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Data-dependent acquisition-mass spectrometry guided isolation of new benzoxazinoids from the roots of *Acanthus mollis* L

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ABSTRACT

Benzoxazinoids (BXs) are phytochemicals that exert plant-protecting, allelopathic, and human-health-promoting effects. Here, we present a data-dependent acquisition-mass spectrometry based method to locate and annotate BX-conjugates in different fractions of a plant extract. This allows for isolation of compounds present in a much smaller percentage than common for natural products yet still sufficient for subsequent identification through nuclear magnetic resonance (NMR) spectroscopy. The presence of BXs has been extensively studied in monocotyledons, whereas only a limited number of studies have focused on dicotyledons. Here, the presence of hitherto unknown BXs in the roots of the dicotyledonous plant *Acanthus mollis* L. has been determined. Two acetylated glycosylated BXs, compound **1** 2-(6-acetyl-β-D-glucopyranoside)-2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one) and compound **2** (-2-(6-acetyl-β-D-glucopyranoside)-2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one), and a BX-derived glucoside carbamate olide, compound **3** (2-hydroxy-6-(2-deoxy-β-D-glucopyranosyloxy)-phenyl) carbamic acid 2′-olide), were isolated and identified by NMR. These previously unknown BXs may contribute to the reported biological activities of *A. mollis* L.

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1. Introduction

Benzoxazinoids (BXs) are nitrogen-containing indole-derived plant metabolites, found in several members of the Acanthaceae family [1–3] and in other plant families, such as Lamiaceae [4], Ranunculaceae [5], Scrophulariaceae [2], and Poaceae [6,7]. The effects of BXs have been reported to range from health protecting, immunoregulatory, antimicrobial, anticancer, and mood improving in mammals to allelopathic and acting as a defense agent in plants [8–11]. The interest in investigating the bioactivity of BXs has led to a range of publications in which the isolation of BXs from plants [12–14] or the production of BXs through organic synthesis has been reported [15–17]. Some of the published isolation procedures for BXs were dedicated to the isolation of formerly known compounds that were required in substantial amounts for use in specific bioassays [15,18–21]. Other isolation procedures were dedicated to the investigation of a medicinal plant in which the presence of compounds with targeted bioactivity was examined. Among those, one or more BXs were identified [4,22,23].

Till date, only 32 BXs have been extracted from plants [24]. The full names and acronyms of the 15 most common BXs among these are listed in Table 1. BXs can be classified into three groups, namely, lactams, hydroxamic acids, and benzoxazolinones. Commonly known lactams include HBOA, HMBOA, DHBOA, HBOA-glc, HMBOA-glc, DHBOA-glc, and HBOA-glc-hex. Commonly known hydroxamic acids include DIBOA, TRIBOA, DIBOA-glc, DIMBOA-glc, and DIBOA-glc-hex. Commonly known benzoxazolinones include MBOA, BOA, and 6-OH-BOA (for full systematic names see Table 1).

In the case of *A. mollis* L., the main focus has been on the BXs in the aboveground parts (Fig. 1) [3,4,25,26] in spite of the fact that roots are generally known to be a significant source of plant secondary metabolites.

The first aim of this project was to develop a Data Dependent Acquisition (DDA) liquid chromatography–mass spectrometry (LC-MS) technique, capable of detecting new, bioactive compound

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Table 1
Acronyms, systematic names, and structures of 15 most common benzoxazinoids.

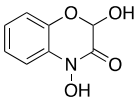
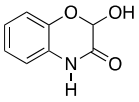
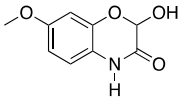
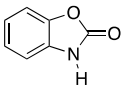
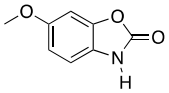
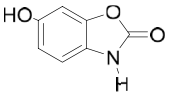
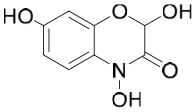
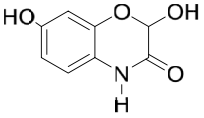
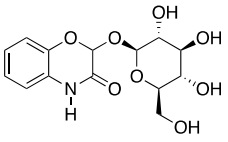
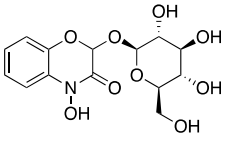
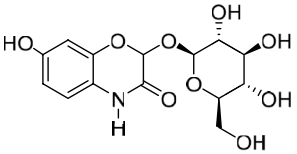
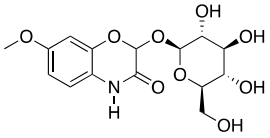
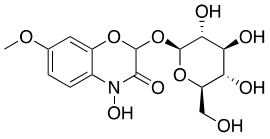
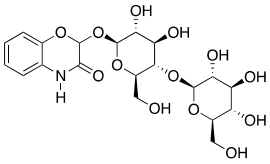
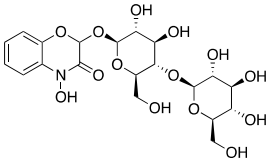
Known BXs	Systematic name	Structure
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one	
HBOA	2-hydroxy-1,4-benzoxazin-3-one	
HMBOA	2-hydroxy-7-methoxy-1,4-benzoxazin-3-one	
BOA	benzoxazolin-2-one	
MBOA	6-methoxybenzoxazolin-2-one	
OH-BOA	6-hydroxy-benzoxazolin-2-one	
TRIBOA	2,4,7-trihydroxy-1,4-benzoxazin-3-one	
DHBOA	2,7-dihydroxy-1,4-benzoxazin-3-one	
HBOA-glc	2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one	
DIBOA-glc	2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one	
DHBOA-glc	2-β-D-glucopyranosyloxy-7-hydroxy-1,4-benzoxazin-3-one	

