N., 2016. Benzoxazolinone detoxification by N-glucosylation: The multi-compartment-network of *Zea mays* L. Plant Signal. Behav. 11.

Schulz, M., Marocco, A., Tabaglio, V., Macías, F. A., Molinillo, J. M. G., 2013. Benzoxazinoids in rye allelopathy - from discovery to application in sustainable weed control and organic farming. J. Chem. Ecol. 39, 154-174.

Schulz, M., Sicker, D., Schackow, O., Hennig, L., Hofmann, D., Disko, U., Ventura, M., Basyuk, K., 2017. 6-Hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one - A degradable derivative of natural 6-hydroxybenzoxazolin-2(3*H*)-one produced by *Pantoea ananatis*. Commun. Integr. Biol. 10, e1302633.

Schulz, M., Wieland, I., 1999. Variation in metabolism of BOA among species in various field communities - biochemical evidence for co-evolutionary processes in plant communities? Chemoecol. 9, 133-141.

Sicker, D., Frey, M., Schulz, M., Gierl, A., 2000. Role of natural benzoxazinones in the survival strategy of plants. Int. Rev. Cytol. - Surv. Cell Biol. 198, 319-346.

Sicker, D., Schneider, B., Hennig, L., Knop, M., Schulz, M., 2001. Glycoside carbamates from benzoxazolin-2(3*H*)-one detoxification in extracts and exudates of corn roots. Phytochem. 58, 819-825.

Śmist, M., Kwiecień, H., Krawczyk, M., 2016. Synthesis and antifungal activity of 2*H*-1,4-benzoxazin-3(4*H*)-one derivatives. J. Environ. Sci. Health Part B - Pestic. Food Contam. Agric. Wastes 51, 393-401.

Søltoft, M., Jørgensen, L. N., Svensmark, B., Fomsgaard, I. S., 2008. Benzoxazinoid concentrations show correlation with Fusarium Head Blight resistance in Danish wheat varieties. Biochem. Syst. Ecol. 36, 245-259.

Tanwir, F., Dionisio, G., Adhikari, K. B., Fomsgaard, I. S., Gregersen, P. L., 2017. Biosynthesis and chemical transformation of benzoxazinoids in rye during seed germination and the identification of a rye *Bx6*-like gene. Phytochem. 140, 95-107.

Tanwir, F., Fredholm, M., Gregersen, P. L., Fomsgaard, I. S., 2013. Comparison of the levels of bioactive benzoxazinoids in different wheat and rye fractions and the transformation of these compounds in homemade foods. Food Chem. 141, 444-450.

Tipton, C. L., Buell, E. L., 1970. Ferric iron complexes of hydroxamic acids from maize. Phytochem. 9, 1215-1217.

Villagrasa, M., Guillamón, M., Labandeira, A., Taberner, A., Eljarrat, E., Barceló, D., 2006. Benzoxazinoid allelochemicals in wheat: Distribution among foliage, roots, and seeds. J. Agric. Food. Chem. 54, 1009-1015.

Virtanen, A. I., Hietala, P. K., 1955. 2(3)-Benzoxazolinone, an anti-Fusarium factor in rye seedlings. Acta Chem. Scand. 9, 1543-1544.

von Rad, U., Hüttl, R., Lottspeich, F., Gierl, A., Frey, M., 2001. Two glucosyltransferases are involved in detoxification of benzoxazinoids in maize. Plant J. 28, 633-642.

Walker, V., Couillerot, O., Von Felten, A., Bellvert, F., Jansa, J., Maurhofer, M., Bally, R., Moenne-Loccoz, Y., Comte, G., 2012. Variation of secondary metabolite levels in maize seedling roots induced by inoculation with *Azospirillum*, *Pseudomonas* and *glomus* consortium under field conditions. Plant Soil 356, 151-163.

Whitney, N. J., Mortimore, C. G., 1961. Effect of 6-methoxybenzoxazolinone on growth of *Xanthomonas stewartii* (Erw. Smith) Dowson and its presence in sweet corn (*Zea mays* var. *Saccharata* bailey). Nature 189, 596-597.

