



Hordatines, dimerised hydroxycinnamoylagmatine conjugates of barley (*Hordeum vulgare* L.): an appraisal of the biosynthesis, chemistry, identification and bioactivities

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Abstract Hydroxycinnamoylamides are specialised metabolites widely distributed in the plant kingdom. These are phenolic moieties covalently linked to mono- or polyamines through amide bonds. Their oxidative coupling (dimerisation) leads to neolignanamides, a group of compounds showing high chemical, structural and functional diversity. Typical to barley, dehydro dimers of hydroxycinnamoylagmatines, hordatines are primarily found in germinated seeds and at the seedling stage. The first step in the biosynthesis of hordatines is catalysed by acyl-coenzyme A-dependent N-hydroxycinnamoyltransferases, and lead to the formation of hydroxycinnamoylagmatines (HCAGms). The oxidative homo- or heterodimerisation of the latter results in different hordatines (A, B, C or D). Hordatines can also undergo various types of conjugation and form hydroxylated, methylated or glycosylated derivatives. Although the research on the bioactivities of the hordatines is still nascent, the *in planta* antifungal properties have long been recognised. While hordatines are naturally and uniquely synthesised in barley plants, these molecules or lead compounds derived therefrom, also exhibit

medicinal and pharmaceutical uses important for human health, stimulating research into the utilisation of biotechnology in alternative production hosts and to enhance agricultural yields and value-added production. This review summarises the older and recent knowledge about hordatines and derivatives and may serve as a springboard for future research on this intriguing class of secondary plant metabolites.

Keywords Agmatine · Biomarkers · Conjugates · Dimerisation · Hordatine · Hydroxycinnamic acid · N-hydroxycinnamoyltransferase, neolignanamide

Abbreviations

4CL	4-Coumaric acid:coenzyme A ligase
ADC	Arginine decarboxylase
AHCT	Anthocyanin O-hydroxycinnamoyltransferase
ARG	Arginase
Asp	Aspartic acid
AtACT	<i>Arabidopsis thaliana</i> agmatine coumaroyltransferase
BEAT	Benzylalcohol O-acetyltransferase
C3H	4-Coumaric acid 3-hydroxylase
C4H	Cinnamic acid 4-hydrolase
COMT	Caffeic acid/5-hydroxyferulic acid O-methyltransferase

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CouAgm-4-O-7'/3-8'-DCouAgm	Hordatine A
CouOHAgm-CouAgm	Hordatine A1
CouOHAgm-CouOHAgm	Hordatine A2
DAT	Deacetylvindoline 4-O-acetyltransferase
F5H	Ferulate 5-hydroxylase
FerAgm-4-O-7'/3-8'-DCouAgm	Hordatine B
FerAgm-4-O-7'/3-8'-DFerAgm	Hordatine C
FerAgm-4-O-7'/3-8'-DSinAgm	Hordatine D
FT-ICR	Fourier transform ion cyclotron resonance
HCAAs	Hydroxycinnamic acid amides
HCAgms	Hydroxycinnamoylagmatines
HCAAs	Hydroxycinnamic acids
HCBT	Anthranilate N-hydroxycinnamoyl/benzoyltransferase
His	Histidine
HvACT	<i>Hordeum vulgare</i> Agmatine coumaroyltransferase
HvLAC1 and HvLAC2	<i>Hordeum vulgare</i> Laccase 1 and 2
Ileu	Isoleucine
MALDI-TOF-MS	Matrix-assisted laser desorption ionisation time-of-flight MS
MS	Mass spectrometry
MSI	Imaging mass spectrometry
N-HCTs	N-hydroxycinnamoyltransferases
NMR	Nuclear magnetic resonance
ODC	Ornithine decarboxylase
OHAgm	Hydroxyagmatine
PAL	Phenylalanine ammonia lyase
Ser	Serine
SLE	Solid-liquid extraction
SPDS	Spermidine synthase
SPMS	Spermine synthase
UHPLC	Ultra-high performance liquid chromatography
Val	Valine

Introduction

As multicellular eukaryotic organisms, plants produce a vast array of structurally and functionally diverse metabolites. These compounds can be synthesised for the plant's primary needs or in response to environmental factors. Central metabolism results in the biosynthesis of primary metabolites or macromolecules such as carbohydrates, lipids, proteins, nucleic acids and hormones; highly conserved in all living organisms (Ferne and Pichersky 2015; Erb and Kliebenstein 2020). Primary metabolites are vital for growth, reproduction and signal transduction, and play a crucial role in photosynthesis, transport, respiration and nutrient assimilation processes. Secondary metabolism, which stems from primary metabolism, results in the biosynthesis of a large number of specialised and structurally diverse metabolites. These are often grouped as phenolic acids, flavonoids, terpenes, nitrogen- and sulphur-containing molecules. They are required for plant survival in the environment and are associated with phenotypic characteristics (Erb and Kliebenstein 2020); hence their biosynthesis varies from plant to plant and from one external pressure to the other. Moreover, the *in vivo* production of specialised metabolites can be genus- and species-specific and involves the activation of intertwined pathways, supported by the central metabolism, and forming complex metabolic networks. Some plant-specific metabolites include the glycoalkaloid tomatin/lycopersicin in tomato (Kozukue et al. 2023) and avenanthranamides in oat (Pretorius and Dubery 2023). This review focuses on distinctive hydroxycinnamoylagmatine conjugates of barley, *Hordeum vulgare* L., the hordatines. Their biosynthetic pathway, structural characteristics and – diversity as well as mass spectral identification and bioactivities are explored to provide an in-depth knowledge on hordatines.

Hordatines, unique phytochemicals of barley

Barley is the fourth most important grain crop in the world, with numerous uses and has great importance in the animal and human food and brewing industries. The sequenced genome of barley, its diploid genetics (seven pairs of nuclear chromosomes), and the close connection among *Triticeae* tribe members, make it

easier to apply the knowledge learned from barley studies to other important cereals. Additionally, the high resistance of barley to several environmental stresses support the use of the plant as a model in crop research (Munns and Tester 2008; Kant et al. 2016; Wiegmann et al. 2019).

Barley is capable of producing phytochemicals possessing a wide range of activities such as anti-oxidant, anti-inflammatory and anti-proliferative properties. These include flavonoids, phenolics, alkaloids, lignins and terpenoids (Shewry 2014). Often categorised as phenolamides or hydroxycinnamic acid amides dimers, hordatines are benzofurans renowned for their anti-fungal properties. Among the 31 species of the *Hordeum* genus, the cultivated barley (*H. vulgare* spp. *vulgare*) and wild barley (*H. vulgare* ssp. *spontaneum*) are the only two that synthesise hordatines (Batchu et al. 2006; Ube et al. 2017). Hordatines have thus been regarded as signature metabolites in cultivated barley (Hamany Djande et al. 2022). It is worth mentioning that other species are also capable of forming dimers of hydroxycinnamic acid amides (Leonard et al. 2021). However, the linkage type is different to that observed in hordatines (Section “Diversity of hordatine structures and associated nomenclature”).

