Metabolism of the Plant Growth Regulator (E)-[3H]2-Ethylhex-2-enoic Acid in Hordeum vulgare

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Metabolism, Plant Growth Regulator, Hydroxylation, Conjugation, Structural Elucidation

The metabolism of (E)-[3H]2-ethylhex-2-enoic acid (EHA) was studied in excised seedlings of barley (Hordeum vulgare) and wheat (Triticum aestivum).

Conjugation with carbohydrates is a common pathway in metabolism of xenobiotics [1]. Especially glucose was found as the conjugating moiety, but there is also an increasing number of examples for conjugation with oligosaccharides [1, 2]. However, complete identification, especially of oligosaccharide conjugates, are rare. In our studies mainly ¹H-NMR spectroscopy was used for detailed structural determination of EHA conjugates.

Beside the complete structure necessary for understanding the biochemical role of carbohydrate conjugates investigations regarding their interconversion are important. Therefore, the time course of EHA metabolism was investigated. Furthermore, [³H]EHA-Glc was applied to barley seedlings to follow its transformation.

Abbreviations: EHA, (E)-2-ethylhex-2-enoic acid; TCL, thin layer chromatography; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry; Glc, β-D-glucosyl; LSC, liquid scintillation counting; TMS, tetramethylsilane; OH-EHA, (E)-2-ethyl-4-hydroxy-hex-2-enoic acid; Gent, β-gentiobiosyl; M, molecular ion peak; m/z, mass/charge; GOD-POD, glucose oxidase – peroxidase.

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In addition to conjugation reactions xenobiotics may undergo oxidation processes in plants [3]. Alkyl hydroxylation, especially of methyl groups, was observed for several xenobiotics. If the alkyl chains longer than methyl groups were involved, hydroxylation takes place at the ω-carbon atom [3]. However, from animals [4] and microorganisms (ω-ν)-hydroxylations are known. In this paper (ω-2)-hydroxylation of EHA in barley plants became evident by ¹H-NMR spectroscopy, indicating simultaneously the potential of this method in aglycon determination.

Material and Methods

Radiochemicals

[³H]EHA was labeled with tritium by means of catalytic exchange of hydrogen corresponding to the method of Pleiss et al. [5] by Isocommerz GmbH, Dresden-Rossendorf, GDR. [³H]EHA-NH₂ was prepared by treating [³H]EHA with aqueous 25% ammonia followed by evaporation. [³H]EHA-Glc was isolated as a metabolite of [³H]EHA from barley and purified by TLC. Specific radioactivities were 840 MBq·mmol⁻¹.

Plant material and application

Seedlings of barley (Hordeum vulgare cv. Vogelsanger Gold) were cultivated in soil in greenhouse at 23–25 °C on a daily photoperiodic regime of 15 h light and 9 h darkness. In each experiment 10 g of 6-day-old excised seedlings were immersed for increas-
ing time intervals (6, 12, 24, 48, 72 h) in 5 ml aqueous solution containing 1.0 mg (1.26 mmol-l⁻¹) [³H]EHA-NH₄. For the aseptic experiment plants were treated for 1.5 min with 1% NaClO₂ solution containing 0.1% Tween 60. Application was carried out for 24 h within a glass apparatus similar to those of Metche and Piffaut [6].

Isolation and quantification of metabolites
Following incubation the plants were rinsed several times with water, cut to small pieces, homogenized with an Ultra-Turrax (Janke u. Kunkel KG, Staufen i. Br., FRG) in 80% aqueous methanol, and washed with 50 ml methanol. The procedure outlined in Fig. 1 was used to fractionate the crude methanol extract. Radioactivity of aliquots of all fractions was determined by LSC with a Tricarb 2660 (Packard Instruments, Chicago, USA). Before LSC the solid residue was combusted with a Micro-Mat BF 5010 sample oxidizer (Berthold-Friesеke GmbH, Karlsruhe, FRG).

The metabolites from the ethyl acetate extract and from the methanol fraction were separated by TLC and purified by TLC and HPLC. For quantification of metabolites the ratios of aglycones, determined after hydrolysis of an aliquot of aqueous phase I, were used, and the integrals of peak areas from radioscans were evaluated.