Table 1 (*continued*)

Known BXs	Systematic name	Structure
HMBOA-glc	2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one	
DIMBOA-glc	2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one	
HBOA-glc-hex*	2-[4-*O*-hexosepyranosyl-β-D-glucopyranosyl]oxy-1,4-benzoxazin-3-one	
DIBOA-glc-hex*	2-[4-*O*-hexosepyranosyl-β-D-glucopyranosyl]oxy-4-hydroxy-1,4-benzoxazin-3-one	

*the structure of the second hexose has not yet been fully elucidated.

analogs from known compound classes in limited amounts of plant material. The second aim was to use the developed method to isolate and identify hitherto unknown BX compounds from the roots of *A. mollis* L. drawing upon chromatographic and mass spectrometric information on available BX reference compounds, and subsequently verify the effectiveness of the DDA methodology by using advanced NMR techniques to rectify the isolated structures.

2. Materials and methods

2.1. Chemicals

Methanol, pentane, dichloromethane, *n*-butanol, and glacial acetic acid used for extraction were of HPLC-grade (Rathburn Chemicals, Walkerburn, UK). Acetonitrile and glacial acetic acid used for LC-MS analysis were of LC-MS grade (Fisher Scientific, Roskilde, Denmark). The water used for sample preparation was obtained from a Milli-Q Advantage A10 instrument equipped with an LC-pack (Merck Millipore, Darmstadt, Germany). The BX reference compounds DIBOA, HBOA, HMBOA, HBOA-glc, DIBOA-glc, HMBOA-glc, DIMBOA-glc, DHBOA-glc, DHBOA, HBOA-glc-hex, and DIBOA-glc-hex were obtained as described by Bhattarai et al. [25]. TRIBOA was a generous gift from Dr. Monika Frey, Technische Universität München, Germany. MBOA, BOA, and 6-OH-BOA were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material

Transplants of *A. mollis* L. were purchased from Plantetorvet (Randers, Denmark). The transplants had been produced by dividing the rhizomes and planting them in individual pots before delivery to our facility where they were transplanted into a bag with soil cut open in one side and grown under greenhouse conditions in soil for three months [25]. Whole plants (12 plants) were removed from the soil, shaken to remove excess soil, separated into roots and shoots, wrapped in aluminum foil, dipped in liquid nitrogen, and lyophilized. The dried roots were ground using a Geno/Grinder (SPEX Sample Prep, Rickmansworth, UK), vacuum-packed, and stored at −20 °C until further analysis.

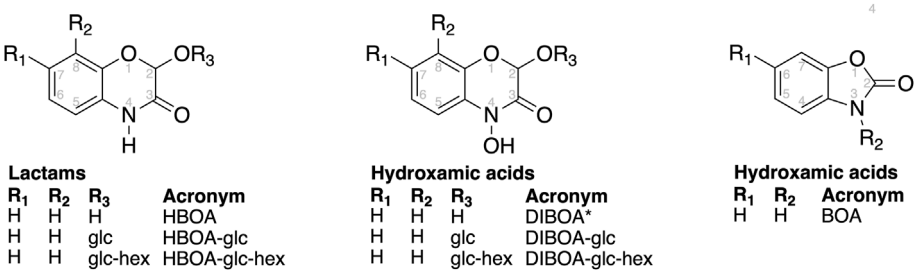


Fig. 1. Structure of benzoxazinoids hitherto found in aboveground parts and roots of *Acanthus mollis* L. [2, 25, 27]. *: DIBOA is the only BX reported in the roots. The full names of the benzoxazinoids are given in Table 1.

2.3. Extraction

The dried root powder of *A. mollis* L. (57 g, 12 plants combined) was extracted by shaking with a mixture of methanol/water/acetic acid (80:19:1, v/v/v). Two consecutive extractions were performed, each using 1.8 L of solvent (3.6 L in total). The combined extract was filtered, dried (using a rotary evaporator), reconstituted in water (2.4 L), and divided into eight portions. Each aqueous portion (300 mL) was subjected to successive extraction with pentane, dichloromethane, and *n*-butanol (100 mL each). Extraction with pentane and dichloromethane removed unwanted hydrophobic compounds, whereas *n*-butanol was used to extract as many BXs as possible. The *n*-butanol phases were combined, dried (993 mg), and reconstituted in water (30 mL).

2.4. Isolation and purification

2.4.1. Solid-phase extraction (SPE)

The water-reconstituted combined *n*-butanol extracts were fractionated via SPE using Oasis HLB 35 cc LP (Waters A/S, Taastrup, Denmark) extraction cartridges (two in sequence). The SPE fractionation was performed by successively adding five eluents (5, 20, 40, 60, and 80% methanol in water, v/v; 20 mL each) to the cartridge. Elution was repeated three times with each eluent, resulting in 15 fractions. All the fractions were screened for the presence of BXs using LC-MS/MS (section 2.5) and subsequently evaporated to dryness.

2.4.2. Semi-Preparatory high-Performance liquid chromatography (semi-prep HPLC)

The SPE fractions containing potential new BXs (as determined using DDA-LC-MS as described in section 2.5) were reconstituted in 2 mL of water/methanol (95:5, v/v) and further fractionated using a semi-prep setup in an Agilent 1260 Infinity HPLC system equipped with a diode array detector. The absorbance was recorded at the wavelengths of 240, 254, 260, and 280 nm. Water/acetic acid (99:1, v/v) was used as the eluent A and methanol/water (95:5, v/v) as the eluent B. The solvent gradient was as follows: 0–2 min at 30% B, 2–17 min ramped to 50% B, 17–18 min ramped to 100% B, 18–22 min at 100% B, 22–23 min ramped to 30% B, and 23–50 min at 30% B. The flow rate was 4.7 mL/min, the injection volume was 100 μL, and the temperature of the column was 30 °C. The fractions were collected at 30 s intervals.