Biosynthesis of hordatines

Hydroxycinnamic acid amides or phenolamides

Hydroxycinnamic acid amides (HCAAs) or phenolamides or phenylamides are metabolites found ubiquitously in the plant kingdom and are recently attracting considerable attention (Zeiss et al. 2021). HCAAs are structurally diverse phenolic compounds and often categorised as phenolic acid conjugates or alkaloids because of a N-containing sub-structure. They arise from phenolic moieties, primarily hydroxycinnamic acids (HCAs) and derivatives, forming covalent amide bonds with either an aromatic or aliphatic amine (Fig. 1) (Bassard et al. 2010; Macoy et al. 2015a; Zeiss et al. 2021; Roumani et al. 2021, 2023). HCAs (*p*-coumaric, caffeic, ferulic, and sinapic acids) are synthesised in the early phenylpropanoid pathway from L-phenylalanine, with the help of the enzymes phenylalanine ammonia lyase (PAL; EC 4.3.1.5) and cinnamic acid 4-hydrolase

(C4H; EC 1.14.13.11) (Gray et al. 2012). To a lesser extent, L-tyrosine can also be a precursor, producing 4-coumaric acid in presence of an ammonia-lyase (Young et al. 1966; Petersen 2016). The activation of HCAAs as coenzyme A thioesters necessary for downstream events is performed in the presence of hydroxycinnamate:coenzyme A ligase or 4-coumaroylCoA ligase (4CL; EC 6.2.1.12) in a two-step reaction (Petersen 2016). Polyamines on the other hand, are low molecular weight polycations, resulting from the decarboxylation of amino acids, mainly L-arginine. The amino acid can be converted into the non-proteinogenic amino acid ornithine, which in turn is decarboxylated into putrescine by the mitochondrial enzyme arginase (ARG; EC 3.5.3.1) and ornithine decarboxylase (ODC; EC 4.1.1.17) respectively. Alternatively, L-arginine can be decarboxylated by arginine decarboxylase (ADC; EC 4.1.1.19) to produce agmatine from which several polyamines may result. Plant amines include putrescine, spermidine, spermine, (collectively known as polyamines or low molecular weight aliphatic nitrogenous bases that contain two or more amino groups), agmatine, tyramine, anthranilate, tyramine, tryptamine, etc. (Bagni and Tassoni 2001; Mehta et al. 2002; Takahashi and Kakehi 2010).

Since the discovery of the first HCAA in the late 1940s, years of phytochemical research have demonstrated a significant structural diversity in the family and a widespread distribution throughout the plant world. The physicochemical diversity of HCAAs is credited to the possible combinations of different HCA and amine moieties, as well as the potential for N-substitution on the aliphatic polyamines (Roumani et al. 2020). More than 800 HCAAs and derivatives were generated in silico with the most encountered building blocks (Li et al. 2018). Chemically, the hordatines are the products of the oxidative dimerisation of two HCAAs derived from the polyamine agmatine and the coenzyme A derivatives of HCAAs (Burhenne et al. 2003; Gorzolka et al. 2014; Piasecka et al. 2015) (Fig. 2).

Hordatines A and B were the first barley specific benzofurans to be characterised (Stoessl 1965, 1967) and their biosynthesis involves the participation of two enzymes: *H. vulgare* agmatine coumaroyltransferase (*HvACT*, E.C. 2.3.1.64) and an oxidase. *HvACT* is a CoA-dependent transacylation system responsible for the formation of hydroxycinnamoylagmatines

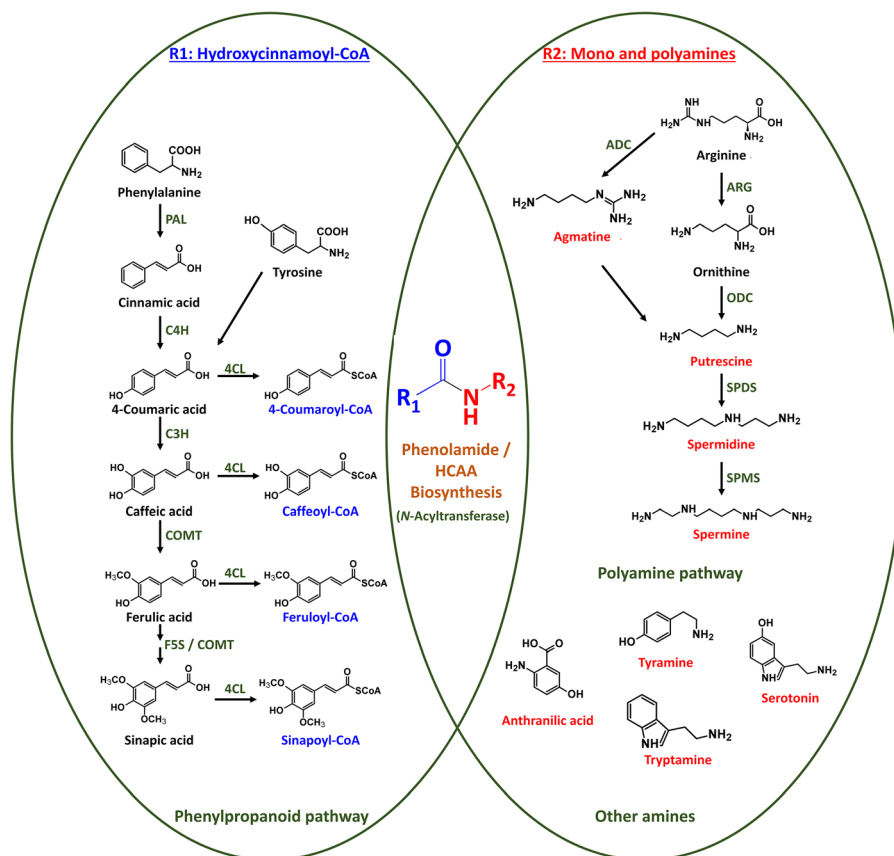


Fig. 1 Biosynthesis of phenolamides / hydroxycinnamic acid amides in plants. The formation of HCAAs requires the participation acyl-activated coenzyme A thioesters (donors of acylated moieties) and amines (acceptors) from the phenylpropanoid – and amine pathways respectively. The reactions are catalysed by plant acyl-CoA dependent *N*-acyltransferases. *PAL* phenylalanine ammonia lyase, *C4H* cinnamic acid 4-hydrolase,

4CL 4-coumaric acid:coenzyme A ligase, *C3H* 4-coumaric acid 3-hydroxylase, *F5H* coniferaldehyde/ferulate 5-hydroxylase, *COMT* caffeic acid/5-hydroxyferulic acid O-methyltransferase, *ADC* arginine decarboxylase, *ARG* arginase, *ODC* ornithine decarboxylase, *SPDS* spermidine synthase, *SPMS* spermine synthase. The double arrows indicate the implication of more than one step

(HCAgms, *e.g.* *p*-coumaroylagmatine) from the corresponding coenzyme A derivatives of the HCAAs with agmatine. The enzyme was first purified from barley (GenBank: AY228552) and its cDNA cloned (Burhenne et al. 2003). Since then, ACTs have also been identified in *Arabidopsis thaliana* (*AtACT*), resulting in *p*-coumaroylagmatine biosynthesis as the major product (Muroi et al. 2009). An oxidase, long believed to be a peroxidase, is responsible of the oxidative phenol coupling of HCAAs to produce hordatines (Fig. 2) (Burhenne et al. 2003).