TLC
Silica gel 60 HF₂₅₄ was used from Merck (Darmstadt, FRG). The plates (20 x 20 cm, 0.9 mm thick for preparative TLC and 0.3 mm thick for comparative TLC) were developed successively with the following solvent systems:
1. n-hexane:acetone 75:25 (v:v)
2. benzene:methanol:acetic acid 96:8:4 (v:v:v)
The TLC plates were analyzed for radioactive zones with a Radioscanner II (Berthold, Wildbad, FRG).

HPLC
A Serva Si 100 polyol RP 18 column (5 μm, 4.6 x 250 mm) was fitted with an RCT HPLC eluent supply and a PYE Unicam PU 4020 detector set on 254 nm. Isocratic elution with 80% aqueous methanol (1 ml-min⁻¹) was used.

Hydrolysis and derivatization
For hydrolysis samples of conjugates (2 to 8 μg supposed glucose, calculated from specific radioactivity) were dissolved in 1 ml McIlvan buffer, pH 3.0, and incubated for 20 h at 37 °C with 250 μg dialyzed cellulase.
Quantification of glucose was carried out by the GOD-POD method [7].
Acetylation was carried out with acetic anhydride : pyridine 2:1 (v:v). For methylation diazomethane was used.

Spectrometric methods
¹H-NMR spectra were recorded on a Bruker WP 200 spectrometer (Karlsruhe, FRG) at 200.13 MHz in CDCl₃. Chemical shift values are related to TMS.
Electron impact (2 to 4 eV) and electron attachment (10 to 16 eV) mass spectra were obtained with a “Manfred von Ardenne” mass spectrometer (Dresden, GDR).

Results
Metabolism
The applied [³H]EHA was rapidly absorbed by the excised barley plants. In a characteristic experiment the radioactivity inside the plants increased from 15% of the offered radioactivity 6 h after the beginning of the experiment to 67% after 72 h. In contrast, at the same time the recovered radioactivity (total radioactivity in the plants and the medium) slowly decreased from 82% to 68%. The radioactivity was distributed to the ethyl acetate extract, methanol fraction, insoluble residue, and precipitates (cf. Fig. 1). Table I demonstrates that the main

<table>
<thead>
<tr>
<th>Fractions</th>
<th>% of radioactivity absorbed</th>
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<tbody>
<tr>
<td>Time intervals</td>
<td>6 h</td>
</tr>
<tr>
<td>insoluble residue</td>
<td>0.5</td>
</tr>
<tr>
<td>precipitate I</td>
<td>3.0</td>
</tr>
<tr>
<td>ethyl acetate extract</td>
<td>59.8</td>
</tr>
<tr>
<td>precipitate II</td>
<td>2.2</td>
</tr>
<tr>
<td>methanol fraction</td>
<td>34.5</td>
</tr>
</tbody>
</table>

Table I. Distribution of radioactivity in fractions obtained from barley plants treated with [³H]EHA for increasing time intervals.
This assumption was confirmed by TLC of the metabolites from ethyl acetate and methanol. Characteristic radioscans for the time interval of 12 h are shown in Fig. 2. After further purification of the radiopeaks by TLC and HPLC the structures of the corresponding metabolites were determined as described below. Thus the radioactivity in the ethyl acetate extracts was shown to be associated with EHA, OH-EHA, and EHA-Glc. The methanol fractions contained another portion of EHA-Glc and the more polar conjugates OH-EHA-Glc, Glc-O-EHA, EHA-Gent, EHA-Glc₂ (a conjugate containing a glucosyl glucose which is different from β-gentiobiose), and non-identified higher conjugates. The time course of metabolism of EHA, which is shown in Fig. 3, was obtained by quantification of metabolites, formed after different time intervals. Fig. 3 shows that EHA was metabolized very rapidly. After 24 h only 1.8% were still detectable. The amount of EHA-Glc which was the initially formed metabolite after passing a maximum (6 h) significantly decreased. The disaccharide ester fraction after increase in concentration up to 48 h also slightly decreased. However, the concentration of higher con-

Fig. 2. TLC radioscans of (A) ethyl acetate extract and (B) methanol fraction from barley plants treated with [³H]EHA for 12 h. The TLC plates were developed successively with (A) solvent system 1 and 2 (two times) and (B) solvent system 2, 3, and 4 respectively.