Wieland, I., Kluge, M., Schneider, B., Schmidt, J., Sicker, D., Schulz, M., 1998. 3-*β*-d-glucopyranosyl-benzoxazolin-2(3*H*)-one - a detoxification product of benzoxazolin-2(3*H*)-one in oat roots. Phytochem. 49, 719-722.

Woodward, M. D., Corcuera, L. J., Schnoes, H. K., Helgeson, J. P., Upper, C. D., 1979. Identification of 1,4-benzoxazin-3-ones in maize extracts by gas-liquid chromatography and mass spectrometry. Plant Physiol. 63, 9-13.

Wouters, F. C., Gershenzon, J., Vassão, D. G., 2016. Benzoxazinoids: Reactivity and modes of action of a versatile class of plant chemical defenses. J. Braz. Chem. Soc. 27, 1379-1397.

Wouters, F. C., Reichelt, M., Glauser, G., Bauer, E., Erb, M., Gershenzon, J., Vassão, D. G., 2014. Reglucosylation of the benzoxazinoid DIMBOA with inversion of stereochemical configuration is a detoxification strategy in lepidopteran herbivores. Angew. Chem. - Int. Ed. 53, 11320-11324.

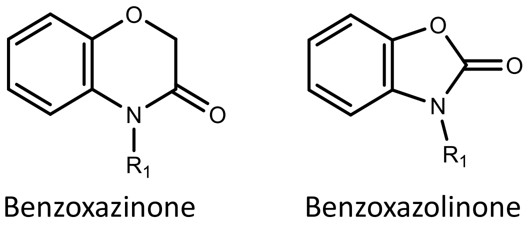
Wu, W. H., Chen, T. Y., Lu, R. W., Chen, S., Chang, C. C., 2012. Benzoxazinoids from *Scoparia dulcis* (sweet broomweed) with antiproliferative activity against the DU-145 human prostate cancer cell line. Phytochem. 83, 110-115.

Yalcin, I., Tekiner, B. P., Oren, I. Y., Arpaci, O. T., Aki-Sener, E., Altanlar, N., 2003. Synthesis and antimicrobial activity of some novel 2,6,7-trisubstituted-2*H*-3,4-dihydro-1,4-benzoxazin-3-one derivatives. Indian J. Chem. Sect. B - Org. Chem. Incl. Med. Chem. 42, 905-909.

Zasada, I. A., Rice, C. P., Meyer, S. L. F., 2007. Improving the use of rye (*Secale cereale*) for nematode management: Potential to select cultivars based on *Meloidogyne incognita* host status and benzoxazinoid content. Nematology 9, 53-60.

Zhao, D., Xie, L. J., Yu, L., An, N., Na, W., Chen, F., Li, Y. B., Tan, Y. F., Zhang, X. P., 2015. New 2-benzoxazolinone derivatives with cytotoxic activities from the roots of *Acanthus ilicifolius*. Chem. Pharm. Bull. 63, 1087-1090.

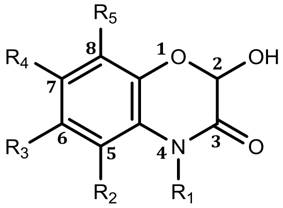
Figures and tables

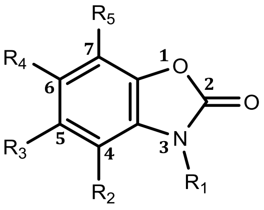


Benzoxazinone Benzoxazolinone

Figure 1. The backbones of the two classes of benzoxazinoids: benzoxazinones (1,4-benzoxazin-3-one) and benzoxazolinones (1,3-benzoxazol-2-one). R_1 substituent determines subclass, see **Table 1**.