N-hydroxycinnamoyltransferases, members of the BAHD acyltransferase family

Plant acyl-CoA dependent acyltransferases are members of the BAHD superfamily that catalyse reactions in which acylated moieties (RC(O)R') of acyl-activated coenzyme A thioesters are transferred to an acceptor molecule (St-Pierre and De Lucas 2000; D'auria 2006; Yuan et al. 2022). In these reactions, the phenolic acyl donor is transferred to an amine acceptor compound (Luo et al. 2009), and produce secondary metabolites which act functionally as phytoanticipins and/or phytoalexins. The term BAHD was coined from the first letter of the first four biochemically characterised group of enzymes in the

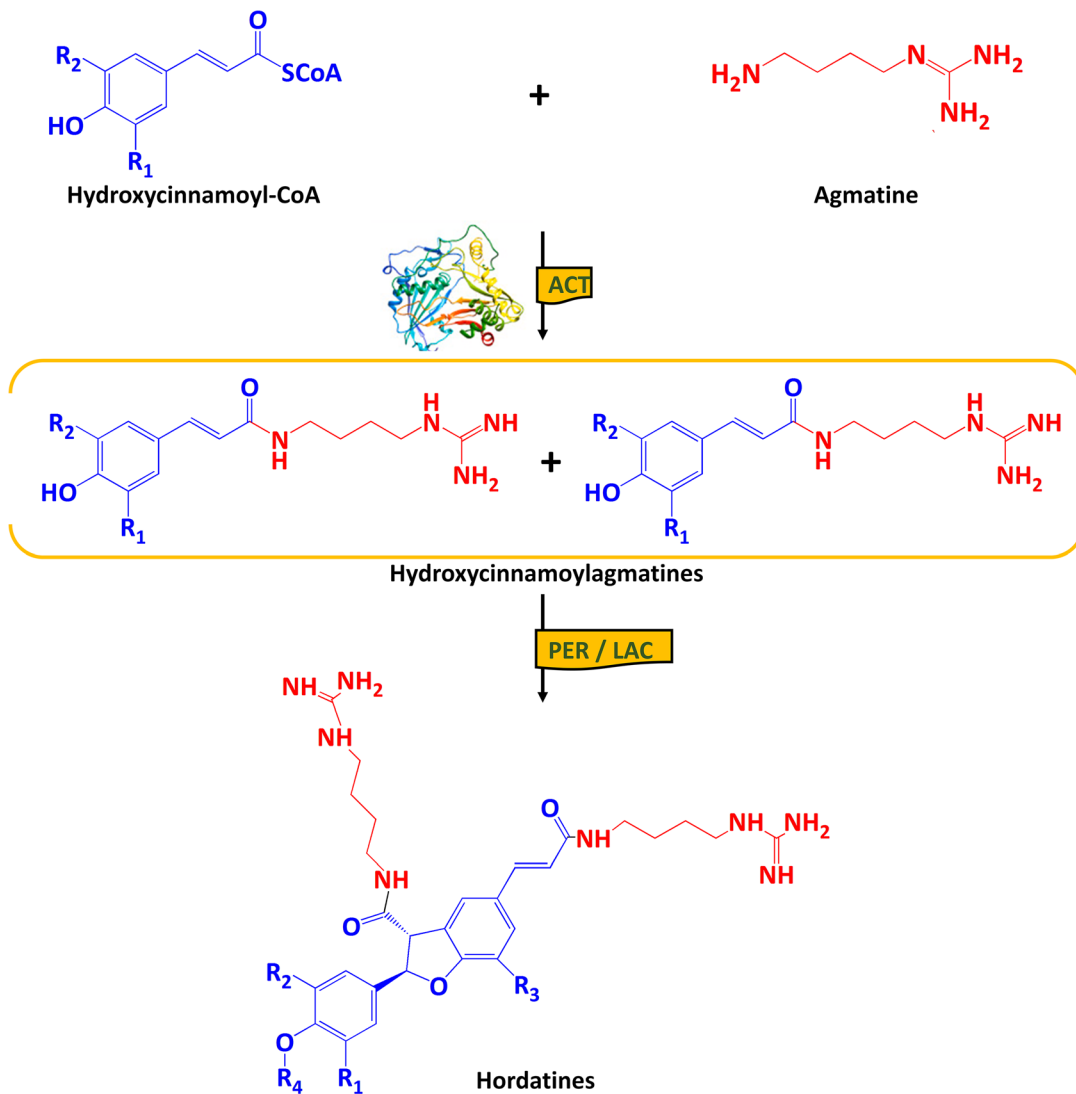


Fig. 2 Biosynthesis of hordatines in barley. Hydroxycinnamoyl-CoA and agmatine react in the first step, catalysed by agmatine coumaroyltransferase (ACT), to generate a hydroxycinnamoylagmatine (HCAgm). For *p*-coumaroylagmatine: R_1 , R_2 and $R_4 = H$; feruloylagmatine: $R_1 = OCH_3$, R_2 and $R_4 = H$; sinapoylagmatine: R_1 , $R_2 = OCH_3$ and $R_4 = H$. The oxidative

family (St-Pierre and De Lucas 2000; D’auria 2006). These were benzylalcohol O-acetyltransferase (BEAT) (Dudareva et al. 1998), anthocyanin O-hydroxycinnamoyltransferase (AHCT) (Fujiwara et al. 1998), anthranilate N-hydroxycinnamoyl/benzoyltransferase (HCBT) (Yang et al. 1997), and

coupling of two hydroxycinnamoylagmatine molecules in the presence of an oxidase or peroxidase (PER) or laccase (LAC) constitute the second stage. The diversity of the hordatine structures as a result of different R groups and associated nomenclature are described in Section “Diversity of hordatine structures and associated nomenclature”

deacetylindoline 4-O-acetyltransferase (DAT) (St-Pierre et al. 1999). In vitro, the substrate specificity of some family members varies from narrow to broad, and the products generated *in planta* are thus often dictated by the relative availability of substrates. In addition, motif enrichment analysis and molecular

dynamics simulations performed on these BAHD acyltransferases put forward the fact that the specialisation may result from variations in amino acid sequences in specific motifs instead of those uniformly distributed throughout the enzyme structure (Weng et al. 2021).

Several protein sequence alignments demonstrated that BAHD superfamily proteins share high sequence identity (up to 34%) (D'Auria 2006). The BAHD superfamily is characterised by two conserved amino acid motifs. Found in the central region of the enzyme, the first motif comprises a consecutive amino acids string that includes histidine and aspartic acid (HXXXD), where the former plays a function as a catalytic residue and the latter associated with a structural role. The catalytic activity of histidine consists of removing a proton (deprotonation) from the acyl acceptor (the nucleophile), leading to a nucleophilic attack on the carbonyl carbon of the Co-A thioester (donor) to form a tetrahedral intermediate. Sequentially, the protonation of the intermediate results in the formation of an acylated amide and free CoA (Ma et al. 2005; D'Auria 2006; Kong et al. 2020). The second conserved motif DFGWG (Asp, Phe, Gly, Try, Gly), is located next to the C-terminal end, away from the active site of the enzyme (D'Auria et al. 2007; Morales-Quintana et al. 2015). Unlike the first motif, DFGWG is not involved in the catalytic mechanism; instead, it is assumed to play a structural role such as involvement in CoA binding (El-Sharkawy et al. 2005; Molina and Kosma 2015; Petersen 2016). The conserved motifs in the BAHD superfamily allowed the discovery of several acyltransferase genes in different plants notably *A. thaliana* and *Oryza sativa* (D'Auria et al. 2007; Yu et al. 2009). In a recent study conducted by Yuan et al. (2022), 116 putative *Hv*BAHD acyltransferase genes were found in the barley genome with 113 mapped across seven chromosome groups.

Regarding the biosynthesis of hordatines from barley, *HvACT* is one of the phylogenetically unique enzymes among the BAHD acyltransferases, responsible of the biosynthesis of *p*-coumaroylagmatine and feruloylagmatine. Building on the classification scheme of D'Auria (2006), Kruse et al. (2022) grouped BAHD acyltransferases into 8 (from 0–7) clades based on structural similarities between acceptor substrates. N-Hydroxycinnamoyltransferases (N-HCTs) are found in all the clades, except in clades 2 and 7. The

acceptors used by clade 4 N-HCTs are agmatine, putrescine, tryptamine, tyramine, and serotonin. Among the acyltransferase in barley, *HvACT* was the first amine N-HCT characterised and was placed in clade 4 (Yuan et al. 2022; Moghe et al. 2023). In that clade, only enzymes from the Gramineae/Poaceae family (monocot flowering plants / grasses) have been characterised thus far.

