

Metabolism of the Herbicide Bromoxynil in *Hordeum vulgare* and *Stellaria media*

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The metabolism of $[3,5-^{14}\text{C}]3,5$ -dibromo-4-hydroxybenzonitrile (bromoxynil) was studied in *Hordeum vulgare* and *Stellaria media*.

In excised barley seedlings the time course of metabolism was followed over 48 h and the distribution of radioactivity to various metabolic fractions measured. The metabolites, separated by TLC and HPLC, were identified by chemical and spectrometric methods. Bromohydroquinone and 2,6-dibromohydroquinone were found, indicating partly debromination and replacement of the nitrile group by hydroxyl. The major parts of bromoxynil and of the metabolites were recovered in glucosidic conjugates.

In *Stellaria media* the same metabolites as in *Hordeum vulgare* were determined. Additionally, metabolites of unknown structures without bromine were detected.

Introduction

In the first step of metabolism the herbicide bromoxynil, a photosynthetic inhibitor in plants, is converted by hydrolysis of the nitrile group to the corresponding benzoic acid [2]. Further metabolic reactions are debromination and replacement of bromine against hydroxyl. Due to these reactions, theoretically a great variety of metabolites may originate. However, in earlier metabolic studies only a limited number of metabolites were detected in small concentrations [3]. Furthermore, till now possible conjugates of bromoxynil were deduced in an indirect way only by hydrolytic experiments [2].

Studies referring to selectivity and metabolism of the title compound showed that the susceptibility of a plant species decreased with increasing rate of metabolism of bromoxynil [1]. *Hordeum vulgare* and *Stellaria media*, two species with little susceptibility metabolized bromoxynil to a great extent.

The subject of this study was isolation, identification, and characterization, respectively, of the metabolites found after application of $[3,5-^{14}\text{C}]$ -bromoxynil to this species.

Materials and Methods

Radiochemicals and synthetic standards

The specific radioactivity of $[3,5-^{14}\text{C}]$ -bromoxynil [4] was $127 \text{ MBq} \times \text{mmol}^{-1}$. For some experiments specific radioactivity was adjusted to $5.5 \text{ MBq} \times \text{mmol}^{-1}$ before application by mixing with nonlabeled compound.

2,3,4,6-Tetra-O-acetyl- β -D-glucopyranosylester of bromoxynil was prepared by the Koenigs-Knorr method (m.p. 152–155 °C).

Plant material and application

Hordeum vulgare cv. Salome and *Stellaria media* were cultivated in soil in the greenhouse at 23–25 °C (*H. vulgare*) and 15–17 °C (*S. media*), respectively, on a diurnal photoperiodic regime of 15 h light and 9 h darkness. 5 day old excised seedlings of barley and about 7 cm long excised segments of 5–8 week old plants of *S. media* were immersed in an aqueous solution of the potassium salt of bromoxynil at pH 8. The concentrations were $5 \times 10^{-5} \text{ M}$ for kinetic experiments with *H. vulgare* and experiments with *S. media*, and $3 \times 10^{-3} \text{ M}$ for assays to isolate metabolites from *H. vulgare*.

Abbreviations: LSC, liquid scintillation counting; TMS, trimethylsilyl; *m/z*, mass/charge; CI, chemical ionization; EI, electron impact; glc, glucosyl; MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide.

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Extraction and measurement of radioactivity

Following incubation the plant material was rinsed with water, homogenized with an Ultra-Turrax, and extracted with 80% methanol and methanol. The extracts were evaporated and separated by silica gel chromatography (TLC, column) and reversed phase chromatography (HPLC).

The radioactivity of liquid samples was measured by LSC (Tricarb 2660, Packard Instruments, Chicago). Solid samples (bound residues) before LSC were incinerated with a Micro-Mat BF 5010 (Berthold-Frieseneke, Karlsruhe).