The first round of semi-prep fractionation was conducted using a Synergi Fusion-RP 80 Å (150 × 10 mm, 4 μm particle size) chromatographic column. The Synergy-Fusion-selected fractions were then dried, reconstituted in 100–400 μL of water/methanol (95:5, v/v), and re-fractionated using a Kinetex 5 μM Biphenyl 100 Å (150 × 10 mm) chromatographic column. The purity of the final isolates of the prospective BX compounds were assessed through DDA-MS in full scan mode prior to nuclear magnetic resonance (NMR) analyses.

2.5. LC-MS analysis

The extracts and fractions were analyzed using a 4500 QTrap from Sciex coupled with an Agilent 1260 HPLC system equipped with a Synergi Polar-RP chromatographic column (250 × 2.1 mm, 5 μm, 80 Å, Phenomenex). All LC-MS analyses were performed in negative ion mode for enhanced selectivity and the trap functionality was utilized for enhanced sensitivity whenever possible. Two LC-MS methods (methods 1 and 2) were developed (see subsequent sections) and used for analyzing the extracts and fractions from SPE and the semi-prep fractions to screen for potential BXs. The detected ions were considered potential BXs if they featured an even *m/z* (indicating an odd number of nitrogen atoms) and at least two of the characteristic MS fragments generated from the BX reference compounds listed in **2.1 Chemicals** (for fragments see Table 2).

2.5.1. DDA-MS method 1 (EMS-EPI)

Method 1 combined a full scan (EMS) as the survey scan with product ion scans (EPI) as the dependent scans (EMS-EPI). The instrument settings used for the EMS and EPI are presented in Table S1.

The DDA criteria were set to acquire one to two ions exceeding three times the noise level to exclude the acquisition of the same ion for 10 s after three occurrences. The EPI experiment was conducted at unit resolution for quadrupole 1 (Q_1) with a step size of 0.12 Da. The linear ion trap (LIT) fill time was adjusted throughout the experiment. A longer fill time was necessary when analyzing the raw extracts, and a shorter fill time was required when analyzing cleaner fractions. The compound-dependent parameters for the BX reference compounds are presented in Table S1. The characteristic MS/MS fragmentation patterns of the BX reference compounds listed in **2.1 Chemicals** were recorded (Table 2) and the occurrence of the precursor and product ions in the known BX reference compounds were used as the basis for including a fraction for further analysis.

2.5.2. DDA-MS method 2 (MRM-EPI)

Method 2 (MRM-EPI) was operated using MRM for the survey scan and EPI as the dependent scan. MRM was operated at unit resolution for Q_1 and Q_3. The instrument and compound-dependent parameters are listed in Table S1 and Table S2. The *m/z* values of the potential BXs were hypothesized based on the initial EMS-EPI investigations supplemented by data reported by de Bruijn et al. and Pihlava and Kurtelius [28,29]. The hypothesized *m/z* values were a)312/150/122, b)358/150/122, c)368/164/136, d)384/180/134, and e)384/180/124. The DDA criteria were set to acquire one to two ions with peak intensities exceeding three times the noise level to exclude the acquisition of the same ion for 30 s after two occurrences. The LIT fill time was adjusted throughout the experiment. A longer fill time was necessary when analyzing the raw extracts, and a shorter fill time was required when analyzing cleaner fractions.

2.5.3. Chromatographic method for DDA-MS methods 1 and 2

Analysis was performed using water/acetonitrile (93:7, v/v)

Table 2
Characteristic MS/MS fragments of known BXs in negative ion mode. The data were generated based on BX reference compounds using data-dependent acquisition. The product ions are presented in the order of relative intensity.

Known BXs	Precursor ion	Product ions
DIBOA	180	134, 108, 91.1
HBOA	164	136, 108, 118, 92
HMBOA	194	138, 166, 123, 179, 148
BOA	134	91.1, 78.3
MBOA	164	149, 121
OH-BOA	150	122.2, 106, 82.1
TRIBOA	196	150, 160.8, 122.2, 178
DHBOA	180	124, 152, 134, 90
HBOA-glc	326	164, 136, 108, 118
DIBOA-glc	342	134, 162, 180, 108, 118
DHBOA-glc	342	180, 124, 152, 162, 134, 90
HMBOA-glc	356	194, 166, 138, 123
DIMBOA-glc	372	149, 164
HBOA-glc-hex	488	164, 136, 108, 118
DIBOA-glc-hex	504	134, 162, 108, 118

Full names of benzoxazinoids are given in Table 1.

containing 20 mM acetic acid as eluent A and acetonitrile/water (78:22, v/v) containing 20 mM acetic acid as eluent B. The gradient was as follows: 0 min at 0% B, 0–0.6 min ramped to 8% B, 0.6–1.8 min ramped to 10% B, 1.8–7.8 min ramped to 70% B, 7.8–8.4 min ramped to 90% B, 8.4–9.6 min at 90% B, 9.6–10.20 min ramped to 0% B, and 10.20–13.80 min at 0% B. The temperature of the auto-sampler tray was 10 °C and the flow rate was 500 μL/min. During the experiment, the chromatographic method was adjusted by lowering the flow rate to 300 μL/min and increasing the run time to 23 min to resolve the coeluting peaks. The adjustment allowed us to obtain more data points across the peak.

