Occurrence of 2-(2-Hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one)-β-D-glucopyranoside in *Triticum aestivum* Leaves and Its Conversion into 6-Methoxy-benzoxazolinone

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Triticum aestivum, Gramineae, 2-(2-Hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one)-β-D-glucopyranoside, 6-Methoxy-benzoxazolinone

Cyclic hydroxamic acids occurring in the Gramineae have been reported to be involved in the resistance of cereals to fungi and insects. Here we describe the occurrence, in wheat leaves, of 1,4-benzoxazinone derivatives and their glucosylated forms, a major representative of which was found to be 2-(2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one)-β-D-glucopyranoside. Owing to its spontaneous conversion into 6-methoxy-benzoxazolinone the corresponding aglucone could not be isolated following enzymic cleavage. We propose a plausible mechanism for this reaction which also explains the higher stability of other members of the 1,4-benzoxazinone family.

Introduction

The glucosides of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA), 2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (HMBOA), 2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one (HDIBOA) and of a number of related cyclic hydroxamic acids have been found to occur in gramineous plants [1–4]. It has been suggested that DIMBOA plays a role in plant resistance against insects [5–7] and several pathogens [8–12]. Whilst the 1,4-benzoxazinones occur only as glucosides in plants, the occurrence of the free aglucones has been questioned [13].

In aqueous solution near neutral pH, free DIMBOA decomposes to 6-methoxy-benzoxazolinone (MBOA) [14]. The yield varies with temperature and pH: at 28 °C, the half-life is 5.3 h at pH 6.75 and about 50 h at pH 5.0 [15]. Niemeyer *et al.* [16] suggested that the hydroxamic hydroxyl oxygen acts as a nucleophile in the rate-determining step of the reaction.

The present investigation is part of a programme aimed at elucidating the role of cyclic hydroxamic acids in host-pathogen interactions. Here we report on the occurrence of these acids in wheat leaves and the rapid formation of MBOA as a consequence of the enzymic cleavage of HDIBOA glucoside.

Results

During our examination of 1,4-benzoxazinones in wheat plants we encountered, in addition to glucosides of DIMBOA and HMBOA, a substance which occurred in high concentrations and which, owing to its spectroscopic characteristics and instability, was reminiscent of a hitherto little-considered benzoxazinone glucoside; Hofman *et al.* [2] had proposed for this the structure 2-(2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one)-β-D-glucopyranoside but they had not found it possible to isolate the corresponding aglucone.

In reexamining the problem we were able to confirm that the substance in question is cleaved by

Abbreviations: DIMBOA, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one; HDIBOA, 2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one; HMBOA, 2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3-one; HPLC, high performance liquid chromatography; MBOA, 6-methoxy-benzoxazolinone.

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β-glucosidase. By applying aliquots of the reaction mixture directly to the HPLC column we were able to observe the intermediate appearance of a substance 'X_1' (Fig. 1 – I) which was apparently converted to a product 'X_2' after prolonged reaction time; 'X_2' showed the same retention time as MBOA (**14**). As a rule, attempts to isolate 'X_1' from the enzyme mixture (*e.g.* by shaking out, with resulting concentration of the organic phase, or by means of preparative HPLC and concentration of the eluate) failed. 'X_2' was that product always found and 'X_1', at most, in only trace concentrations.

Mass spectroscopic analysis confirmed that the conversion product 'X_2' was indeed MBOA (**14**). The spectra in both cases were obviously identical (Fig. 2 – I + II).