Silica gel chromatography

Preparative TLC was carried out on TLC plates coated with silica gel 60 HF₂₅₄, Merck, 0.9 mm thick. For comparative TLC silica gel plates "Silufol UV₂₅₄", Kavalier, C.S.F.R., were used. The following solvent systems were used:

1. chloroform:ether:acetic acid 40:9:1 (v:v:v),
2. ethyl acetate:i-propanol:water 65:24:12 (v:v:v),
3. ethyl acetate:toluene:methanol 75:50:75 (v:v:v).

Detection of radioactive zones on TLC plates was performed with the Radioscanner II (Berthold, Wildbad).

Elution of the silica gel columns (silica gel 60, 0.063–0.2 mm, 30 ml volume) was performed with chloroform followed by ethyl acetate and ethyl acetate:methanol 1:1 (v:v).

Hydrolyses and derivatization

For alkaline hydrolysis, an aliquot was treated with 1 ml 1 N NaOH (24 h, room temperature). Acid hydrolysis was carried out by treatment of an aliquot with 1 ml 1 N HCl (1 h, 100 °C).

Acetylation was performed with acetanhydride:pyridine 1:1 (v:v). Silylation was carried out with MSTFA:acetonitrile 1:1 (v:v, 1 h, 70 °C).

HPLC

HPLC was performed with LiChrosorb RP 18, 5 µm, column 4.6 × 250 mm, which was fitted with a RCT HPLC eluent supply and a PYE Unicam PU 4020 detector set at 254 nm.

Isocratic elution was carried out with methanol:water 25:75 (v:v) for preparative separation

and with methanol:phosphoric acid 0.1% 30:70 (v:v) for analytical separation.

MS

GC/MS (EI) was carried out with HP 5970 with GC 5880 A (Hewlett-Packard). MS (CI, reactant gas NH₃) was performed with a Finnigan 8230.

Results and Discussion

Kinetic experiments with barley

To achieve rapid uptake and a sufficient rate of metabolism, [3,5-¹⁴C]bromoxynil was applied by immersing excised barley seedlings to an aqueous solution of the active ingredient (5×10^{-5} M). As expected for excised plants, a rapid absorption of more than 80% of the offered bromoxynil takes place within the first 24 h. Accordingly, the radioactivity within the medium decreased. After 24 h the rate of uptake became significantly slower.

The major part of the radioactivity absorbed by the plants was extractable with methanol (80% and 100%). However, the radioactivity found in bound residues during the experiment (48 h) slowly increased. After 6 h, for instance, 6.3% of the absorbed radioactivity were bound to insoluble residues, 18.7% after 24 h, and 25.0% after 48 h. Bound residues were not further studied.

Thin layer chromatographic separation of the extract (preparative TLC in solvent system 1, 2 times developed) yielded 3 fractions named H 1 (R_f 0.7 in solvent system 1; bromoxynil and 1 non-polar metabolite), H 2 (R_f 0.65 in solvent system 2; glucosides), and H 3 (R_f 0.05 in solvent system 2; higher conjugates) (Fig. 1). The percentages changed during the experiments in favour of the hydrophilic fractions H 2 and H 3. Simultaneously, the lipophilic fraction H 1 decreased. The portion of bromoxynil within the fraction H 1 also decreased after application while a new non-polar metabolite appeared.

Isolation and identification of metabolites from barley

Because of the low concentrations of the applied bromoxynil the kinetic experiments did not yield amounts of metabolites sufficient for identification. Therefore in additional experiments 180 mg bromoxynil ($5.5 \text{ MBq} \times \text{mmol}^{-1}$) were applied to 1700 g barley seedlings. Despite the high concen-