Table 1. Overview of monomeric natural benzoxazinoids of the classes benzoxazinones (1,4-benzoxazin-3-one backbone with *C*2 hydroxylation) and benzoxazolinones (1,3-benzoxazol-2-one backbone) reported in literature, with their systematic name and abbreviation. All benzoxazinones are displayed as aglycones. Note that some of these compounds have, so far, only been detected as their 2-*O*-glycosides. n.a., no abbreviation assigned in literature.

Benzoxazinones	**R$_2$**	**R$_3$**	**R$_4$**	**R$_5$**	**Systematic name**	**Abbreviation [a]**	**Natural source [b]**	**Ref.**
N-hydro ***(R$_1$ = H): lactams***								
	H	H	H	H	2-hydroxy-1,4-benzoxazin-3-one	HBOA	Poaceae	(Hofman and Hofmanová, 1969; Tanwir et al., 2013)
							Acanthaceae	(Baumeler et al., 2000; Pratt et al., 1995)
							Calceolariaceae	(Bravo et al., 2005)
							Plantaginaceae	(Pratt et al., 1995)
							Lamiaceae	(Alipieva et al., 2003)
	H	H	OH	H	2,7-dihydroxy-1,4-benzoxazin-3-one	DHBOA	Poaceae	(de Bruijn et al., 2016; Woodward et al., 1979)
							Acanthaceae	(Bravo et al., 2004)
							Calceolariaceae	(Bravo et al., 2005)
							Lamiaceae	(Alipieva et al., 2003)
	H	H	OCH_3	H	2-hydroxy-7-methoxy-1,4-benzoxazin-3-one	HMBOA	Poaceae	(Cambier et al., 1999; Tanwir et al., 2013)
							Acanthaceae	(Baumeler et al., 2000; Pratt et al., 1995)
							Plantaginaceae	(Pratt et al., 1995)
	H	H	OCH_3	OCH_3	2-hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one	HM$_2$BOA	Poaceae	(Cambier et al., 1999; Hofman and Masojídková, 1973)
	Cl	H	OCH_3	H	5-chloro-2-hydroxy-7-methoxy-1,4-benzoxazin-3-one	Cl-HMBOA	Poaceae	(Le-Van and Wratten, 1984)
	H	H	Cl	H	7-chloro-2-hydroxy-1,4-benzoxazin-3-one	n.a.	Acanthaceae	(Kanchanapoom et al., 2001a)
	OH	H	H	H	2,5-dihydroxy-1,4-benzoxazin-3-one	n.a.	Acanthaceae	(Kanchanapoom et al., 2001a)
	H	OH	H	H	2,6-dihydroxy-1,4-benzoxazin-3-one	n.a.	Lamiaceae	(Alipieva et al., 2003)
N-hydroxy ***(R$_1$ = OH)****:* ***hydroxamic acids***								
	H	H	H	H	2,4-dihydroxy-1,4-benzoxazin-3-one	DIBOA	Poaceae	(Cambier et al., 1999; Tanwir et al., 2013)
							Acanthaceae	(Baumeler et al., 2000; Bravo et al., 2004)
							Ranunculaceae	(Özden et al., 1992)
							Calceolariaceae	(Bravo et al., 2005)
							Plantaginaceae	(Pratt et al., 1995)
							Lamiaceae	(Alipieva et al., 2003)
	H	H	OH	H	2,4,7-trihydroxy-1,4-benzoxazin-3-one	TRIBOA	Poaceae	(Woodward et al., 1979)
							Acanthaceae	(Pratt et al., 1995)
	H	H	OCH_3	H	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one	DIMBOA	Poaceae	(Cambier et al., 1999; Tanwir et al., 2013)
							Acanthaceae	(Baumeler et al., 2000; Pratt et al., 1995)
							Plantaginaceae	(Pratt et al., 1995)
	H	H	OH	OCH_3	2,4,7-trihydroxy-8-methoxy-1,4-benzoxazin-3-one	TRIMBOA	Poaceae	(Handrick et al., 2016)
	H	H	OCH_3	OCH_3	2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one	DIM$_2$BOA	Poaceae	(Cambier et al., 1999; Niemeyer, 2009)
	H	H	Cl	H	7-chloro-2,4-dihydroxy-1,4-benzoxazin-3-one	7-Cl-DIBOA	Acanthaceae	(Kanchanapoom et al., 2001b)
N-methoxy ***(R$_1$= OCH$_3$): methyl derivatives***								
	H	H	H	H	2-hydroxy-4-methoxy-1,4-benzoxazin-3-one	4-*O*-Me-DIBOA	Poaceae	(de Bruijn et al., 2016; Oikawa et al., 2002)
	H	H	OCH_3	H	2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one	HDMBOA	Poaceae	(Cambier et al., 1999; Tanwir et al., 2013)
	H	H	OCH_3	OCH_3	2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one	HDM$_2$BOA	Poaceae	(Handrick et al., 2016; Köhler et al., 2015)