Fig. 3. Time course of metabolism of [³H]EHA in barley. Percentages are related to the radioactivity which was absorbed by the plants (○ = EHA, □ = EHA-Glc, △ = EHA-Gent and EHA-Glc₂, ● = OH-EHA, ■ = OH-EHA-Glc, ▲ = Glc-O-EHA, ▽ = higher conjugates, × = insoluble residue, = = = = = total OH-EHA, free and conjugated).
Fig. 4. The metabolism of (E)-[^3]H2-ethylhex-2-enolic acid (EHA) in barley.

jugates increased during the whole incubation time. Corresponding to this time course of EHA metabolism, the formation of the higher conjugates goes on most probably by sugar chain elongation, but also the reaction of EHA with activated oligosaccharides cannot be ruled out.

Although there were only traces of free EHA present in the plants after 24 h, also in the later time the increase of total OH-EHA (free and conjugated) was about 4% in 24 h. Therefore, conversion of conjugated EHA into ester conjugates of OH-EHA must be assumed either by direct oxidation or via hydrolytically released EHA as an intermediate.

To decide between these two possibilities biochemically prepared [3H]EHA-Glc was fed back to excised barley plants. After 24 h the plants contained a pattern of metabolites which were present also after application of EHA. Thus 2% free EHA were detected, indicating certain reversibility of the conjugation process. Furthermore, 4% total OH-EHA were present in the plants as has been determined after hydrolysis of aqueous phase I. These percentages agreed with the increase of the OH-EHA content within 24 h obtained after application of EHA as mentioned above. Consequently the formation of OH-EHA and its conjugates from EHA-Glc very probably proceeded via free EHA. The presence of a normal level of Glc-O-EHA after application of EHA-Glc also indicates hydroxylation of intermediate EHA rather than direct hydroxylation of EHA-Glc since the direct way would imply transformation of OH-EHA-Glc into Glc-O-EHA.

Additional experiments were carried out to examine the formation of OH-EHA under aseptic conditions. The total content of free and conjugated OH-EHA within the plant extracts 24 h after application of EHA was 3.8%. This was not appreciably different from the results of the previous experiments (Fig. 3) and demonstrated that OH-EHA was a product of plant and not of microbial metabolism.

The pathways of EHA metabolism are summarized in Fig. 4.

Structural elucidation of OH-EHA

Chromatographic properties and results of derivatization (methylaration, acetylation) indicated another hydrophilic group (beside the carboxyl) in the molecule of OH-EHA. The presence of a hydroxyl group was confirmed by electron impact MS of its methyl ester (OH-EHA-Me, M' + 1 = m/z 173 (76%)) and other peaks which agreed with the suggested structure.

The position of the hydroxyl group was determined by comparing the 1H-NMR spectrum of OH-EHA-Me (Fig. 5B) with that of EHA-Me (Fig. 5A). The vinylic proton, which appears in the spectrum of EHA-Me as a triplet (6 6.72), has changed
its pattern into a doublet (δ 6.58), indicating the loss of one proton at C-4. Consequently the hydroxyl group should have occurred at this carbon atom. The double triplet at δ 4.38 was assigned to 4-H. Its coupling connectivities with 3-H and 5-H were established by decoupling experiments (Fig. 5C and D). The downfield shift of this proton in comparison to the corresponding signal of EHA-Me (δ 2.16) as well as its integral step are additional evidences that the hydroxyl group must be situated at C-4. The assignments of 6-H₃ and 8-H₃ signals were also supported by a decoupling experiment (Fig. 5E). The signal of the hydroxyl proton is located within the multiplet of the 5-H₂ protons, centered at δ 1.60. It disappeared after H/D exchange (Fig. 5F). Concerning the stereochemistry at the double bond the chemical shift of 3-H proves the maintenance of the E configuration [8]. From the above data the metabolite is considered to be (E)-2-ethyl-4-hydroxy-hex-2-enoic acid.