2.6. NMR analysis

NMR spectra were acquired in DMSO-d_6 at 300 K using a Bruker Avance III NMR spectrometer (^{1}H resonance frequency of 600.13 MHz). The ^{1}H and ^{13}C NMR chemical shifts were referenced to the residual solvent signal of DMSO-d_6 (δ_H = 2.50 ppm, δ_C = 39.52 ppm). The ^{1}H NMR spectra were recorded with a spectral width of 20 ppm using 30° pulses and 65 k data points. For the 2D NMR experiments, phase-sensitive DQF-COSY, NOESY, and ROESY spectra were recorded using gradient-based pulse sequences with a spectral width of 9 ppm and 2 k × 512 data points (processed with forward linear prediction to 1 k data points in F1). Multiplicity-edited HSQC spectra were acquired with the following parameters: $^1J_{C,H}$ = 145 Hz, a spectral width of 12 ppm for ^{1}H and 140 ppm for ^{13}C, 1730 × 192 data points (processed with forward linear prediction to 512 data points and zero-filling to 1 k data points in F1), and 1.0 s relaxation delay. Low-pass-filtered HMBC experiments were optimized for $^nJ_{C,H}$ = 8.0 Hz (long-range), $^1J_{C,H\ min}$ = 125 Hz, and $^1J_{C,H\ max}$ = 160 Hz and recorded using a spectral width of 12 ppm for ^{1}H and 180 ppm for ^{13}C, 2k × 256 data points (processed with forward linear prediction to 512 data points and zero-filling to 1 k data points in F1), and 1.0 s relaxation delay.

2.7. Method validation/validation of experiments

The developed DDA methods were validated based on their ability to detect the BXs, known to be present in extracts of seeds and green plant materials from rye, wheat, and maize (Table 1) as demonstrated by our group [25,30–32] on several occasions.

The authenticity of the newly identified acetylated BXs was tested: freshly prepared root extracts of *A.mollis* were prepared with and without acetic acid, and with the acetic acid substituted with formic acid. The root extracts were analyzed using LC-DDA-MS as described in section 2.5 with two different eluent systems: one as described, and the other substituting acetic acid with formic acid. The authenticity of the newly identified BXs were confirmed if they were present in all the extracts that were extracted using either acetic acid, formic acid, or no acid, and in all the LC-MS analyses performed with or without acetic acid.

The authenticity of the newly identified olide was confirmed by comparing the MS spectrum of the purified compound with that of a newly prepared raw extract.

3. Results and discussion

3.1. Method development and verification (testing the fit for purpose)

The DDA methods were developed based on the available BX reference compounds listed in Table 2. The fractions containing at least two characteristic MS fragments of the BX reference compounds (hereafter referred to as *compounds of interest*) were included in the study.

Both the developed full scan (EMS) and targeted scan (MRM) based DDA methods demonstrated the presence of all the 15 BXs mentioned in Table 2 which were isolated from wheat and rye extracts where their presence has previously been established on several occasions [13,33–35].

To test whether the detected compounds were indeed novel compounds or artifacts of the isolation and purification process, we performed a combination of tests. The root materials of *A. mollis* were investigated using conditions with and without acetic acid. This test confirmed that the acetylated BX-conjugates were present regardless of the presence of acetic acid in either the extraction solvents or eluents and, hence, must be concluded to originate from the plant material. The authenticity of compound **3** was examined by comparing the MS spectrum and retention time of the purified compound to those of the newly prepared raw extract, which confirmed that the compound originated from the plant.

3.2. Using mass spectrometry guided fractionation for elucidation of structures of new BXs

A common approach to fractionation in natural product chemistry is bioactivity-guided fractionation. This is a suitable method if the aim is to identify a new bioactive compound which may be responsible for the effects of a traditional medicinal herb. In this study, however, the main bioactive compounds are readily known, namely the BXs. The aim of the present work was to identify new forms in which the BXs are stored/present in the plant material, and since these forms do not have any known biological activity, we chose to proceed with developing a DDA-MS guided fractionation method. The dried root extract of *A. mollis* was initially partitioned between pentane, dichloromethane, n-butanol, and water using liquid-liquid extraction. The pentane and dichloromethane phases did not show any BX-like compounds but helped remove the more lipophilic substances. Both the n-butanol and aqueous phases showed *compounds of interest*. However, since the number of *compounds of interest* was higher in the n-butanol phase, it was subjected to further fractionation using reverse phase solid phase extraction (RP-SPE).

The dried n-butanol phase was sequentially extracted via RP-SPE with 5, 20, 40, 60, and 80 % methanol to enable the compounds to elute according to their polarity and remove interfering substances. With 5 and 80 % methanol, no known BXs or potential BXs were eluted. With 20 and 40 % methanol, readily known double hexose and single hexose forms of DIBOA and HBOA were eluted in high quantities as confirmed by comparison with our reference compounds. Several potential BX compounds displaying MS fragments characteristic of HBOA and DIBOA were eluted with 60% methanol together with the BX aglycons DIBOA, HBOA, and BOA. This fraction was dried and further fractionated using semi-preparative HPLC.