Benzoxazolinones	**R_2**	**R_3**	**R_4**	**R_5**	**Systematic name**	**Abbreviation [a]**	**Natural source [b]**	**Ref.**
N-hydro ***(R_1 = H)***								
	H	H	H	H	1,3-benzoxazol-2-one	BOA	Poaceae Acanthaceae Ranunculaceae Calceolariaceae Plantaginaceae	(Cambier et al., 1999; Hanhineva et al., 2011) (Bravo et al., 2004; Pratt et al., 1995) (Özden et al., 1992) (Bravo et al., 2005) (Pratt et al., 1995)
	H	H	OH	H	6-hydroxy-1,3-benzoxazol-2-one	BOA-6-OH	PDP [c]	(Schulz and Wieland, 1999; Wieland et al., 1998)
	H	OH	H	H	5-hydroxy-1,3-benzoxazol-2-one	BOA-5-OH	PDP	(Hofmann et al., 2006)
	OH	H	H	H	4-hydroxy-1,3-benzoxazol-2-one	n.a.	Acanthaceae	(Huo et al., 2005; Zhao et al., 2015)
	H	NO_2	OH	H	5-nitro-6-hydroxy-1,3-benzoxazol-2-one	Nitro-BOA-6-OH	MDP [c]	(Schulz et al., 2017)
	H	H	OCH_3	H	6-methoxy-1,3-benzoxazol-2-one	MBOA	Poaceae Acanthaceae Plantaginaceae	(Cambier et al., 1999; Niemeyer, 2009) (Pratt et al., 1995) (Pratt et al., 1995)
	H	H	OCH_3	OCH_3	6,7-dimethoxy-1,3-benzoxazol-2-one	M_2BOA	Poaceae	(Anai et al., 1996; Cambier et al., 1999)
	H	Cl	OCH_3	H	5-chloro-6-methoxy-1,3-benzoxazol-2-one	Cl-MBOA	Poaceae	(Kato-Noguchi et al., 1998)
	Cl	H	OCH_3	OCH_3	4-chloro-6,7-dimethoxy-1,3-benzoxazol-2-one	Cl-M_2BOA	Poaceae	(Anai et al., 1996)
	H	H	OGlc	H	6-β-D-glucopyranosyloxy-1,3-benzoxazol-2-one	BOA-6-*O*-Glc	PDP	(Hofmann et al., 2006; Wieland et al., 1998)
	H	OGlc	H	H	5-β-D-glucopyranosyloxy-1,3-benzoxazol-2-one	BOA-5-*O*-Glc	PDP	(Hofmann et al., 2006)
	$COCH_3$	H	H	H	4-acetyl-1,3-benzoxazol-2-one	4-ABOA	Poaceae	(Fielder et al., 1994)
N-hydroxy ***(R_1 = OH)***								
	H	H	OCH_3	H	3-hydroxy-6-methoxy-1,3-benzoxazol-2-one	3-OH-MBOA	Plantaginaceae	(Wu et al., 2012)
N-methoxy ***(R_1 = OCH_3)***								
	H	H	OCH_3	H	3,6-dimethoxy-1,3-benzoxazol-2-one	DMBOA	Plantaginaceae	(Wu et al., 2012)
N-glucosyl ***(R_1 = Glc)***								
	H	H	OCH_3	H	3-β-D-glucopyranosyl-6-methoxy-1,3-benzoxazol-2-one	MBOA-*N*-Glc	IDP [c] PDP	(Maag et al., 2014) (Hofmann et al., 2006; Wieland et al., 1998)