In a recent study by Yamane et al. (2020), *HvACT* was crystallised for the first time, and the apo form structure was elucidated. The overall folding of *HvACT* was comparable to that of other known members of the BAHD superfamily (Walker et al. 2013; Levsh et al. 2016; Yamane et al. 2020). The enzyme contains 13 α -helices and 18 β -strands, and similarly to other enzymes in the BAHD superfamily, the structure can be divided in two domains (I and II). Both domains are connected by lengthy crossover, interacting with α 11 and β 14 in domain II. β 16 takes part in the core β -sheet of domain I which is stringed over by the loop between β 15 and β 16. According to the class, architecture, topology and homologous superfamily (CATH) classification, these domains adopt a two-layer $\alpha\beta$ -sandwich architecture. In barley, one of the two highly conserved motifs (described above) corresponds to ¹⁵²HIVSD¹⁵⁶ (His, Ileu, Val, Ser, Asp), with ¹⁵²His located on the solvent channel surface. The other conserved region ³⁸⁵DFGWG³⁹⁰ is located between β 15 and β 16 (Burhenne et al. 2003; Yamane et al. 2020).

Dimerisation of hydroxycinnamoylagmatine conjugates and isomerisation of hordatines

The dimerisation of secondary metabolites *in planta* occurs as an effective biochemical mechanism, to increase the diversity of compounds through the creation of new carbon skeletons, and potentially resulting in stronger biological activities (Pretorius and Dubery 2023). Considering the precursors and the type of linkage occurring, a wide variety of phytochemical dimers can hypothetically be formed; this is without taking into consideration stereoisomers and larger oligomers. These dimeric products of HCAAs also often carry names deriving from the plant in which they were first identified (van Zadelhoff et al. 2021). Accordingly, hordatines are neolignanamides found in *H. vulgare*. As part of the reaction leading to the biosynthesis of hordatines, a peroxidase enzyme

was suggested to catalyse the oxidative phenol coupling or dimerisation of agmatine conjugates, *e.g.* by linking coumaroylagmatine and feruloylagmatine. The implication of the peroxidase-encoding barley gene *Prc7* in the biosynthesis of hordatines was highlighted by their synchronised upregulation following a powdery mildew (*Blumeria* [syn. *Erysiphe*] *graminis* f.sp. *hordei*) infection (Kristensen et al. 1999). Unfortunately, little is known about the dimerisation step and the characteristics of the peroxidase involved, except that a radical coupling reaction in presence of the enzyme led to the formation of hordatines *in vitro* and that the catalysis involves an oxidative phenol coupling mechanism (Stoessl 1966, 1967; Burhenne et al. 2003; Ube et al. 2017; van Zadelhoff et al. 2022). In the *in vitro* reactions using peroxidase in presence of H₂O₂, not only hordatines were produced, but also compounds with different linkage types (van Zadelhoff et al. 2022). Unlike hordatines synthesised *in vivo*, the laboratory-produced compounds were optically inactive. Hence, in recent studies, Ube et al. (2023) pointed out to another oxidase as the catalyst of the oxidative coupling of *p*-coumaroylagmatine. The proposed enzyme was laccase, encoded by *HvLAC1* and *HvLAC2* genes. Contrary to peroxidase, *HvLAC1* and *HvLAC2* were shown to catalyse the stereospecific formation of the enantiopure hordatine A, and may not require a dirigent protein (Ube et al. 2023). Laccases are multicopper glycoprotein oxidases implicated in the biosynthesis of phenolic compound dimers such as the polymerisation of HCA derivatives (Li et al. 2020). Their participation in the oxidative coupling of *p*-coumaroylagmatine for the formation of hordatines is therefore not unexpected. These *HvLAC1* and *HvLAC2* were identified in the apoplast and the vacuole (Ube et al. 2023). It was then suggested that the precursor *p*-coumaroylagmatine, synthesised in the cytosol by *HvACT* enzymes (Yuan et al. 2022), would be transported to the vacuole and the extracellular space (apoplast) where the formation of hordatines will occur (Burhenne et al. 2003; Ube et al. 2023). So far, the peroxidase route remains the most investigated one regarding the dimerisation of two HCAgms, but the revelation of laccases as oxidative enzymes in the biosynthesis of hordatines opens new fields of study.

Diversity of hordatine structures and associated nomenclature

In a recent study by van Zadelhoff et al. (2022), the oxidative coupling of HCAgms by horseradish peroxidase was investigated and different linkage types, not only found in barley but also in related *Hordeum* species, or other (neo)lignanamides were revealed. Five linkage types were reported to lead to HCAgm dimers: 4-O-7'/3-8', 2-7'/8-8', 8-8'/9-N-7', 8-8' and 4-O-8'; three of which were previously reported (Stoessl 1967; Ube et al. 2017). These linkage types descriptors were based on the systematic nomenclature for (neo)lignanamides proposed by van Zadelhoff et al. in (2021) and the only linkage type detected in barley was 4-O-7'/3-8' (Ube et al. 2017; van Zadelhoff et al. 2021, 2022). The position of the linkage has previously been reported although the numbering system was different (Kageyama et al. 2011; Pihlava 2014; Hamany Djande et al. 2022). In Kageyama et al. (2011), the corresponding linkage was 3-3a/2-O-7' (Fig. 3A).

As explained earlier, HCAs are important constituents that contributes to the respective hordatine structures. When exposed to UV radiation, cinnamic acids have been known to undergo *trans* (*E*) to *cis* (*Z*) isomerisation due to the presence of a double bond (Salum et al. 2013). A case in hand is transgenic *Torenia hybrida* plants transformed with agmatine coumaroyltransferase from Arabidopsis, which accumulated substantial amounts of *p*-coumaroylagmatine, that isomerised from the *trans*-form to the *cis*-form *in planta* (Muroi et al. 2012). A similar photo-isomerisation occurs in barley, leading to the formation of isomeric pairs of the corresponding hordatines (van Zadelhoff et al. 2022). Several authors previously proposed (Stoessl 1966; Yamaji et al. 2007; Kageyama et al. 2011) that the *cis* and *trans* isomerisation of hordatines occur at the two chiral centres on carbons 2 and 3, or positions 7' and 8' of the dihydrobenzofuran residue found in their core structure (Kageyama et al. 2011). Similarly to HCAs, an isomerisation site was also reported at positions 1'' and 2'' (Kageyama et al. 2011, 2012; Hamany Djande et al. 2022) or positions 7 and 8 (van Zadelhoff et al. 2022 nomenclature) (Fig. 3A and B) and the chemical structures of the *cis* isomers of the 1''–2'' double bond of the cinnamic acid moiety of hordatines A and B were elucidated by mass spectrometry (MS) and

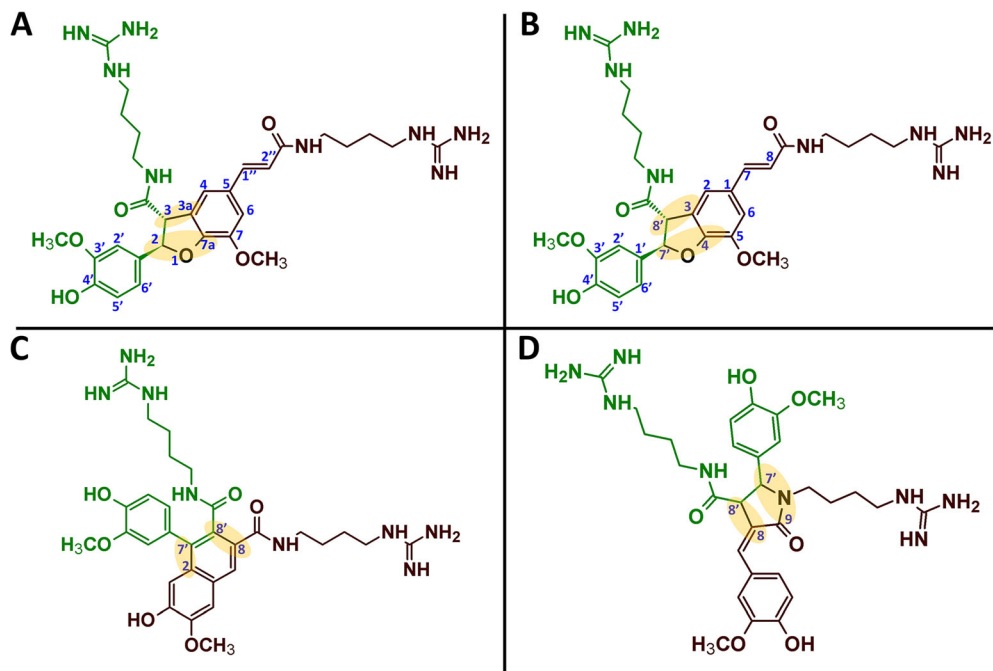


Fig. 3 Linkage bond types and nomenclature of homodimers of feruloylagmatines. (A) and (B): Hordatine C following the nomenclatures of Kageyama et al. (2011) and van Zadelhoff et al. (2022) respectively; (C) and (D): Murinamides A and B