The initial round of HPLC purification was performed using an RP column with embedded polar functionalities. Fractions were collected at intervals of 30 s (F1 to 36 beginning at 2 min) and analyzed in the DDA-MS setup. The HPLC fractions showed DIBOA (found in F17-19) in high amounts, together with DHBOA (F6), DIBOA-glc (F10), HBOA (F19), and HBOA-glc (F10) as confirmed by comparison with the BX reference compounds (See Table 2). Along with these known BXs, the HPLC fractions contained *compounds of interest* at individual retention times displaying MS fragments at the lower *m/z* range of the spectrum similar to those of DIBOA, DHBOA, HBOA, and OH-BOA: 444 (Rt 5.72 min, F6), 312 (Rt 5.77 min, F6-7), 594 (Rt 6.42 min, F10), 384 (6.75 min, F14), 368 (Rt 7.11 min, F18), 384 (Rt 7.12 min, F18-19), and 522 (Rt 7.94 min, F33). The fractions containing the possible BXs were subjected to a second round of purification using a biphenyl RP-column to exploit the

different selectivities toward the separation of the formerly co-eluting peaks. This resulted in seven pure compounds, 444 (Rt 5.72 min, F6), 312 (Rt 5.77 min, F6-7), 594 (Rt 6.42 min, F10), 384 (6.7 min, F14), 368 (Rt 7.11 min, F18), 384 (Rt 7.12 min, F18-19), and 522 (Rt 7.94 min, F33). Three of these compounds, *m/z* 384 (compound **1,** Rt 7.12 min), 368 (compound **2,** Rt 7.11 min), and 312 (compound **3,** Rt 5.77 min) contained enough material (sub-milligrams) for structural elucidation through NMR analyses in micro tubes.

The molecular ion *m/z* 384 was observed at 7.12 min. The MS analysis of compound **1** (molecular ion *m/z* 384, Rt = 7.12 min) showed MS fragments that are characteristic of DIBOA aglycone and DIBOA-glc (Table 1). The loss of 42 [M-H-342] indicated an acetyl group, and the loss of 42 + 162 indicated the presence of an acetyl-hexose. The structure of compound **1** was confirmed using NMR (See section 3.3 and Fig. 2). A small peak with the same molecular ion and a similar mass spectrum was eluted shortly before compound **1** at 6.7 min. However, this compound appeared over time in the solution of the pure compound **1**. Hence, it is most likely the diastereomer of compound **1** with the position C2 as the center of inversion since it is α to the carbonyl group. A similar pattern has been observed for the standard of DIBOA-glc and other BX-glucosides (unpublished data). Since the quantity of this compound was insufficient for NMR analysis, it was not investigated further.

The MS analysis of compound **2** (molecular ion *m/z* 368, Rt = 7.11 min) showed MS fragments that are characteristic of HBOA aglycone and HBOA-glc (Table 1). The peak with *m/z* 326 points to the neutral loss of 42 most likely corresponding to an acetyl group, and the loss of 42 + 162 is related to an acetyl-hexose (See Fig. 3). Thus, the molecular ion *m/z* 368 was provisionally identified as acetylated HBOA-glc and this was confirmed through NMR as discussed in detail in section 3.3. A minor peak of the likely diastereomer was also detected for compound **2** as for compound **1**.

The compound **3** (molecular ion *m/z* 312, Rt = 5.77 min) exhibited MS fragments at *m/z* 150 and *m/z* 122 that are characteristic ions of OH-BOA. The peak with *m/z* 312 points to the neutral loss of 162 corresponding to a hexose sugar. The MS fragment at *m/z* 122 corresponds to the loss of CO from *m/z* 150 or the loss of hexose and CO from *m/z* 312. Based on the MS fragmentation, compound **3** was assumed to be a hexose conjugate of OH-BOA, which upon NMR analysis was found to be a rearrangement product of the glucosylated 4-OH-BOA, as discussed in detail in section 3.3. (See Fig. 4 and Fig. 6).

3.3. NMR analysis

Compound **1**, assumed to be acetylated DIBOA-glc or one of its isomers, was further analyzed using NMR. In addition to the characteristic signals for BXs (Table 2), the COSY spectrum revealed correlations between H-1′, H-2′, H-3′, H-4′, H-5′, and H-6′A/H-6′B of the glycoside. H-1′ appeared as a doublet with $J_{H\text{-}1',H\text{-}2'}$ = 7.8 Hz at δ 4.62. The remaining coupling constants were axial–axial, i.e., $J_{H\text{-}2',H\text{-}3'}$ = 8.9 Hz, $J_{H\text{-}3',H\text{-}4'}$ = 8.9 Hz, and $J_{H\text{-}4',H\text{-}5'}$ = 9.6 Hz, which revealed the glycoside to be a β-D-glucopyranoside. The downfield shifts of H-6′A (δ 4.0, dd, $J_{H\text{-}6'A,H\text{-}6'B}$ = 11.8 Hz, $J_{H\text{-}6'A,H\text{-}5'}$ = 6.5 Hz) and H-6′B (δ 4.30, dd, $J_{H\text{-}6'A,H\text{-}6'B}$ = 11.8 Hz, $J_{H\text{-}6'B,H\text{-}5'}$ = 1.9 Hz) indicated acetylation at C-6′, which was confirmed by the HMBC correlations from H-6′A, H-6′B, and H-1″ to C-2″ as well as the NOE correlation between H-6′A/H-6′B and H-1″. Lastly, the HMBC correlation from H-1′ to C-2 and the NOE correlation between H-1′ and H-2 revealed that the glucopyranoside was attached to C-2. All the 2-glycosylated BXs isolated from the plants have the 2*R* configuration [35]; thus, **1** is tentatively assigned the 2*R* configuration based on biosynthetic arguments. Compound **1** is thus (2*R*)-2-(6-acetyl-β-D-glucopyranoside)-2,4-dihydroxy-2*H*-1,4-benzoxazin-