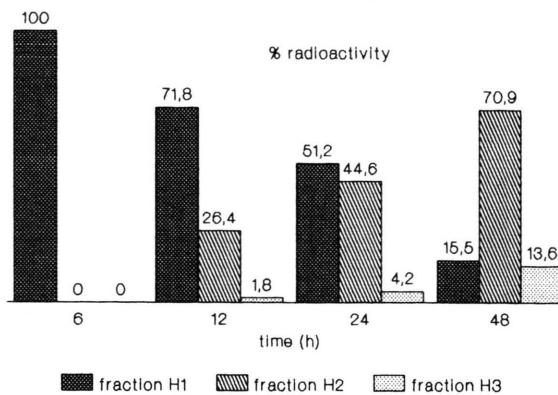


Fig. 1. Distribution of radioactivity in fractions obtained from excised barley seedlings treated with $[3,5-^{14}\text{C}]$ bromoxynil for increasing time intervals. Fraction H1: bromoxynil and 1 non-polar metabolite; fraction H2: glucosides; fraction H3: higher conjugates. The radioactivity, extracted with methanol (80% and 100%) was set to 100%.

tration of 3×10^{-3} M bromoxynil the metabolic pattern was very similar to that of kinetic experiments.

After 48 h incubation time the plants were extracted and the extracts were separated by silica gel column chromatography to the fractions H1, H2, and H3, followed by several times purification of the single fractions by TLC (solvent system 1 and solvent system 2) and HPLC. Beside the parent compound within the fraction H1, a non-polar compound occurred. This spectrum [m/z 268 ($^{79}\text{Br}/^{81}\text{Br}$, relative intensity 100, parent peak); m/z 186 (^{79}Br , 8, M-HBr); m/z 158 (^{79}Br , 14); m/z 131 (^{79}Br , 6)] was obtained. This spectrum is due to a dibromodihydroxybenzene. However, by means of GC/MS the position of the new hydroxyl group could not be established. Comparing the metabolite by HPLC with different isomeric dibromodihydrobenzenes as synthetic standards, the identity was demonstrated with authentic 2,6-dibromohydroquinone.

The fraction H2 contained 3 compounds H2-1, H2-2, and H2-3 as was shown by HPLC. After acetylation H2-1 was proved to be identical with synthetic 2,3,4,6-tetra-O-acetyl- β -D-glucoside of bromoxynil (TLC in solvent system 1, HPLC).

The mass spectra (GC/MS analysis of TMS derivatives) of H2-2 and H2-3 showed fragments of the respective aglycone as well as fragments char-

acteristic for a hexose. As expected, the parent peaks were not present within the spectra of this sugar conjugates. The double peaks [m/z 332 (^{79}Br , 53) and m/z 260 (^{79}Br , 12)] in the mass spectrum of H2-2 indicated the presence of one bromine atom inside the molecule. The peak m/z 260 was due to a bromodihydroxybenzene moiety (mono-TMS) and m/z 332 was attained by transfer of TMS to m/z 260. Several peaks, *e.g.* m/z 378 (12), m/z 361 (30), and m/z 217 (26) were characteristic for silylated hexose.

The mass spectrum of H2-3 [TMS derivative, m/z 412 (^{79}Br , 19)] confirmed the structure of a dibromodihydroxybenzene (transfer of TMS according to m/z 332 at H2-2). Some characteristic fragments of the silylated sugar moiety, *e.g.* m/z 450 (7), m/z 361 (95), m/z 271 (18), and m/z 217 (29) also occurred.

The GC/MS analysis confirmed the results of hydrolysis. At acid conditions bromoxynil, 2,6-dibromohydroquinone (already detected as a free metabolite), and bromohydroquinone were obtained. The latter was identified by comparison with several isomeric bromodihydrobenzenes (2-bromoresorcine, 4-bromoresorcine, bromohydroquinone) by HPLC.

The fraction H3 during hydrolysis yielded the same aglycones as H2 did. According to the behaviour during TLC and hydrolysis it was supposed that the metabolites of fraction H3 were higher conjugates with 2 or 3 sugar moieties. Earlier such conjugates were found for other xenobiotic compounds in barley [5, 6]. At alkaline conditions the compounds of the fractions H2 and H3 were not hydrolyzed, indicative of their glucosidic structure.

In Fig. 2 the supposed metabolic pathway of bromoxynil in barley is shown including the percentages of the metabolites. From bromoxynil only small amounts remained unchanged. 2,6-Dibromohydroquinone as a "free" metabolite also occurred only in minor concentrations. The major parts of the metabolites and the parent compound were found as glucosides and higher conjugates.