[a] Most commonly used abbreviation reported in literature, for some compounds multiple different abbreviations are in use.

[b] Natural source (plant family or detoxification product) in which the benzoxazinoid has been detected. Note that genera Scoparia and Calceolaria, formerly members of Scrophulariaceae, have been moved to Plantaginaceae and Calceolariaceae, respectively.

[c] PDP, plant detoxification product; IDP, insect detoxification product; MDP, microbial detoxification product.

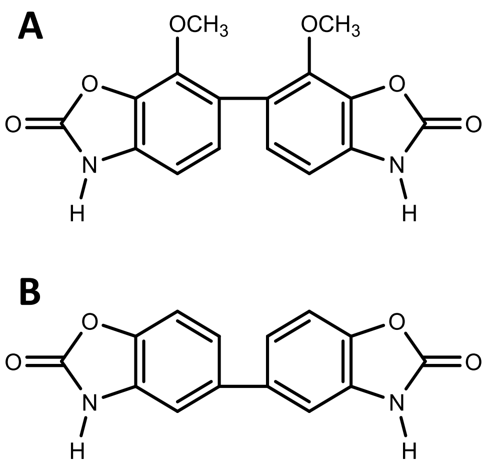


Figure 2. Structures of zeaoxazolinone (**A**), a 7-methoxy-1,3-benzoxazol-2-one dimer from *Zea mays* (Mohamed et al., 2014), and 5,5′-bis-benzoxazoline-2,2′-dione (**B**), a 1,3-benzoxazol-2-one dimer from *Acanthus ilicifolius* (D'Souza et al., 1997).