respectively, following van Zadelhoff et al. (2022). The linkage bond type is highlighted in yellow

nuclear magnetic resonance (NMR) spectroscopy, and reported as minor components in barley malt (Kageyama et al. 2012).

Following van Zadelhoff et al. (2021) nomenclature which is more descriptive, hordatine A and B are CouAgm-4-O-7'/3-8'-DCouAgm and FerAgm-4-O-7'/3-8'-DCouAgm respectively, showing the precursors involved and the linkage type. The former results in the dimerisation of two molecules of *p*-coumaroylagmatine, and the latter in a heterodimer of feruloylagmatine and *p*-coumaroylagmatine (Gorzolka et al. 2014, 2016; Pihlava et al. 2016). Hordatine C, consisting of two feruloylagmatines (FerAgm-4-O-7'/3-8'-DFerAgm) and hordatine D (FerAgm-4-O-7'/3-8'-DSinAgm), a dimer of feruloylagmatine and sinapoylagmatine, were also reported by Gorzolka et al. (2014) and Hamany Djande et al. (2022). Up to now, caffeoylagmatine has not been reported as a component of a hordatine, and while homodimers of *p*-coumaroylagmatine and feruloylagmatine have been reported (hordatine A and hordatine C respectively), it does not appear to be the case with sinapoylagmatine.

Of special interest is a collection of compounds resulting from the dimerisation of HCAgms but with different linkage types to that of the hordatines (2-7'/8-8', and 8-8'/9-N-7', as in Fig. 3C and D). Murinamides A and B were discovered during a study of evolutionary changes in specialised defensive metabolism in the genus *Hordeum*. Murinamides consist of two molecules of feruloylagmatine (similarly to hordatine C) and were identified in the shoot and root tissues of related *Hordeum* species, *H. murinum* and *H. bulbosum* (Ube et al. 2017; van Zadelhoff et al. 2021). Their absence in the cultivated and wild barley, which are close relatives, led to the speculation that murinamides were replaced by hordatines during speciation (Ube et al. 2017). It was suggested that the activation of precursor supply, the expression of the coupling enzyme, as well as a collection of factors regulating the stereo- and regioselectivity of cyclisation reactions occurring subsequent to the coupling reaction, are responsible for the differences in the accumulation of dehydrodimers of agmatine HCAAs.

The complexity of hordatines thus derives not only from the origin of the carbon skeletons from two major

metabolic pathways, but also the hydroxylation level, distinct conjugations, substitutions and polymerisation level. Figure 4 and Table 1 shows the core structure of hordatines and derivatives. The hydroxylation of hordatines was reported by Gorzolka et al. (2014); Heuberger et al. (2014) and Pihlava et al. (2016) and characterised by the presence of one or two molecules of hydroxyagmatine (OHAgm). Hordatine A1 or A2 for example, corresponded to the compound with only one or two OHAgm(s) respectively (CouOHAgm-CouAgm or CouOHAgm-CouOHAgm). In

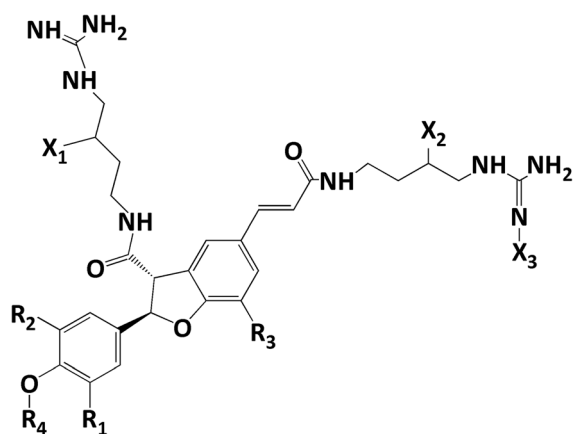


Fig. 4 Examples of the chemical structures of hordatine and derivatives. (Adapted from Pihlava (2014), Pihlava et al. (2016)). The various substitutions are listed in Table 1. The positions of X_1 and X_2 were suggested based on MS analysis alone and are thus not conclusive as yet. X_3 can occur on either of the two guanidino moieties Further NMR analyses may be required to provide more information regarding these positions

the context of conjugation, hordatines (A-D) can occur attached to one or more hexosyl group (up to 9 units) (Heuberger et al. 2014; Gorzolka et al. 2014; Pihlava 2014; Piasecka et al. 2015; Gorzolka et al. 2016). Glycosylation might exhibit a level of tissue and developmental specificity. For example, although higher in germinated seed, hordatine A β -D-glucopyranoside was also found in ungerminated mature seeds of barley. Hordatines can be stored in the glycosylated form in mature grains and partially synthesised or generated from the hydrolysis of the glycosides during/following germination (Kohyama and Ono 2013). Gorzolka et al. (2016) also reported hordatines that occur in both hydroxylated and glycosylated forms. In addition, monomethylated hordatines A, B, and C were found in barley samples (Pihlava 2014). Older literature mentions hordatine M, which is a mixture of the glycosylated versions of hordatines A and B (Smith and Best 1978).

Mass spectrometric analysis of hordatines and diagnostic ions

Such structural diversification as described above supports functionalisation and increases the ‘chemical space’ occupied by hordatines in the barley plant. Assessing the functional characteristics of bound phenolic acids often requires an understanding of their stereoisomeric configuration. Furthermore, the complexity of profiling hordatines found in barley extracts is influenced by differences in substitution

Table 1 Possible substitution patterns corresponding to Fig. 4, exemplifying the chemical diversity of hordatines

Hordatines	R ₁	R ₂	R ₃	R ₄	X ₁	X ₂	X ₃
A	H	H	H	H/Hexose	H	H	H
B	H	H	OCH ₃	H/Hexose	H	H	H
C	H	OCH ₃	OCH ₃	H/Hexose	H	H	H
D	OCH ₃	OCH ₃	OCH ₃	H/Hexose	H	H	H
A1	H	H	H	H/Hexose	H	OH	H
B1	H	H	OCH ₃	H/Hexose	H	OH	H
C1	H	OCH ₃	OCH ₃	H/Hexose	H	OH	H
A2	H	H	H	H/Hexose	OH	OH	H
B2	H	H	OCH ₃	H/Hexose	OH	OH	H
C2	H	OCH ₃	OCH ₃	H/Hexose	OH	OH	H
Met-A	H	H	H	H/Hexose	H	H	CH ₃
Met-B	H	H	OCH ₃	H/Hexose	H	H	CH ₃
Met-C	H	OCH ₃	OCH ₃	H/Hexose	H	H	CH ₃

patterns as well as geometric isomerisation that results in differences in retention times in the course of reverse phase chromatographic separation procedures.