Metabolism in *Stellaria media*

In the experiments with *Stellaria media* $[3,5-^{14}\text{C}]$ bromoxynil was taken up by the excised plants from a 5×10^{-5} M solution during 24 h.

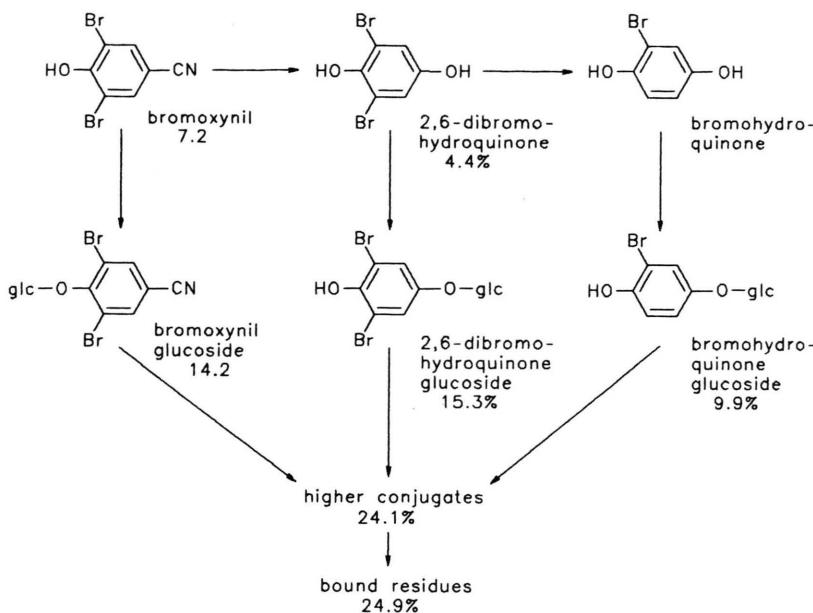


Fig. 2. Metabolic pathway of $[3,5-^{14}\text{C}]$ bromoxynil in excised barley seedlings and percentages of metabolites. The radioactivity absorbed by the plants was set to 100%.

After another 24 h the plants were extracted. 81.9% of the incorporated radioactivity were found in the extracts and 18.1% were bound to insoluble residues. The bound residues were not investigated in detail.

By TLC the extract (solvent system 3, 2 times developed) was separated into five radioactive fractions S1–S5. The percentages were calculated from the areas of the radiochromatogram peaks and given in Table I. Fraction S3 by HPLC was further separated yielding the components S3-1 and S3-2.

Identification of the metabolites was performed, as far as possible, by cochromatography with

bromoxynil, with authentic standards, and with metabolites from barley. Furthermore, GC/MS analysis was performed.

S1 was shown to be the parent compound (TLC in solvent system 3, 2 times developed). S2 was proved to be identical with 2,6-dibromohydroquinone glucoside from barley (HPLC; MS in Fig. 3). The mass spectrum (CI of the TMS derivative) showed the parent peak m/z 808 ($^{79}\text{Br}/^{81}\text{Br}$, $\text{M}+\text{NH}_4$) as the only signal with intact aglycone–sugar bond. There were several peaks of the silylated sugar moiety in the spectrum, *e.g.* m/z 469, m/z 450, m/z 778, m/z 361 and m/z 288, confirming the structure of a glucoside.

Table I. Percentages of metabolites isolated after application of $[3,5-^{14}\text{C}]$ bromoxynil to excised chickweed plants. The radioactivity absorbed by the plants was set to 100%.