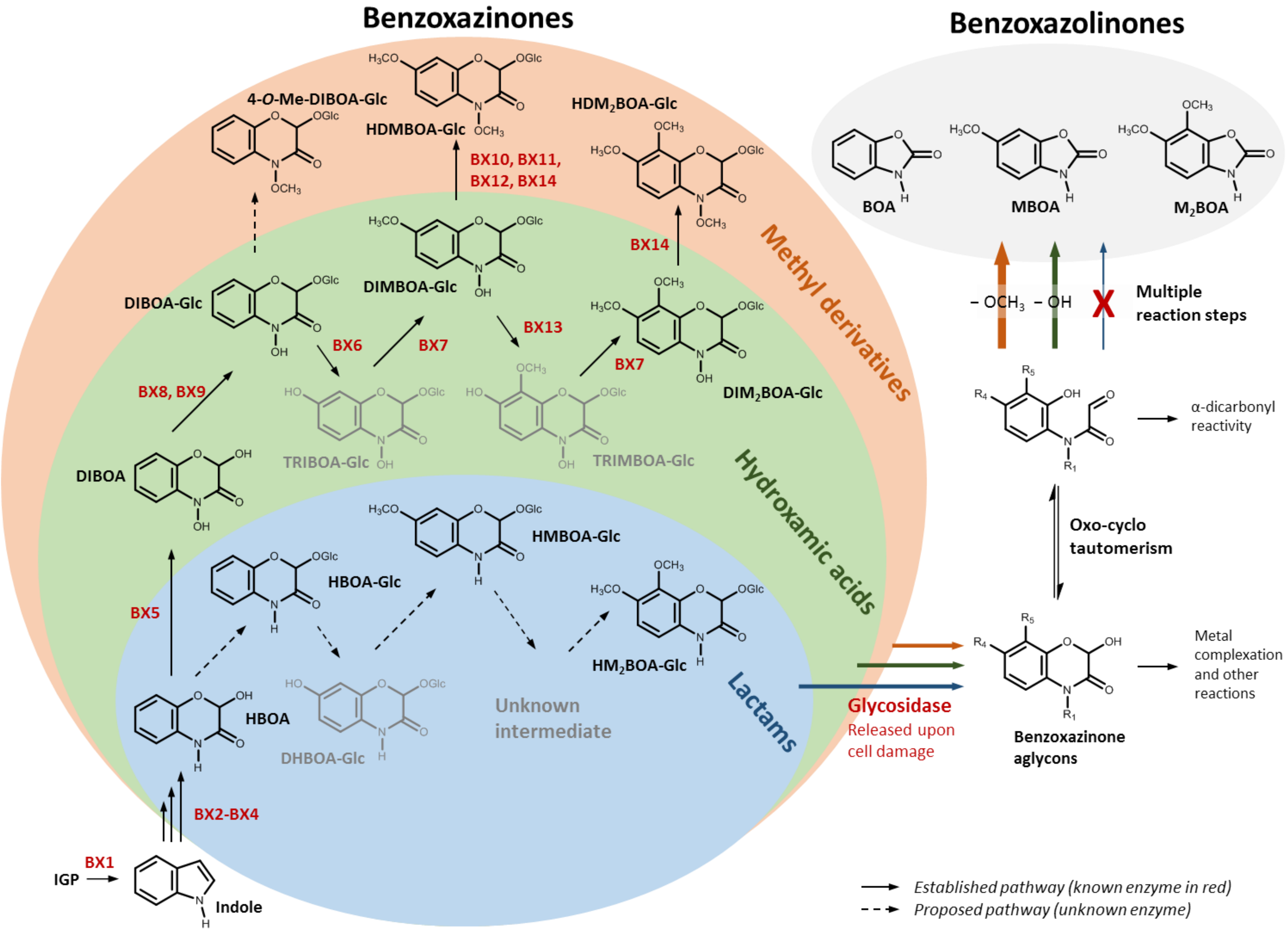

Benzoxazinones
Benzoxazolinones
4-O-Me-DIBOA-Glc
HDMBOA-Glc
HDM$_2$BOA-Glc
BX10, BX11, BX12, BX14
BX14
DIBOA-Glc
DIMBOA-Glc
BX6
BX7
BX13
BX7
DIM$_2$BOA-Glc
BX8, BX9
TRIBOA-Glc
TRIMBOA-Glc
DIBOA
BX5
HBOA-Glc
HMBOA-Glc
HM$_2$BOA-Glc
HBOA
DHBOA-Glc
Unknown intermediate
BX2-BX4
IGP
BX1
Indole
Methyl derivatives
Hydroxamic acids
Lactams
BOA
MBOA
M$_2$BOA
– OCH$_3$
– OH
X
Multiple reaction steps
α-dicarbonyl reactivity
Oxo-cyclo tautomerism
Glycosidase
Released upon cell damage
Benzoxazinone aglycons
Metal complexation and other reactions
Established pathway (known enzyme in red)
Proposed pathway (unknown enzyme)

Figure 3. The biosynthetic pathway of the three subclasses of benzoxazinones in plants (Dutartre et al., 2012; Frey et al., 1997; Handrick et al., 2016; Jonczyk et al., 2008; Meihls et al., 2013; Niemeyer, 2009; Oikawa et al., 2002) and their transformation into benzoxazolinones (Wouters et al., 2016). Structures of biosynthetic intermediates are shown in grey. Different mechanisms have been proposed for the multi-step degradation from the ring-opened configuration of benzoxazinones to the corresponding benzoxazolinone (Wouters et al., 2016). IGP, indole-3-glycerol phosphate.