Structural elucidation of conjugates

¹H-NMR spectroscopy is a convenient method for identification of sugar conjugates. The spectra of EHA-GlcAc₄ and GlcAc₄-O-EHA-Me showed the acetyl groups of the acetylated hexose (four singlets each, δ 1.98 to 2.07 and δ 2.00 to 2.10, resp.). The spectrum of EHA-GentAc₇ confirmed the presence of seven acetyl groups, indicating two hexose units. The chemical shifts of the carbohydrate protons agreed well with those of the model compound GentAc₈ (Table II) and with ¹H-NMR data for related triglucoses, published by Rychener et al. [9, 10]. The ester linkages of EHA-GlcAc₄ and EHA-

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Table II. ¹H-NMR shifts (δ) of the carbohydrate moieties of acetylated EHA conjugates and vicinal coupling constants ³J (Hz) (CDCl₃, TMS, 200.13 MHz).

<table>
<thead>
<tr>
<th></th>
<th>EHA-GlcAc₄</th>
<th>GlcAc₄-O-EHA-Me</th>
<th>EHA-GentAc₇</th>
<th>GentAc₈</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ</td>
<td>HZ</td>
<td>δ</td>
<td>HZ</td>
<td>δ</td>
</tr>
<tr>
<td>1'-H</td>
<td>5.76</td>
<td>7.9</td>
<td>4.45</td>
<td>8.0</td>
</tr>
<tr>
<td>2'-H, 3'-H, and 4'-H</td>
<td>5.00 to 5.30</td>
<td>4.95 to 5.25</td>
<td>5.00 to 5.30</td>
<td>5.00 to 5.25</td>
</tr>
<tr>
<td>5'-H</td>
<td>3.85</td>
<td>3.64</td>
<td>3.82</td>
<td>3.79</td>
</tr>
<tr>
<td>6e'-H</td>
<td>4.10</td>
<td>4.14</td>
<td>3.61</td>
<td>3.57</td>
</tr>
<tr>
<td>6a'-H</td>
<td>4.31</td>
<td>4.23</td>
<td>3.93</td>
<td>3.94</td>
</tr>
<tr>
<td>1''-H</td>
<td>4.57</td>
<td>8.0</td>
<td>4.54</td>
<td>7.8</td>
</tr>
<tr>
<td>2''-H, 3''-H, and 4''-H</td>
<td>5.00 to 5.30</td>
<td>5.00 to 5.25</td>
<td>5.00 to 5.25</td>
<td>5.00 to 5.25</td>
</tr>
<tr>
<td>5''-H</td>
<td>3.72</td>
<td>3.67</td>
<td>4.10</td>
<td>4.12</td>
</tr>
<tr>
<td>6e''-H</td>
<td>4.26</td>
<td>4.27</td>
<td>4.26</td>
<td>4.27</td>
</tr>
<tr>
<td>6a''-H</td>
<td>4.26</td>
<td>4.27</td>
<td>4.26</td>
<td>4.27</td>
</tr>
</tbody>
</table>
GentAc7 were proved by the chemical shift values of the anomeric protons 1'-H (Table II). In contrast the anomeric proton 1'-H of GlcAc7-O-EHA-Me was shifted upfield due to the glucosidic linkage. Similarly, the glucosidic linkage between the two monosaccharide units in EHA-GlcAc7 was established by upfield shifts of 1'-H, 6a'-H and 6e'-H, respectively. The β configuration at the anomeric centres of all conjugates studied by 1H-NMR was proved by the coupling constants of the anomeric protons (Table II).

The aglycon signals of EHA-GlcAc4, EHA-GentAc7, and GlcAc7-O-EHA-Me were in good agreement with those of EHA-Me (Fig. 5A) and OH-EHA-Me (Fig. 5B), respectively. In the mass spectra (electron attachment and electron impact) of these acetylated and methylated conjugates (EHA-GlcAc4, GlcAc7-O-EHA-Me, and EHA-GentAc7) the parent peaks and/or aglycon fragments occurred. Characteristic fragmentation of the acetylated carbohydrate moieties corresponded with that of other sugar esters [2, 11] and with the suggested structures. By means of the GOD-POD reaction glucose was identified as the only sugar, released from purified conjugates by enzymatic hydrolysis. Ratios corresponded to the results of 1H-NMR and MS and no other sugars were detected.