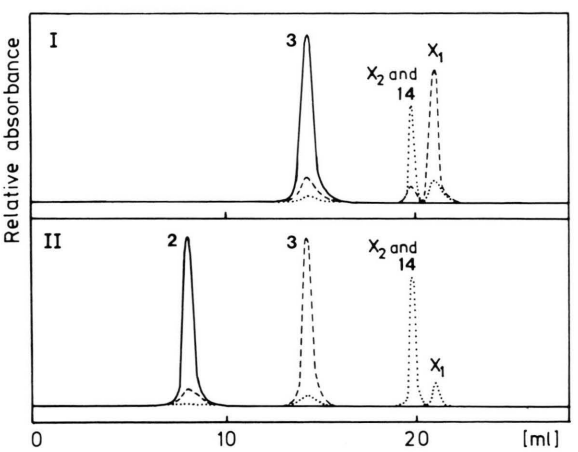


Fig. 1. HPLC elution profiles of DIMBOA glucoside (**2**), HDIBOA glucoside (**3**) and products obtained after chemical or enzymic conversions. Numbers of the compounds refer to structures shown in Fig. 5. Reversed phase column C_{18} 250×8 mm, H_2O/MeOH (70:30), isocratic elution 1.8 ml/min, UV detection at 210 nm.
I: Products formed from **3** following enzymic cleavage by β-D-glucosidase. Solid line = **3** before treatment. Dashed line = formation of 'X_1' which can be detected when the reaction mixture is directly applied to the HPLC column without preliminary partitioning or concentration. Dotted line = 'X_1' is converted into 'X_2' when the reaction mixture is left to stand for a short while after formation of 'X_1' (see also Fig. 3) or when it is tried to prepurify and concentrate 'X_1' prior to the application to the column. The retention volume of 'X_2' corresponds to that of MBOA (**14**).
II: The isolated material supposed to be HDIBOA glucoside (dashed line, **3**) was produced by methylation of DIMBOA glucoside (solid line, **2**) with diazomethane. Product **3** was then treated with β-glucosidase to form 'X_1' and, finally, 'X_2'. This result confirms that the isolated material, in fact, was HDIBOA glucoside (**3**).

We were able to confirm by the following experiments that the isolated material was HDIBOA glucoside (**3**): methylation of **2** by diazomethane apparently led to the formation of **3** (Fig. 1 – II). The mass spectra obtained from **3** isolated from leaves and the material produced by methylation were identical (Fig. 2 – III, only one spectrum is shown). Also, glucosidase treatment of the methylated product led directly to the formation of **14** *via* 'X_1' (Fig. 1 – II; result confirmed by mass spectroscopy). An attempt to obtain **6** by methylation of **5** also led to the formation of **14**, in addition to several unidentified products (results confirmed by HPLC and mass spectroscopy).

Any attempt to identify 'X_1' by mass spectroscopy failed even when it was tried to introduce a sample directly into a mass spectrometer within 30 min after preparation. The resulting spectrum (not shown) was generally similar to the spectrum of **14** in that all characteristic fragments were present. It should be mentioned, however, that it may be difficult to differentiate between the mass spectra of **13** and **14**.

Although neither the isolation nor the concentration of 'X_1' from the HPLC eluate proved successful, the substance was still, in the non-concentrated eluate, adequately stable. Its conversion to MBOA could therefore be followed UV spectroscopically (Fig. 3), when the instability of 'X_1' (curves a–e) was seen to be markedly increased compared to **5** (curve f) which, in fact, was rather stable under the same conditions at room temperature. As may be expected there is a pronounced similarity between the absorption spectra of **2**, **3**, and **5**. However, the spectrum of 'X_1' is different in that it exhibits a bathochromic shift of about 60 nm. Since it is difficult to imagine that the absorption spectrum of **6** differs significantly from the spectra mentioned above, it is suggested that 'X_1' is identical with an intermediate in the reaction pathway **6** → **14**.

The thermally-induced degradation reaction **5** → **14** has been studied in the past (see Introduction). In agreement with the literature, we too were able, with the aid of HPLC techniques, to observe such a temperature dependent conversion. **14** here appeared as the major product in addition to several unknown side products. In this case, however, it was not possible to detect an intermediate like 'X_1' with similar properties with respect to instability and UV absorption.