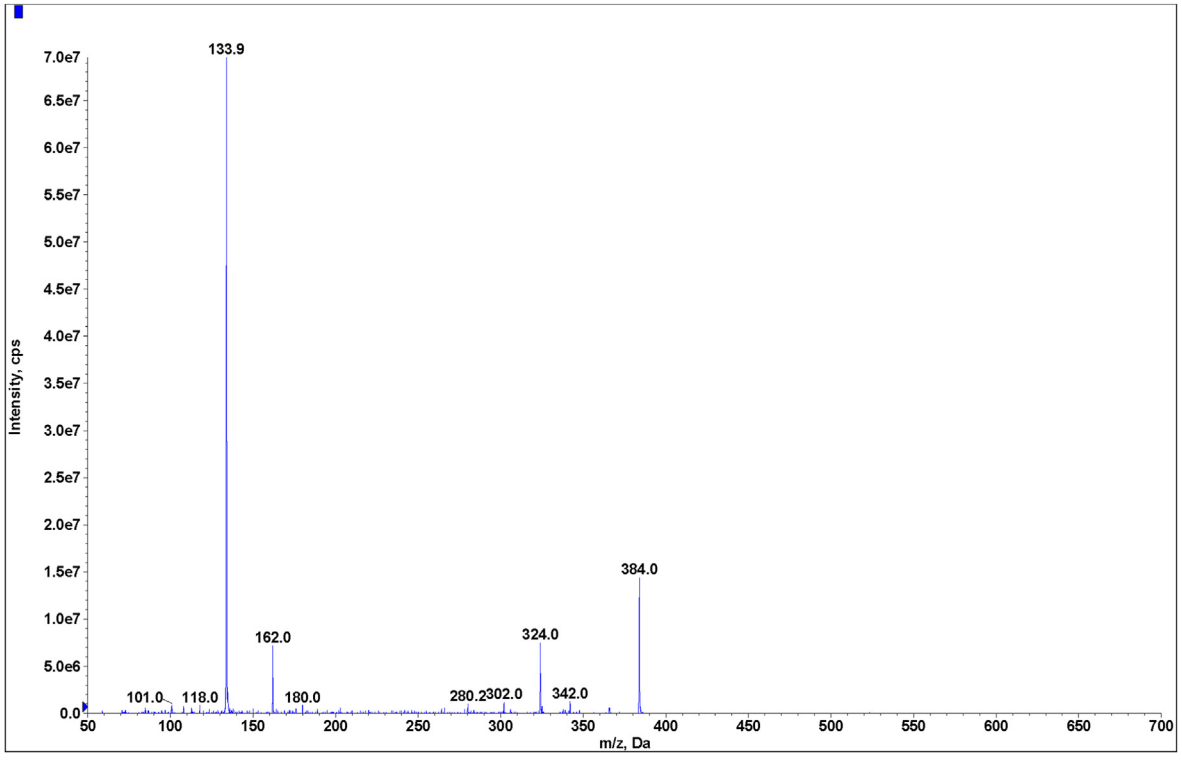


Fig. 2. MS/MS spectrum of potential DIBOA-glc-acetyl (compound **1**).

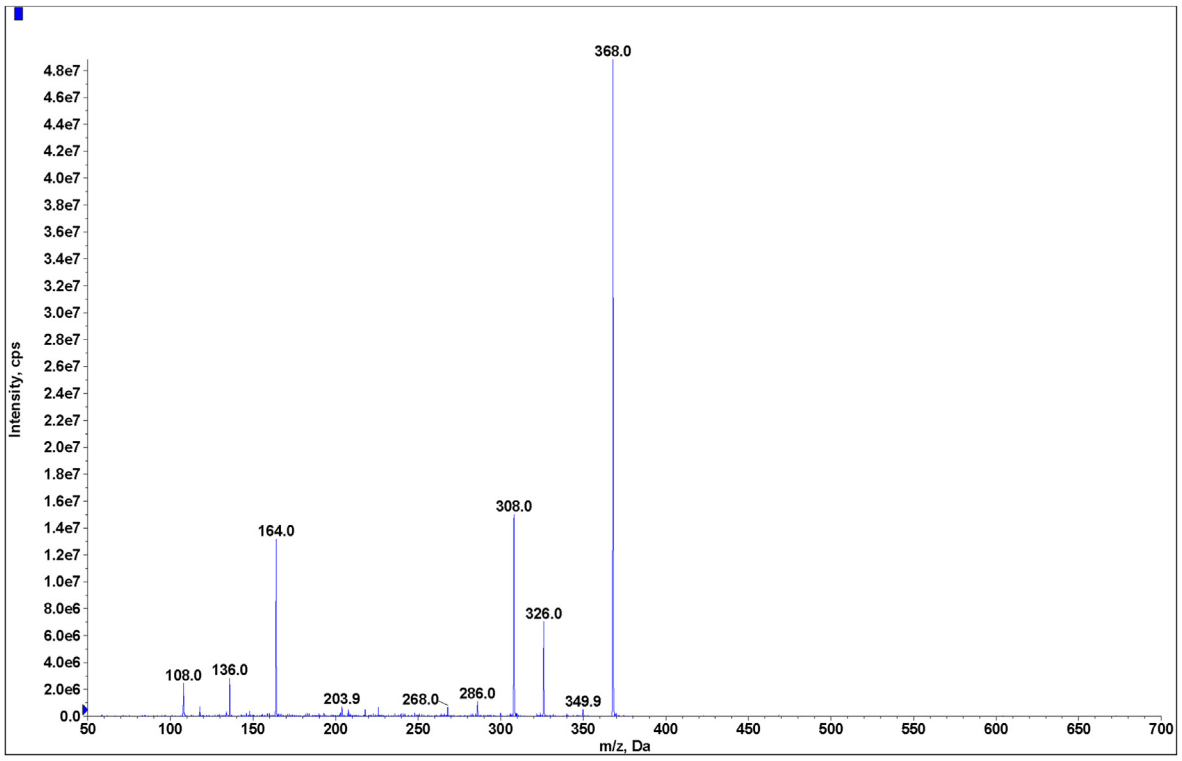


Fig. 3. MS/MS spectrum of potential HBOA-glc-acetyl (compound **2**).