Not sufficient purified material of EHA-Glc2Ac7 and Ac-O-EHA-GlcAc4 was available for 1H-NMR. The structures of these two conjugates were confirmed by the results of hydrolysis, derivatization, glucose quantification by means of GOD-POD reaction, and mass spectral data. The GOD-POD reaction after hydrolysis of EHA-GentAc7 and EHA-Glc2Ac7 indicated in both cases two glucose units per aglycone molecule. Furthermore, the electron attachment mass spectra of both conjugates, which have different TLC and HPLC properties, showed identical fragmentation, e.g. the parent peak M' = m/z 760 (56, 28), the aglycone peak m/z 141 (100, 100), fragments of the Glc2Ac7 moiety m/z 635 (69, 49), m/z 347 (63, 34) and characteristic fragments of GlcAc4 [2, 11]). However, the per cent abundance relative to the base peak, given in parantheses for EHA-GentAc7 (first value) and EHA-Glc2Ac7 (second value), were different. Therefore, isomerism inside the diglucose moieties of EHA-GentAc7 and EHA-Glc2Ac7 was supposed.

The structure of OH-EHA-Glc was proved by MS of its peracetylated derivative, Ac-O-EHA-GlcAc4. The electron attachment MS showed the parent peak M' − 1 = m/z 529 (12) and characteristic fragments of the aglycone and GlcAc4 moiety [11].

Discussion

Investigations on the metabolism of new phytoeffectors, e.g. plant growth regulators, are necessary before using them in agriculture in a large scale. However, beside that aim of metabolism studies more general aspects of xenobiotic transformation in plants are of interest.

For understanding the biochemical role of xenobiotic conjugates, including oligosaccharide conjugates, investigations on their interconversion and complete identification are necessary. However, there are a lot of difficulties in metabolism research which often result from insufficient methods of purification and structural elucidation of the small amounts of metabolites available. Therefore, new methods and new applications of known methods are desired in this field [12].

The results of our investigations demonstrate that the metabolism of EHA in barley does not lead to a steady state. During the short time interval the metabolism was followed, dramatic changes in the concentrations of transformation products were observed. The initially formed EHA-Glc most probably underwent sugar chain elongation. The resulting disaccharide esters also seem to be no final products of metabolism and may be incorporated into higher conjugates and insoluble residues.

β-D-Gentiobiose as a conjugating moiety of EHA was not unexpected. However, another (not completely characterized) disaccharide ester of EHA was isolated (EHA-Glcβ). This example suggested that with increasing sugar chain length the mixture of oligosaccharide conjugates becomes more complex. Furthermore, it cannot be excluded that during purification of the polar carbohydrate conjugate fraction some sugar conjugates in minor concentration were lost. Considering the 1H-NMR data in connection with the results of MS and other methods, the structures of the different conjugates of EHA from barley were determined. Thus, beside other structure elements of the carbohydrate moiety, the glucosidic linkage between the two sugar units of EHA-Gent was determined by the upfield shifts of the 1H-NMR signals of the protons at the linkage sites of the acetylated derivatives.
In addition to conjugation reactions oxidation is another fundamental metabolic pathway. In barley for EHA there seems to exist a competition between these two possibilities of metabolism. However, there are reasons why OH-EHA in free and conjugated form constantly accumulated during the time intervals up to 72 h. Hydroxylation is not reversible and, furthermore, the concentration of free OH-EHA was reduced by permanent formation of its glucoside (Glc-O-EHA) and glucosyl ester (OH-EHA-Glc). Therefore, the equilibrium between the substrate for hydroxylating enzymes, EHA, and EHA-Glc was disturbed, resulting in release of EHA from its glucosyl ester (EHA-Glc). The latter was supported experimentally by the formation of OH-EHA after application of EHA-Glc to the plants.

The structural elucidation of OH-EHA demonstrated that 'H-NMR spectroscopy is able to solve special problems also of aglycone determination. Hydroxylation of EHA at C(4) was not expected because no examples of (ω-2)-hydroxylation by higher plants were known. Evidence that 4-OH-EHA is a real product of plant metabolism was given by an aseptic experiment.