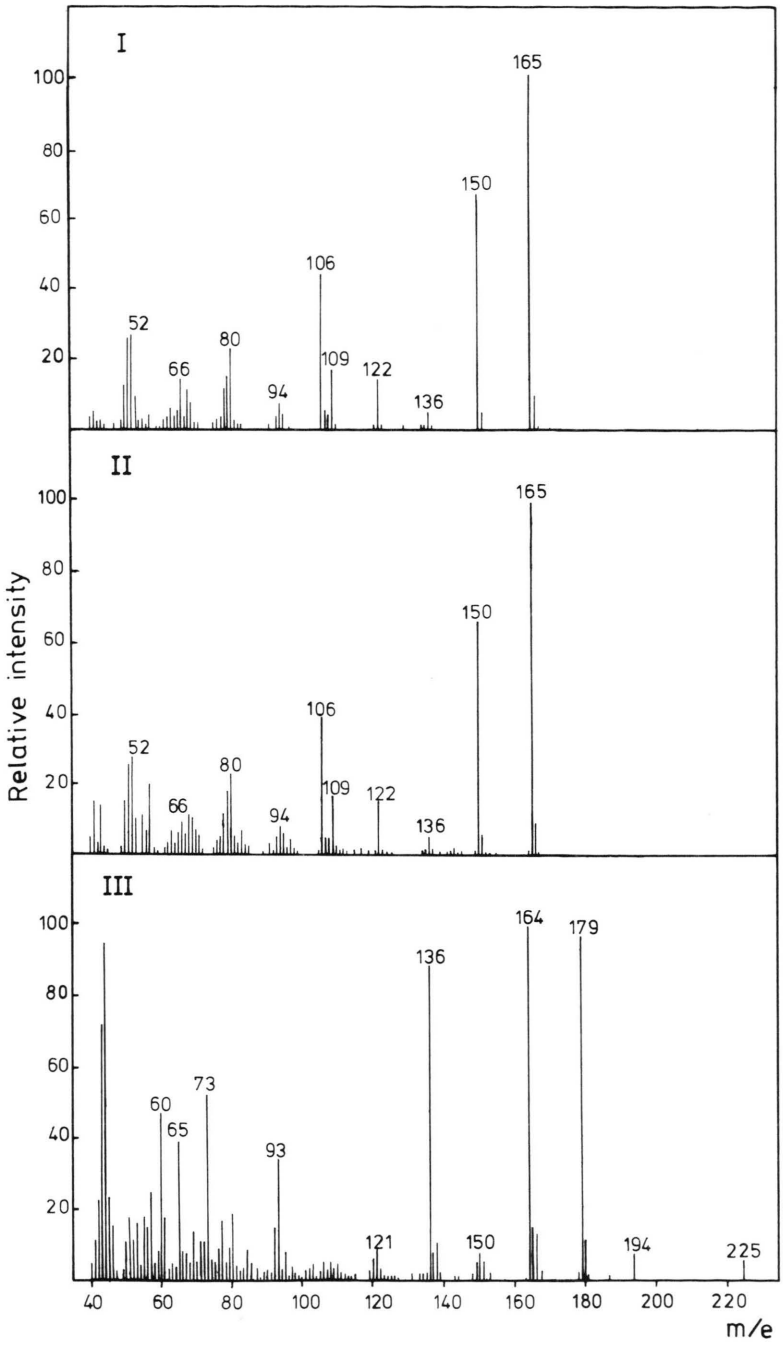


Fig. 2. I: Mass spectrum of MBOA (**14**). II: Mass spectrum of 'X_2' which, apparently, is identical with the spectrum of **14**. III: Mass spectrum of the isolated material supposed to be HDIBOA glucoside (**3**) obtained after direct application of the compound to the mass spectrometer. The spectrum of the material obtained by methylation of DIMBOA glucoside was identical with that shown here (*m/e* 179 apparently is derived from the glucose and *m/e* 225 with the corresponding aglucone moiety).

Discussion

HMBOA glucoside (**1**), DIMBOA glucoside (**2**) and HDIBOA glucoside (**3**) are the main 1,4-benzoxazinone derivatives in leaves of *Triticum aestivum*, cv. 417/65. In contrast to HMBOA (**4**) and DIMBOA (**5**) free HDIBOA (**6**) could not be detected in extracts and in aqueous solutions of the corresponding glucoside, containing β-glucosidase. Instead, glucosidase action resulted in the formation of MBOA (**14**).