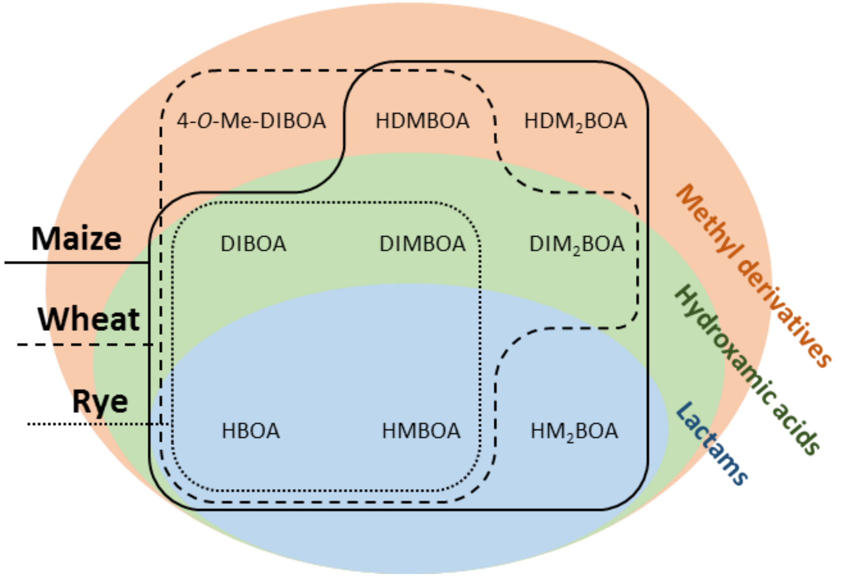


Figure 4. Distribution of individual benzoxazinones amongst the three major benzoxazinoid producing species of the family Poaceae (Cambier et al., 1999; de Bruijn et al., 2016; Le-Van and Wratten, 1984; Søltoft et al., 2008; Tanwir et al., 2013; Woodward et al., 1979). The biosynthetic intermediates DHBOA, TRIBOA, and TRIMBOA are not included.

Table 2. Antimicrobial activity of natural benzoxazinoids against the pathogens *S. aureus* (gram-positive bacterium), *E. coli* (gram-negative bacterium) and *C. albicans* (yeast, fungus). Colour of abbreviation indicates benzoxazinone subclass, green is hydroxamic acid whereas blue is lactam.

		Antimicrobial activity, MIC (μg mL^{-1})^{a}			
Abbreviation	**Class**	***S. aureus***	***E. coli***	***C. albicans***	**Ref.**
DIMBOA	Benzoxazinone	250 - 500	666	500	(Bravo and Lazo, 1993, 1996; Gleńsk et al., 2016)
DIBOA	Benzoxazinone	500	1250	666	(Bravo and Lazo, 1993, 1996)
MBOA	Benzoxazolinone	>1000	>1000	450	(Bravo et al., 1997)
BOA	Benzoxazolinone	>1000	>1000	650	(Bravo et al., 1997)
DIBOA-2-*O*-Glc	Benzoxazinone	n.a.	n.a.	>1000	(Bravo and Lazo, 1996)
DIMBOA-2-*O*-Glc	Benzoxazinone	n.a.	n.a.	>1000	(Bravo and Lazo, 1996)
HMBOA	Benzoxazinone	n.a.	n.a.	1000	(Bravo and Lazo, 1996)
HBOA	Benzoxazinone	n.a.	n.a.	>1000	(Bravo and Lazo, 1996)

n.a., no data available.

[a] Minimum inhibitory concentration, range indicates that different values were reported in literature.