As described above, hordatines are structurally complex polar molecules within the class of HCAAs dimers. Their extraction has mostly been done with 70–80% methanol or 80% 2-propanol solutions, and solid–liquid extraction (SLE) with 60–75% of acetone, sometimes followed by purification and fractionation steps. Different analytical platforms have been employed to elucidate the structural diversity of these classes of compounds. These include ultra-high performance liquid chromatography (UHPLC) for separation, coupled to MS for detection and structural elucidation and NMR for final structural verification. In addition, matrix-assisted laser desorption ionisation time-of-flight MS (MALDI-TOF-MS)-profiling and imaging mass spectrometry (MSI) have been used (Kohyama and Ono 2013; Pihlava 2014; Piasecka et al. 2015; Sarabia et al. 2018; Spreng and Hofmann 2018; Becker et al. 2022; Hamany Djande et al. 2021, 2022; Laupheimer et al. 2023). Both hordatines and precursors have been identified and characterised using these protocols. Due to their structural similarities, all four hordatine aglycones cluster closely to each other during chromatographic separation, and poor baseline resolution of peaks frequently does not allow the generation of clear spectra that only feature the compound of interest (Hamany Djande et al. 2022). A similar observation was made by Pihlava et al. (2016) who noted co- or close eluting compounds in addition to several precursor ions being very low. Hence, the necessity to improve chromatographic methods capable of clearly separating the hordatines, related derivatives and isomers (Gorzolka et al. 2014; Pihlava 2014; Piasecka et al. 2015; Hamany Djande et al. 2022).

In general, the hordatine precursors *p*-coumaroylagmatine (MW = 276 g/mol), feruloylagmatine (MW = 306 g/mol) and sinapoylagmatine (MW = 336 g/mol) are characterised by a neutral loss of m/z 130 corresponding to agmatine and the presence of a dehydroxylated hydroxycinnamoyl moiety (Fig. 5) (Gorzolka et al. 2014; Piasecka et al. 2015; Pihlava et al. 2016). For *p*-coumaroylagmatine for example, in the positive ionisation mode, the protonated parent ion has a m/z of 277 and the fragment ion m/z 147 resulting from the loss of a residue of agmatine (130) and corresponding to the dehydroxylated *p*-coumaric acid

(164–18 = 147). The similar pattern is observed with ferulic acid and sinapic acid. Gorzolka et al. (2014) used MALDI coupled with Fourier transform ion cyclotron resonance (FT-ICR) MS (MALDI-FT-ICR) imaging, or with TOF (time-of-flight) MS and UHPLC-electrospray ionisation (ESI) tandem MS (MS/MS) to elucidate the chemical structures of the HCAgms and derivatives. Hordatines A (MW = 550 g/mol) and B (MW = 580 g/mol) as well as two glycosylated derivatives were identified in the ‘Metlin’ (Metabolite and Chemical Entity, <https://massconsortium.com/>; Smith et al. 2005) database. In addition, two non-identified peaks with a similar fragmentation pattern as hordatines A and B were identified and named hordatine C (MW = 610 g/mol) and D (MW = 640 g/mol). The neutral losses and the in silico fragmentation of known compounds were assessed using ‘Mass Frontier’ spectral interpretation software from ThermoFischer (www.thermofisher.com). Neutral losses to keep in mind during the interpretation of mass fragmentation data of hordatines are m/z 130, corresponding to one agmatine moiety, 260 corresponding to the loss of two agmatine side chains, 276 (130 + 146) matching the elimination of one agmatine and a modified agmatine via hydroxylation (130 + 16 = 146), 42 for the head group (NH–C = NH) of the side chains, 17 for the loss of one of the four amines and 162 for the loss of a hexoside. A mass difference of 30 corresponding to the methoxyl group could be noted between hordatine A, B, C and D (Gorzolka et al. 2014; Hamany Djande et al. 2022). This observation was also evident with the precursors *p*-coumaroylagmatine, feruloylagmatine and sinapoylagmatine (Gorzolka et al. 2014; Piasecka et al. 2015; Pihlava et al. 2016; Hamany Djande et al. 2022).

Using a quadrupole time-of-flight (qTOF) MS instrument, following a reverse phase chromatographic separation of 30 min, Hamany Djande et al. (2022), also identified hordatine A, B, C and D, and glycosylated derivatives, with two isomers of hordatine B and hordatine C hexose. Similarly to Gorzolka et al. (2014), no glycosylated derivative of hordatine D was detected. The structural information was interpreted using a chemically intelligent structure elucidation tool, ‘Massfragment’ within the MassLynx MSTM software from Waters (www.waters.com) (Hamany Djande et al. 2022). In negative ESI mode, deprotonated ([M-H][−]) hordatine A

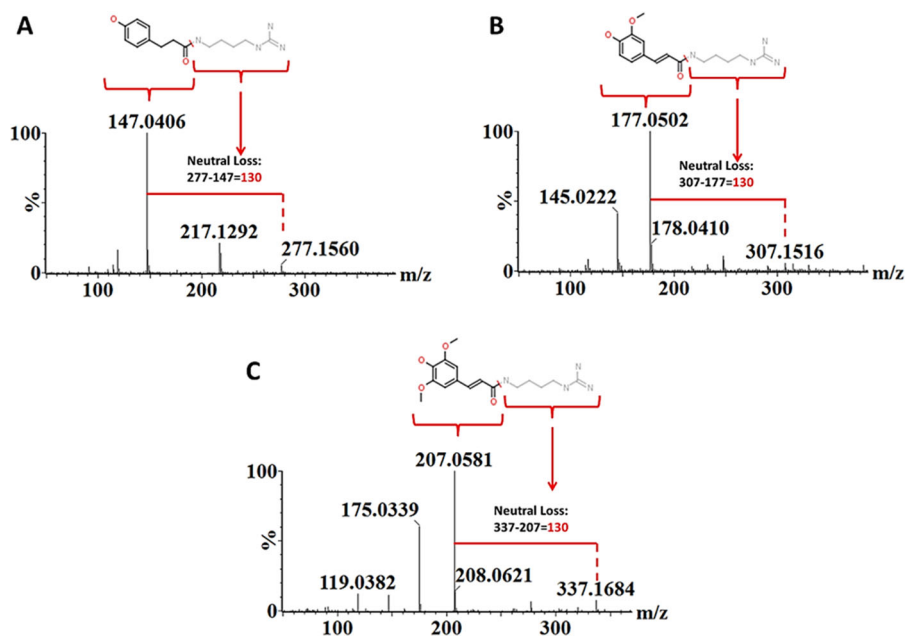


Fig. 5 Illustration of the fragmentation patterns (ESI +) of *p*-coumaroylagmatine (m/z 277), feruloylagmatine (m/z 307) and sinapoylagmatine (m/z 337) and their corresponding structures showing the fragmentation site. The grey side of the structure

correspond to the neutral loss as indicated with the red arrows. The dotted lines are just an extension of the fragment helping to illustrate the neutral loss. (Modified from Hamany Djande et al. 2022)

(m/z 549), B (m/z 579) and C (m/z 609), yielded fragment ions with m/z 237, 267 and 297, respectively, corresponding to their acidic cores (Piasecka et al. 2015; Hamany Djande et al. 2022). Here the presence of formic acid adducts in the negative ionisation mode can add to the characteristic ions and also interfere with the structural identification (Piasecka et al. 2015; Hamany Djande et al. 2022). In the positive ESI mode ($[M + H]^+$), hordatine A (m/z 551), B (m/z 581), C (m/z 611) and D (m/z 641) were characterised by the presence of diagnostic fragment ions m/z 157 ($C_6H_{13}N_4O$), 131 ($C_5H_{15}N_4$), 114 ($C_5H_{12}N_3$) deriving from agmatine molecules. In addition, double protonated ($[M + 2H]^{2+}$) base peaks with m/z 276, 291 and 306 for hordatine A, B and C respectively, are usually the main ions detected (Fig. 6). The double charge observed is due to the easy protonation of the two guanidino moieties. As a consequence, the positive ionisation of these compounds is more favourable as opposed the negative ionisation.