The occurrence of **3** in Gramineae has already been reported once [2], when it was also recognized

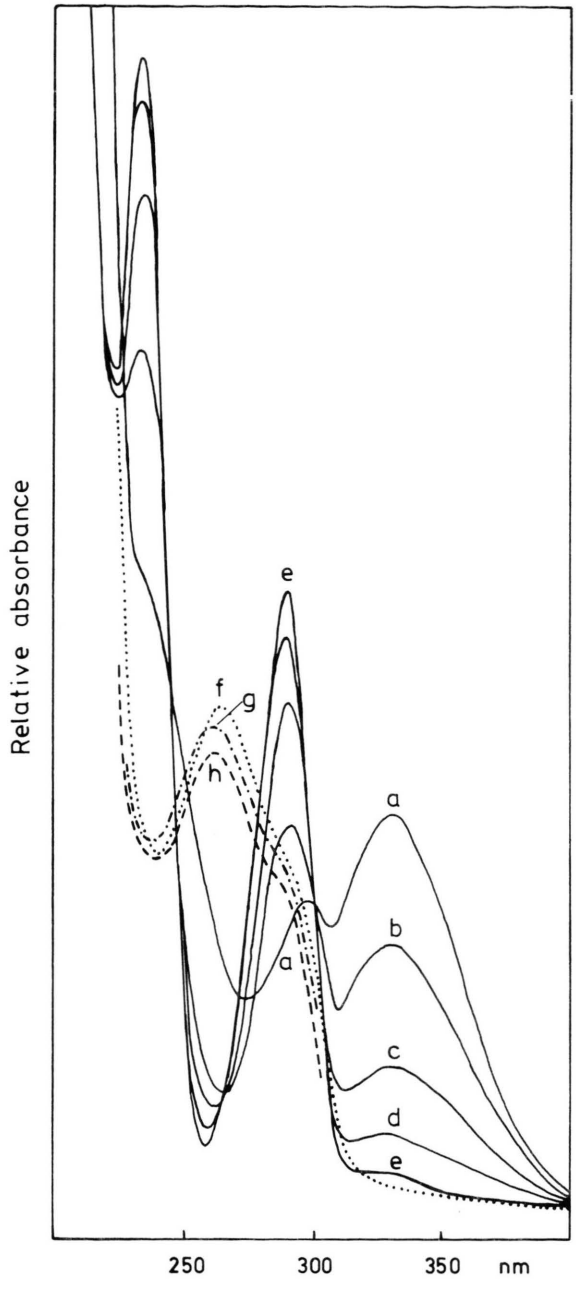


Fig. 3. UV absorption of the reaction products obtained after enzymic cleavage of HDIBOA glucoside (**3**) and direct application of the reaction mixture to the HPLC column (reverse phase C_{18}; H_2O/MeOH (70:30)) without preliminary partitioning or concentration.
a) UV absorption of compound 'X_1' in H_2O/MeOH (70:30) measured immediately after HPLC elution.
b–e) UV absorption spectra after 'X_1' in the solvent system had been allowed to stand at room temperature for 30, 60, 90, or 120 min. Under these conditions the conversion of 'X_1' into **14** (MBOA, curve e) was virtually complete after 120 min.
f) UV absorption of DIMBOA (**5**) which, under the same conditions, did not markedly change within 120 min.
g) UV absorption spectrum of DIMBOA glucoside (**2**).
h) UV absorption spectrum of HDIBOA glucoside (**3**).

that following hydrolysis, no corresponding aglucone could be detected but rather a product which exhibited the same absorption characteristics in the UV region as **14**. That these two were in fact identical was not shown. In contrast, the (slower) rearrangement of **5** into **14** is a well-known reaction which has been the subject of several investigations [1, 15, 17] and has formed the basis of suggestions regarding possible intermediates [16, 18].

The observations on the chemical relationship between benzoxazinone derivatives and **14** which we have described above, as well as those reported in the literature, are summarized in Fig. 4. The extremely low stability of **6** which had, up till now, prevented the detection of the free aglucone raises the question of the cause for the different stability of the various benzoxazinone aglucones.