Metabolite	%
S1 Bromoxynil	18.8
S2 Glucoside of 2,6-dibromohydroquinone	16.7
S3-1 } Glucosides of unknown aglycones without bromine	17.2
S3-2 } Glucosides of unknown aglycones with bromine	17.2
S4 Higher conjugate of unknown aglycone	6.7
S5 Higher conjugates of unknown aglycones S1, 2,6-dibromohydroquinone, and bromohydroquinone	26.3
S6 Bound residues	14.3

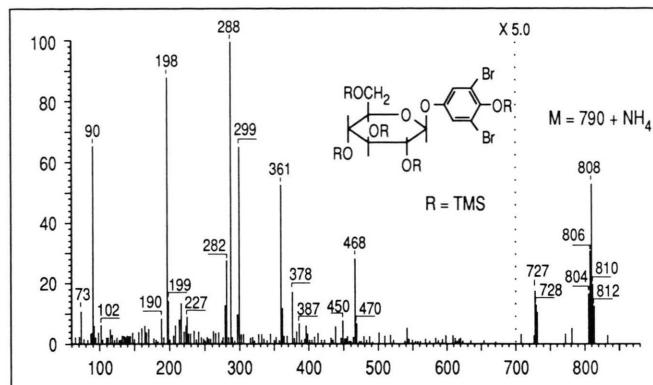


Fig. 3. Mass spectrum (TMS derivative, Cl/NH_3) of 2,6-dibromohydroquinone glucoside isolated from excised barley seedlings after application of $[3,5\text{-}^{14}\text{C}]$ bromoxynil.

Because of their chromatographic properties (R_f 0.33 in solvent system 3, 2 times developed) S 3-1 and S 3-2 were considered to be conjugates. GC/MS analysis did not indicate any bromine in the molecules of both metabolites.

S 4 and S 5 by their chromatographic behaviour were characterized as higher conjugates. Chromatographic separation was not possible since they were extensively hydrophilic compounds and were not mobile in the solvent systems used.

Acid hydrolysis of S 4 yielded an aglycone of unknown structure (R_f 0.67 in solvent system 3). This compound was obtained also from S 5 after acid hydrolysis which apparently was a mixture of conjugates. In addition, by acid hydrolysis bromoxynil, 2,4-dibromohydroquinone, and bromohydroquinone were obtained and identified by HPLC and GC/MS.

Discussion

In the experiments on the metabolism of bromoxynil in *Hordeum vulgare* and *Stellaria media* some new metabolites of the herbicide were identified.

The replacement of the nitrile moiety by hydroxyl at C-1 yielded bromohydroquinone and 2,6-dibromohydroquinone which were not known as yet as metabolites of bromoxynil in plants. The sequence of this reactions may involve hydrolysis of the nitrile group *via* the intermediate 3,5-dibromo-4-hydroxybenzamide to 3,5-dibromo-4-hydroxybenzoic acid in the primary step. This reac-

tion is catalyzed by a nitrilase as was shown for microorganisms and tolerant plant species [2, 7]. Recently, the gene encoding such a bromoxynil-specific nitrilase was transferred from microorganisms to susceptible plants (tobacco), establishing resistance of the target plant against the herbicide [8]. The plant species under study did not accumulate the intermediate amide and carboxylic acid. Therefore they were not detectable in the extracts.

The second step of the metabolic pathway, decarboxylation, is a common degradation reaction of xenobiotics. In experiments with $[^{14}\text{CN}]$ bromoxynil $^{14}\text{CO}_2$ was liberated [2], indicating decarboxylation of the 3,5-dibromo-4-hydroxybenzoic acid intermediate. However, the fate of the aromatic moiety remaining after decarboxylation could not be followed with a ^{14}CN -label.

Cytochrome P₄₅₀ enzymes were shown to catalyze aryl hydroxylation of xenobiotic substances [9]. Such a microsomal mixed function oxidase may also be involved in hydroxylation of bromoxynil and it would be of interest to study this in detail. The position occupied before by the nitrile group may be the sterically preferred for hydroxylation. However, it cannot be excluded that minor products of hydroxylation (or other minor metabolites) were lost during the purification procedure.

As expected, conjugation with carbohydrates is an important way of metabolism of bromoxynil and its metabolites. According to many other examples [10] not only glucose but also oligosaccharides apparently are used as conjugating moieties. Due to experimental difficulties the structures

were not identified in detail. The kinetic experiments indicated an increase of more hydrophilic higher conjugates, and eventually their binding to insoluble residues.

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