Similar signature fragment ions were noted with hordatine hexosides. In addition, a mass difference of m/z 81 was observed between adjacent hexosides due to the double charges (Gorzolka et al. 2014; Pihlava 2014; Piasecka et al. 2015; Hamany Djande et al.

2022). In the case of hydroxylated hordatines (either as aglycones or hexosylated), the compounds were characterised by fragment ions with m/z 129 ($C_5H_{15}N_4$), 147 ($C_5H_{15}N_4O$) and 173 ($C_6H_{13}N_4O_2$), verifying the presence of a hydroxyagmatine residue (Gorzolka et al. 2014; Pihlava 2014; Becker et al. 2022). As for the methylated agmatines, ions with m/z 145 ($C_6H_{17}N_4$), 171 ($C_7H_{15}N_4O$) and 128 ($C_6H_{14}N_3$) were characteristic fragments (Pihlava 2014).

In planta bioactivities of hordatines

Many specialised secondary metabolites have important adaptive significances in protection against adverse conditions. In this context, the biosynthesis of secondary metabolites, and the regulation thereof, play a complex role in the ability of plants to overcome unfavourable growth conditions and to adapt to environmental stressors. Below- and above-ground tissues experience very different environmental conditions and encounter different pathogens. Therefore, the differential composition and tissue-specific synthesis of hordatines and precursor metabolites warrants further investigation to determine their

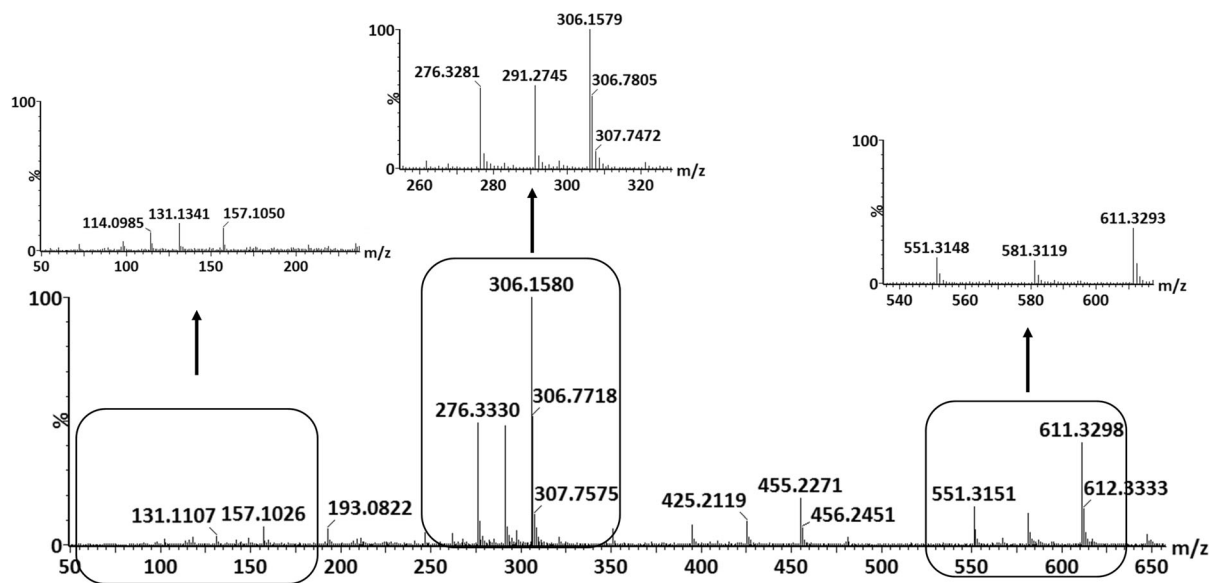


Fig. 6 Illustration of the fragmentation patterns (ESI +) of hordatine A, B and C, and some of the diagnostic fragments. Left: fragments characteristics of the molecule of agmatine, centre: the double protonated fragments of hordatine with m/z

276, 291 and 306 characteristic of hordatine A (m/z 551), B (m/z 581) and C (m/z 611), respectively, right. (Modified from Hamany Djande et al. 2022)

functional significance. Here, the blend of specific hordatines and tissue-specific post-synthesis modifications can support their functional diversification through changes in stability, hydrophobicity, subcellular localization, and thus bioactivity. Likewise, the presence of different hordatines in the cell wall might alter the structural properties of roots and shoots during growth and defence (Gorzolka et al. 2014).

Of late, renewed interest has been directed at the hordatine class of compounds because of their growing importance in diverse areas (Gorzolka et al. 2014; van Zadelhoff et al. 2022; Becker et al. 2022; Ube et al. 2023; Laupheimer et al. 2023). Although the mechanisms behind the biological implication of hordatines are still to be fully uncovered (e.g. germination vs. grain maturation), the most alluring quality of these barley-specific metabolites for plant science and agricultural development research remains their remarkable antifungal properties (Stoessl 1967). As dimerised HCAGms, the hordatines are important and understudied compounds with reported antimicrobial properties. *HvACT* and other enzymes (e.g. ADC) were found to be associated with barley resistance against fungal pathogens (Yuan et al. 2022; Moghe et al. 2023) and it has been reported that the concentration of hordatines in barley leaves increase

after infection, e.g. by powdery mildew (*Erysiphe graminis* f. sp. *hordei*) (Smith and Best 1978; Von Röpenack et al. 1998). Synthesis of hordatines in seedlings in response to fungal infection result in concentrations of hordatines A/B and M greatly in excess of that required to inhibit spore germination of *Erysiphe graminis* (Smith and Best 1978). Hordatines have thus been regarded as signature compounds in cultivated barley, abundant in the seeds of mature plants and the shoots of barley seedlings (Hamany Djande et al. 2021, 2022, 2023a).

Studies conducted *in vitro* have revealed that hordatines and *p*-coumaroylagmatine prevent spore germination in a variety of fungal infections (Stoessl 1967; Stoessl and Unwin 1969; Smith and Best 1978; Macoy et al. 2015b). In our most recent study on the defence response of barley to the necrotrophic fungal pathogen *Pyrenophora teres* f. *teres*, hordatines A and B were identified as discriminant metabolites; while downregulated in shoot tissue of a susceptible cultivar, their involvement in plant defence was reiterated (Hamany Djande et al. 2023a). In another study, barley leaf tissue was treated with dichlorinated derivatives of anthranilic acid, salicylic acid and isonicotinic acid as priming agents or inducers of plant immunity and a positive correlation of coumaroylagmatine and

hordatines A, B and C to the treatments were reported (Hamany Djande et al. 2023b). In addition, their functional significance in plant–pathogen interactions has been observed at an early developmental stage, after germination, and a role as phytoanticipins/phytoalexins has been inferred (Hamany Djande et al. 2021, 2022). Another function ascribed to HCAmgs is cell wall fortification, also associated with hordatines during plant-pathogen interactions (Von Röpenack et al. 1998), perhaps as nucleating agents for the deployment of lignification as a structural defence.

A possibility of involvement of hordatines in plant abiotic stress was previously postulated (Sarabia et al. 2018) and Piasecka et al. (2020) reported the role of hordatine B and its hexoside in the barley response to water deficit. Various internal signals modify metabolism, growth, and development in plants and enhanced biosynthesis of hordatines in response to multiple stresses or combined abiotic and biotic stresses might be due to common upstream response regulators such as reactive oxygen/nitrogen species (ROS/RNS) (Cortese-Krott et al. 2017) and shared signaling circuits to transduce diverse biotic and abiotic signals into the same physiological response.