In the case of **5**, the hydroxamic hydroxyl group has been assigned the role of a nucleophile. The nucleophilic attack has been presumed to occur on the carbonyl group of the ring-opened structure (**16**) and this to lead to the isocyanate intermediate (**13**) and formic acid (Fig. 5, route A). One of the arguments appearing to support this hypothesis is the significant increase in the reaction rate by conversion of **5** into the mono anion **15** [16]. However, the observation that after its release, **6** (HDIBOA) reacts

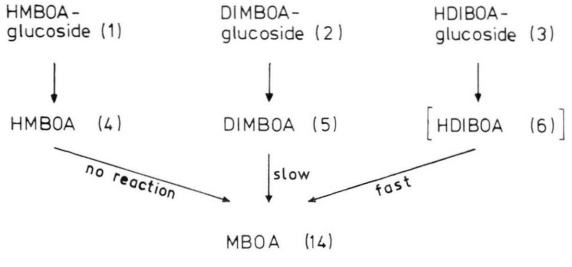
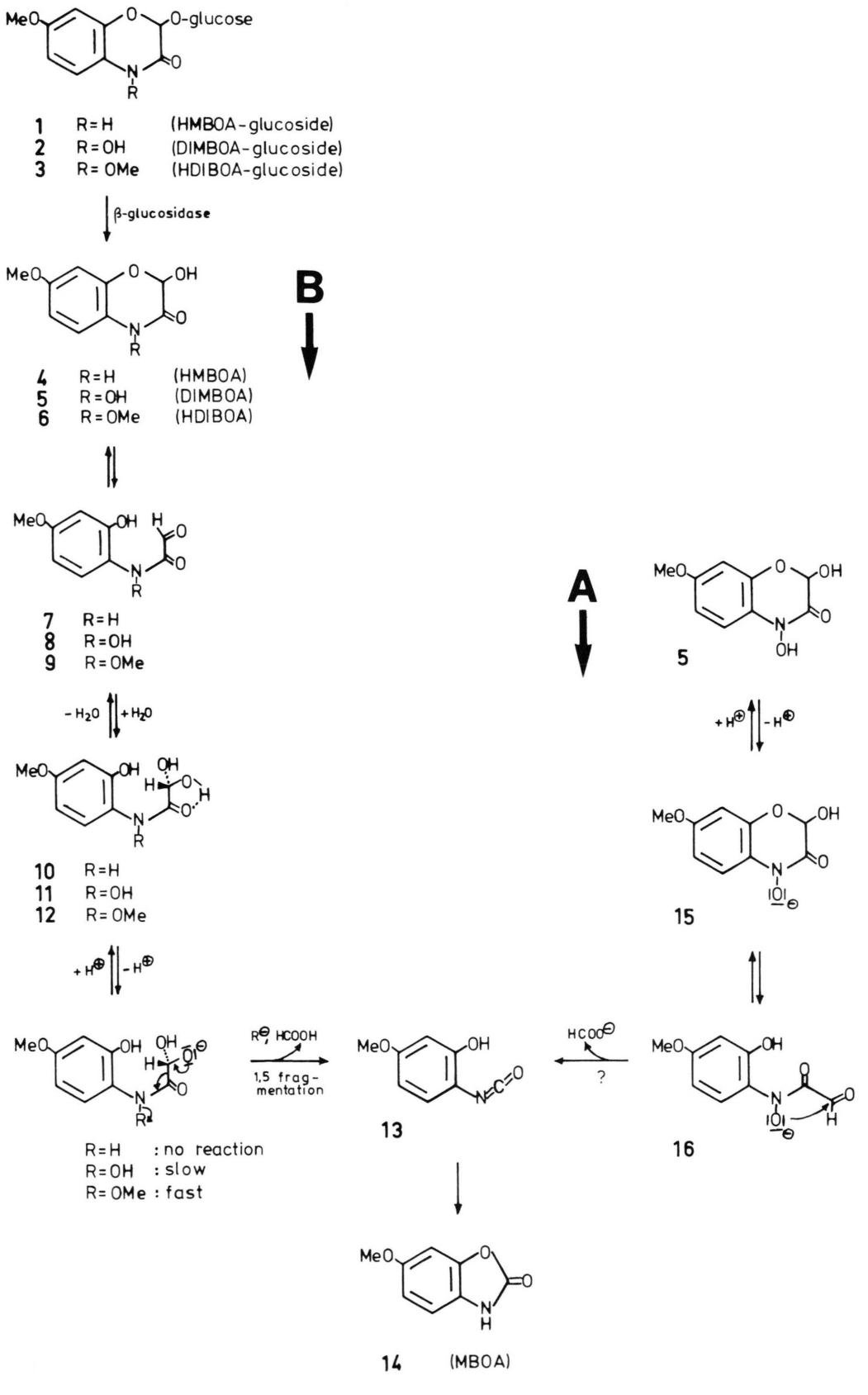