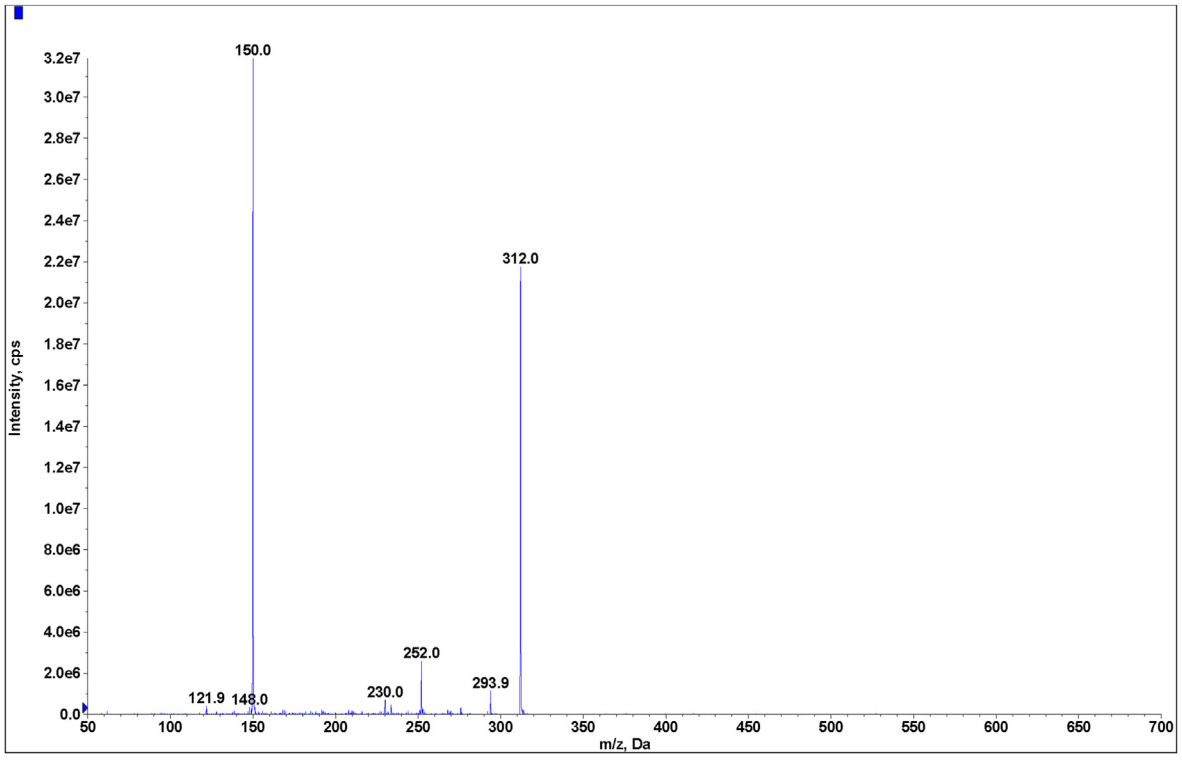


Fig. 4. MS/MS spectrum of potential OH-BOA conjugate (compound **3**).

3(4*H*)-one (Fig. 5). The ^{1}H NMR, COSY, NOESY, HSQC, and HMBC spectra of compound **1** are provided as Figs. S1—S5 in the Supporting Information.

Compound **2** was only available in sufficient amount for ^{1}H NMR. Notably, the ^{1}H NMR spectra of **1** and **2** were almost identical, except for the upfield shift of the H-5 signal from δ 7.48 in **1** to δ 6.97

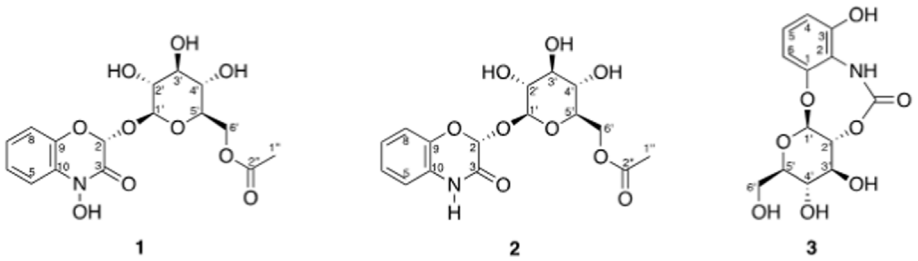


Fig. 5. Structures of compounds **1**–**3**.

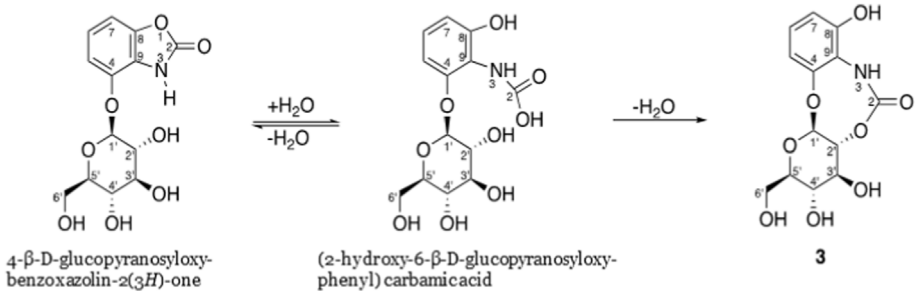


Fig. 6. Proposed rearrangement of 4-β-D-glucopyranosyloxy-benzoxazolin-2(3*H*)-one to form compound **3**.

in **2** (Table S3). Compound **2** is thus (2*R*)-2-(6-acetyl-β-D-glucopyranoside)-2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (Fig. 5). The ^{1}H NMR spectrum of compound **2** is provided as Fig. S6 in the Supporting Information.