Hordatines as pharmacological agents and health beneficial compounds

Extracts from plants that contain important sources of various biologically active compounds are becoming increasingly important in various fields other than agriculture, such as environmental protection, pharmacology and medicine. Barley is known to be rich in recognised nutraceuticals such as glucans and tocopherols (Idehen et al. 2020; Raj et al. 2023). Due to their biological activity as radical scavenging agents / antioxidants (Spreng and Hofmann 2018), hordatines have attracted attention for their possible use in human nutrition. This group of compounds specific to barley possess health beneficial attributes and may have a role as disease preventative agents, supporting their consumption as a component of functional foods (Dahab et al. 2020).

The investigation of hordatines against α -glucosidase, a carbohydrate hydrolase, revealed them as potent inhibitors of the enzyme (Becker et al. 2021, 2022). α -Glucosidase catalyses the hydrolysis of starch to simple sugars (Zhai et al. 2022) and

inhibitors of the enzyme may function to regulate or slow the release of glucose and to avoid hyperglycemia (Bhatnagar and Mishra 2022). Relatedly, hordatine A was found to exhibit muscarinic M3 receptor-binding activity (Yamaji et al. 2007), suggesting a potential role in the regulation of insulin secretion and glucose homeostasis (Gautam et al. 2006) and regulating gastro intestinal smooth muscle function (Yamaji et al. 2007; Kohyama and Ono 2013; Tanahashi et al. 2021). In addition, the compounds also have an antagonistic effect on α 1-adrenergic receptors, located in the peripheral and central nervous system and involved in regulating the contraction of smooth muscle tissue in the lower urinary tract and cardiovascular system (Wakimoto et al. 2009).

Using *in silico* molecular docking studies, hordatine scaffold structures were identified as potential inhibitors of the Severe Acute Respiratory Syndrome Coronavirus 2 that causes coronavirus disease 2019 (COVID-19). Hordatine A and B showed the highest binding affinity to the protease and RNA polymerase targets. This was demonstrated by the strong hydrogen bonds created with the catalytic residues, in addition to noteworthy interactions with other receptor-binding residues. These findings indicated that hordatines could be significant lead candidates for the development of COVID-19 medicines (Dahab et al. 2020).

The guanidine moieties of hordatines can mimic lysine and arginine residues and interact with negatively charged groups or with π -electron-rich aromatic moieties in potential binding sites. The presence of these functional head groups on hordatines may enable their conjugation and derivatisation leading to the development of rationally designed compounds and the investigation of guanidine-based libraries useful in the search of potential pharmacophores. Currently, the hordatines and some structural analogues are being investigated as activating and/or anchoring groups in bioactive compounds and a wide range of related biological activities show the potential to regulate corresponding pathways and control several diseases (Yamaji et al. 2007; Wakimoto et al. 2009; Dahab et al. 2020). However, little is known about the bioavailability, pharmacokinetics and metabolism of hordatines.

Biotechnological approaches for hordatine synthesis

The manipulation of the biosynthesis of bioactive compounds in plants by conventional and biotechnological approaches can provide many opportunities for enhancing concentration levels and yields. Accordingly, the synthesis of valuable secondary plant metabolites in production hosts, such as cultured plant cells and microbial cells, has generated significant interest.

In an early study by Nomura et al. (1999), biosynthesis of hordatines was observed in wheat (*Triticum aestivum*) addition lines carrying the short arm chromosome 2H (2HS) of barley. This revealed the chromosome as responsible of the biosynthesis of hordatines. In a follow up study, the chromosomal locations of *HvACT* and *HvPrx7* stipulated to be responsible for the formation of HCAgms and hordatines respectively, were further investigated. These genes were found located on the long arm of the chromosome 2H (2HL) and not on 2HS. In addition, while HCAgms were detected after addition of both 2HL and 2HS, no hordatines were found in 2HL containing wheat. These results suggested the presence of another *HvACT* (probably an isoform) located on the HS and that another barley peroxidase is responsible for the formation of hordatines (Nomura et al. 2007).

Using a transgenic approach, a 'rational metabolic-flow switching strategy' for the production of exogenous metabolites in suspension cultured bamboo cells as production hosts was proposed as a hordatine-producing system (Nomura et al. 2018). Stable transformants with the *HvACT* gene exhibited metabolic-flow switching, from HCAAs of putrescine to those of the desired agmatine. Relatedly, *Torenia hybrida* plants transformed with agmatine coumaroyl-transferase from *Arabidopsis* (*AtACT*), accumulated significant amounts of *p*-coumaroylagmatine (Muroi et al. 2012). Although high levels of *p*-coumaroylagmatine were found in both cases, no further dimerisation to hordatines was reported, indicating the requirement of additional barley-specific enzymes for the synthesis of hordatines.

In a comparative study of five cultivars of barley, hordatines A and B as well as the hexosylated derivatives were identified as signatory or discriminant metabolites. These compounds may be

considered as potential biomarkers in plant breeding practices for barley cultivar differentiation (Hamany Djande et al. 2021).

Biotechnological advances can also act in support of agroprocessing applications. Originating from barley, hordatines are found in beer and spent grains from the brewing process (Kageyama et al. 2011; Pihlava 2014; Becker et al. 2022). The latter has been investigated as a potential source for agroprocessing purposes, valorisation of waste streams through extraction of hordatines and economic exploitation (Becker et al. 2022).

Concluding remarks and future perspectives

Hordatines play a crucial role as specialised secondary metabolites in barley, especially as multipurpose plant-protective phytochemicals. These phenolamides are the focus of several investigations aiming to elucidate their contribution to plant defence and crop protection. Despite their significance to date, the breadth of hordatine studies is limited and gaps in the literature have created many opportunities for further explorative studies. Precisely, research on the biosynthesis of hordatines in response to pathogens beyond fungi, has been largely unexplored. This presents a potential avenue for gaining deeper insights into their implication in plant defence and crop protection. Thus, hordatines and associated precursors may be used as prospective biomarkers for desirable performance characteristics in crop improvement programmes.

Further investigation of hordatines under various environmental conditions and in response to eliciting agents, is worthwhile being conducted. By adopting a systems biology approach, such studies provide insights into the understanding of interrelationships between genotype-environment-phenotype systems. In this case, thorough genomic and integrated multi-omic data can shed light on the developmental and environmental regulation of hordatine metabolism. The biosynthesis of hordatines exhibit unique features where carbon skeletons derived from the early phenylpropanoid pathway combine with polyamines to generate the co-substrates for the conjugation reaction with hordatines as neolignanamide products. There is limited knowledge regarding the inducibility of the ACT enzyme(s) and regulation of the flux of carbon through pathways supporting the biosynthesis

of hordatine precursors. Filling up those gaps will help to manipulate these pathways to improve crops during contemporary breeding and biotechnological approaches.

Most importantly, as the foundation of future investigations, it is essential to improve analytical platforms and methods for better profiling, characterisation, identification and quantification of the hordatines, precursors and derivatives, as well as for their tissue-specific localisation. The identification and adequate structural elucidation of this complex and diverse class of metabolites and their hydroxylated, methylated or hexosylated derivatives is crucial to elucidate their bioactivities, given the paucity of authentic standards.

Moreover, research into the nutraceutical and beneficial properties related to human health through the consumption of barley and barley-derived products is becoming increasingly relevant. Here, further studies are required to fully address the claimed beneficial effects of hordatines as plant-derived bioactives. Growing interest has been given to these benzofurans which are offering great potential in diverse research areas. Natural products such as hordatines may serve as drug leads in *in silico* molecular docking studies to screen and identify potential therapeutic drugs. This includes characterisation of structural characteristics and mechanisms mediating the pleiotropic effects of hordatines in different models of human diseases.

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Declarations

Competing interests The authors declare no conflict of interest.

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