Fig. 4. Relationship between various derivatives of cyclic hydroxamic acids occurring in wheat leaves and MBOA (**14**). Numbers **1**–**6**, and **14** refer to structures shown in Fig. 5. **6** could not be isolated owing to its high instability.


MeO
O-glucose
1 R=H (HMBOA-glucoside)
2 R=OH (DIMBOA-glucoside)
3 R=OMe (HDIBOA-glucoside)
β-glucosidase
B
4 R=H (HMBOA)
5 R=OH (DIMBOA)
6 R=OMe (HDIBOA)
A
7 R=H
8 R=OH
9 R=OMe
- H2O
+ H2O
10 R=H
11 R=OH
12 R=OMe
+ H⊕
- H⊕
R⊖, HCOOH
1,5 frag-mentation
HCOO⊖
?
13
14 (MBOA)
15
16
R=H : no reaction
R=OH : slow
R=OMe : fast

completely to form **14** does not concur with the postulate of a nucleophilic attack.

Thus we propose a mechanism which may explain the results described in the literature as well as those of our own observations (Fig. 5, route B). Based on this, the compounds HMBOA (**4**), DIMBOA (**5**) and HDIBOA (**6**) which are released following enzymatic hydrolysis of the glucosides exist in equilibrium with the hydrated species **10**, **11** and **12** *via* the open-chain structures **7**, **8** and **9**. Deprotonation of the ring-opened aldehyde hydrate and subsequent heterolytic 1,5-fragmentation (Grob-fragmentation), under formation of formic acid, leads to the isocyanate intermediate **13** which spontaneously cyclises to **14** (MBOA). The different reaction rates thus would result from the likewise different leaving-group properties of the substituents R^- in position **4** ($R^- = OMe^- > OH^- >>> H^-$). In the case of **4** (HMBOA) no more fragmentation takes place.

The pH dependence of the conversion of **5** (DIMBOA) to **14** observed by Niemeyer *et al.* [16], can be qualitatively interpreted on the basis of our proposed reaction mechanism. At low pH values ($pH < 4$) the required deprotonation of the ring-opened aldehyde hydrate (**11**) does not occur to a sufficient extent: thus there is no fragmentation. At high pH values ($pH > 11$) the hydroxamic acid is nearly completely converted into its anion which possesses no leaving-group properties.

The observation that after conversion of **2** to **3** by methylation with diazomethane and subsequent treatment of the reaction product with β-glucosidase, **14** is obtained as the major product, is also in agreement with the concept described here, as indeed is the production of **14** when attempting to methylate **5** with diazomethane. In the latter case, after methylation of the acidic hydroxamic hydroxyl group, **6**, which is formed primarily, rearranges to form the postulated isocyanate. This reaction apparently takes place more rapidly than the additional methylation of the less acidic 2-OH group.

The results clearly show that the aglucone **6** which is to be expected following glucosidic cleavage of **3** is unusually unstable and cannot be readily isolated. The question arose, whether, in cleavage, the substance 'X_1' which appears intermediately on the HPLC chromatogram, is identical with **6** or with an intermediate in the reaction pathway **6** → **14**. Considering the UV absorption spectra the available information appears to exclude the first alternative. In agreement with the proposed reaction mechanism (Fig. 5), instead, it appears to be more likely that 'X_1' is identical with an intermediate following the fragmentation reaction. At present, however, direct evidence to support such an assumption does not appear to be readily available.