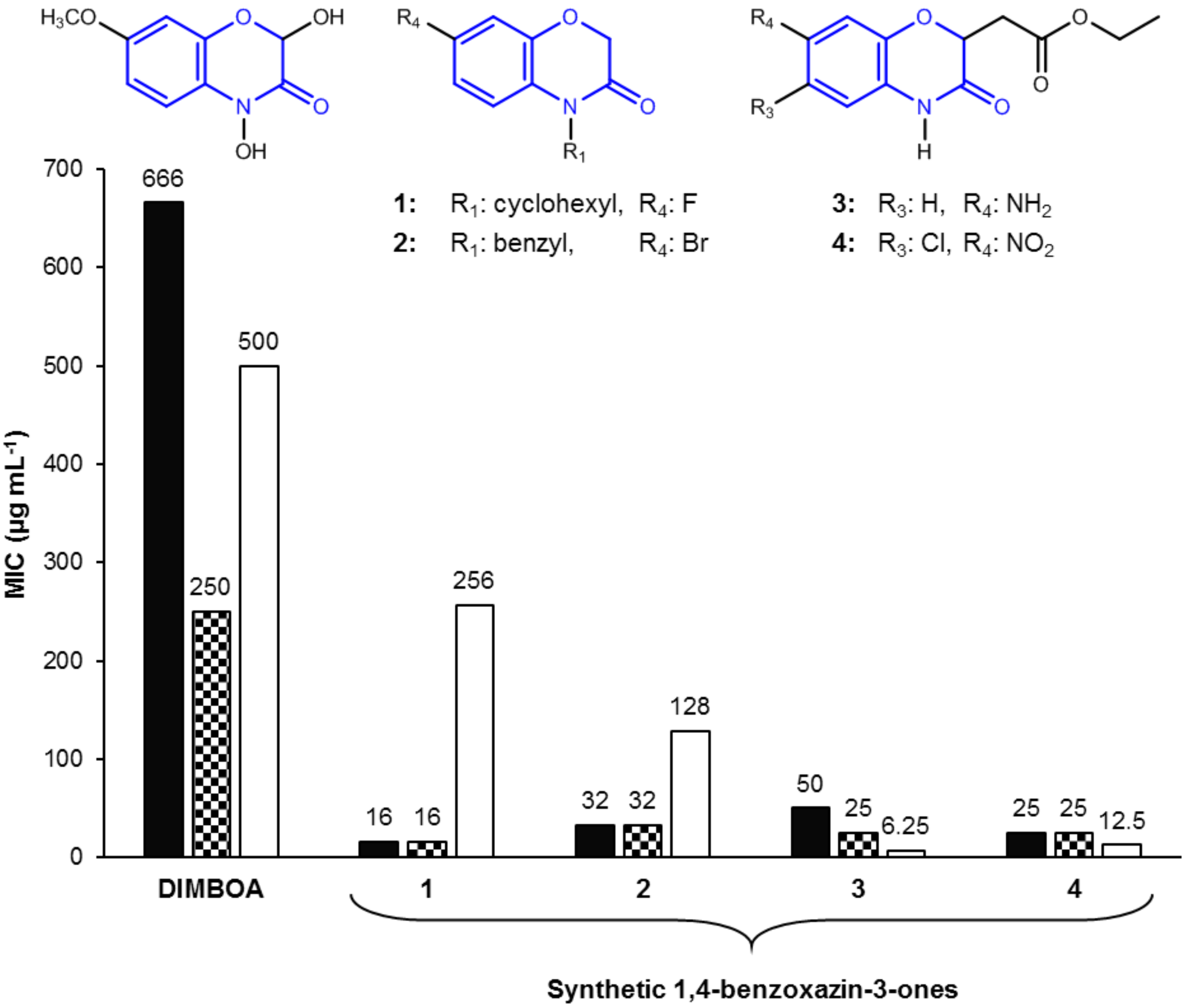


Figure 5. Antimicrobial activity of the most potent monomeric natural benzoxazinone DIMBOA (Bravo and Lazo, 1993; Gleńsk et al., 2016) compared to four synthetic benzoxazinones (Alper-Hayta et al., 2006; Fang et al., 2011). The 1,4-benzoxazin-3-one scaffold is shown in blue. Minimum inhibitory concentration (MIC) displayed in µg mL^{-1} against gram-negative bacterium *E. coli* (black), gram-positive bacterium *S. aureus* (checkered), and fungus *C. albicans* (white).