Compound **3** was further analyzed using NMR, and the ^{1}H and ^{13}C NMR spectral data are presented in Table S4. The ^{1}H NMR spectrum revealed signals consistent with a 1,2,3-trisubstituted benzene ring system, i.e., a doublet for an *ortho*-coupled doublet with $J_{H\text{-}6,H\text{-}7} = 7.7$ Hz at δ 6.61 for H-7, an *ortho*-coupled triplet with $J_{H\text{-}6,H\text{-}7} = J_{H\text{-}5,H\text{-}6} = 7.7$ Hz at δ 6.49 for H-6, and a broad *ortho*-coupled doublet with $J_{H\text{-}5,H\text{-}6} \approx 7.7$ Hz at δ 6.58 for H-5 as well as a β-D glucopyranoside, i.e., H-1′ (δ 4.83, 1H, d, $J_{H\text{-}1',H\text{-}2'} = 7.9$ Hz; δ_C 103.1), H-2′ (δ 3.09, 1H, br dd, $J_{H\text{-}1',H\text{-}2'} \approx 7.9$ Hz, $J_{H\text{-}2',H\text{-}3'} \approx 8.9$ Hz; δ_C 72.9), H-3′ (δ 3.21, 1H, t, $J_{H\text{-}2',H\text{-}3'} = J_{H\text{-}3',H\text{-}4'} = 8.9$ Hz; δ_C 76.6), H-4′ (δ 3.04, 1H, br t, $J_{H\text{-}3',H\text{-}4'} = 8.9$ Hz, $J_{H\text{-}4',H\text{-}5'} \approx 9.2$ Hz; δ_C 69.7), H-5′ (δ 3.17, 1H, m; δ_C 77.3), H-6′A (δ 3.44, 1H, dd, $J_{H\text{-}6'A,H\text{-}6'B} = 11.7$ Hz, $J_{H\text{-}6'A,H\text{-}5'} = 6.0$ Hz; δ_C 60.9), and H-6′B (δ 4.30, dd, $J_{H\text{-}6'A,H\text{-}6'B} = 11.7$ Hz, $J_{H\text{-}6'B,H\text{-}5'} = 1.5$ Hz; δ_C 60.9). The NOE correlation between H-5 and H-1′ as well as the HMBC correlation from H-1′ to C-4 confirmed that the glucoside was positioned at C-4. The downfield shift of C-8 (δ 157.7) suggested a hydroxy group at this position, whereas the chemical shift of 136.1 ppm for C-9 as well as the molecular mass of 313 Da suggested ring closure via a NH–C=O-unit at C-9. Ring closure via anDDA NH–C=O-unit to the oxygen at C-8 would result in the formation of a benzoxazolin-2(3*H*)-one, which is inconsistent with the observed ^{1}H NMR chemical shift values. Thus, based on the downfield shift of H-2′ relative to H-4′, which is opposite to the trend observed for compound **1**, a structure in which an ester is formed between the carboxylic acid and the hydroxy group at C-2′ is suggested, as shown in Fig. 5.

Compound **3** is thus (2-hydroxy-6-(2-deoxy-β-D-glucopyranosyloxy)-phenyl) carbamic acid 2′-olide. This structure can be considered a rearrangement of the 4-*O*-glucopyranoside of 4-hydroxy-1,3-benzoxazolin-2(3*H*)-one (4-OH-BOA), as illustrated in Fig. 6. The ^{1}H NMR, COSY, ROESY, HSQC, and HMBC spectra of compound **3** are provided as Figs. S7–S11 in the Supporting Information.

The formation of related carbamates via the rearrangement of *N*-glucosides of BOA has previously been reported to occur both in planta and in solution [35–37]. The chemical shift of C-1′ (103.1 ppm) in compound **3** is in agreement with that of an *O*-glucoside rather than an *N*-glucoside. Various structural isomers of OH-BOA exist with the hydroxyl group at C-6 (most common), C-4, C-5, or C-7 [38]. It is possible that 4-O-glycosylated 4-OH-BOA underwent rearrangement to form the new glycoside carbamate because the hydroxyl group at the C-4 position provides a greater degree of freedom for ring opening and rearrangement (Fig. 6). Sicker et al. [37] described the polymerization of 4-, 5-, and 6-OH-BOA in corn plant.

4. Conclusion

The isolation of new BXs demonstrates the potential utility of our method. There have been many previous reports on the isolation of BXs from *A mollis* via bioactivity guided fractionation which did not lead to the discovery of new BXs. Till date only acetylated glycosylated forms of HMBOA have been characterized in *Rhizopus*-elicited wheat [28]. Our findings of acetylated DIBOA-glucoside and acetylated HBOA-glucoside (compounds **1** and **2**) in the roots of *A. mollis* L. inspires new studies of whether more naturally occurring acetylated forms of BXs occur in *A. mollis* L. or other plant species. Recent studies have extended the role of BXs in plants from defense and allelopathy to shaping the plant microbiome [39,40]. However, further investigations are required to determine the role and fate of these newly identified BXs and the glucoside olide rearrangement product of 4-OH-BOA.

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Author contributions

Bina Bhattarai: Validation, formal analysis, Investigation, Data Curation, Writing-Original Draft, Writing-Review and editing. Stine K. Steffensen: Methodology, Validation, formal analysis, Data Curation, Writing-Review and editing, Visualization, Supervision. Dan Stærk: Methodology, formal analysis, Resources, Data Curation, Writing-Review and editing, Visualization. Bente B. Laursen: Methodology, formal analysis, Investigation, Supervision. Inge S. Fomsgaard: Conceptualization, Methodology, Resources, Writing-Review and editing, Supervision, Project administraton, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijms.2022.116815.

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