The function of benzoxazine derivatives in Gramineae has been investigated several times with regard to the resistance to pathogens (see Introduction). In wheat rust interactions, **1**, **2**, **3**, **4** and **5** do not appear to play a role owing to their low toxicity to the rust fungus (preliminary results not shown here). However, cleavage of **3** by glucosidase action as a result of pathogen attack would result in the rapid formation of the very toxic MBOA (**14**) which would then interfere with pathogen development. Such a mechanism could well indeed be important in some types of host-pathogen interactions (work in progress).

Experimental

Extraction

Primary and secondary leaves of 10 day old wheat plants (*Triticum aestivum*, cv. 417/65) cultivated under controlled conditions (light period 16 h, 30 WM^{-2} Osram HQJ-E 400 W, humidity 60%, temperature 21 °C/17 °C day/night) were pulverized under liquid N_2 and extracted with MeOH at room temperature. After filtration, the coloured extract was evaporated to dryness, resuspended in a small volume of MeOH (5 ml/10 g leaf material), and refiltered.

TLC separation

The extract was chromatographed on thin layer plates (silica gel with 254 nm fluorescent indicator, thickness 0.5 or 0.25 mm; 5–10 plates 25 × 25 cm/10 g extracted leaf material). The solvent system was $CHCl_3$/MeOH/H_2O (72:25:3). The compounds **1**–**5**

Fig. 5. Proposed mechanism of the conversion of cyclic hydroxamic acids into MBOA (**14**). Route A involves a nucleophilic attack on the carbonyl group of the ring-opened structure **16** as suggested by Niemeyer *et al.* [16] for DIMBOA (**5**). Route B is proposed to explain the effects of the N-substituents on the stability of the various compounds under consideration (see text). The proposed mechanism involves deprotonation of the ring-opened aldehyde hydrate and subsequent heterolytic 1,5-fragmentation (Grob-fragmentation) leading to the isocyanate intermediate **13** (this paper).

and **14** were easily detected as dark zones at 254 nm UV-light: R_f (**1**) = 0.2, R_f (**2**) = 0.15, R_f (**3**) = 0.36, R_f (**4**) = 0.55, R_f (**5**) = 0.51, R_f (**14**) = 0.72. In addition, the N-OH substituted compounds **2** and **5** were rendered visible as blue spots after spraying with an aqueous solution of $FeCl_3$.

HPLC

The semipurified compounds were further purified by the use of the following HPLC systems:

(a) Reverse phase LiChrosorb RP_{18}, 250 × 8 mm and 250 × 4 mm, particle size 7 μm, solvent system H_2O/MeOH (70:30), flow rate 5 ml/min (preparative scale) or 1.8 ml/min (analytical scale), photometric detection at 280 nm or 210 nm. Material from 1.5 g leaf powder, dissolved in 100 μl MeOH, was applied to the preparative column.

(b) Reverse phase, Bondapak phenyl, 300 × 4 nm, solvent system H_2O/MeOH (75:25), flow rate 1.8 ml/min.

After concentration, the compounds were stored at −20 °C. Purity was rechecked before carrying out the experiments.

Chemical and enzymic treatments

To methylate, **2** was treated with excess diazomethane in ether or methanol solution. The formation of **3** was followed by TLC. After completion of the reaction, the product was purified using TLC and HPLC as described above.

To produce enzymic cleavage, the various glucosides were treated with β-glucosidase in aqueous or buffered solution (pH 5). The reaction mixture was applied directly to a disposable C_{18} column (Baker 10 extraction system). After washing with H_2O, the reaction products were eluted with MeOH, concentrated, and further purified by TLC and HPLC. Alternatively, aliquots of the reaction mixture were directly applied to the HPLC column in order to follow the progress of the reaction or the appearance of compound 'X_1' (in the case of compound **3**).

The yields of the reaction products have not been precisely determined in this study. However, it appeared that after glucosidase treatment the conversion of **3** into **14** *via* **6** was quantitative (as judged from the HPLC elution profiles). Similarly, there was a high yield of **3** after methylation of **2**.

Mass spectrometry

The compounds were directly applied in methanolic solution to the mass spectrometer (Finnigan-MAT 212; electron impact ionization; 70 eV; 1 mA; source temperature 200 °C; direct evaporation).

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