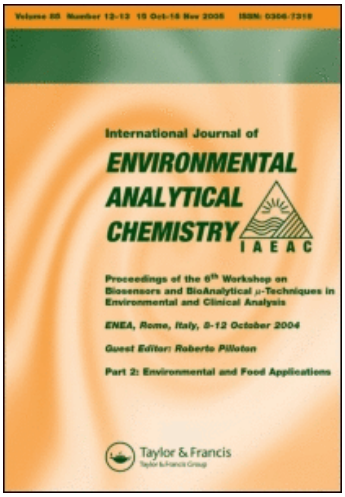

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Chromatographic Purification and Identification of Polar Metabolites of Benazolin-Ethyl from Soybean†

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The metabolism of benazolin-ethyl (4-chloro-2-oxobenzothiazolin-3-ylacetic acid ethyl ester), a post emergence herbicide, has been studied in soybean using (^{14}C)-phenyl labelled compound. Preliminary studies were performed on excised soybean leaves. Following hydrolysis of the ethyl ester to benazolin acid (4-chloro-2-oxobenzothiazolin-3-ylacetic acid), extensive metabolism to polar conjugates was observed. The polar fraction from a Bligh–Dyer extraction was purified by solvent partitioning, preparative TLC and reverse phase HPLC with ion suppression. The two major metabolites were characterised by fast atom bombardment mass spectrometry with accurate mass determination as an aspartate conjugate and a malonyl-β-glucose ester of benazolin acid. Subsequent experiments were performed by spraying intact plants at growth stage V4. The major polar metabolite isolated one month after treatment was identified as the aspartate conjugate by mass spectrometry and high resolution nuclear magnetic resonance spectroscopy.

KEY WORDS: Benazolin, malonyl-glucose ester, aspartic acid, FAB mass spectrometry, soybean.

†Presented at the 2nd Symposium on Handling of Environmental and Biological Samples in Chromatography. October 24–25, Freiburg, FRG.

INTRODUCTION

The herbicidal properties of benazolin acid (4-chloro-2-oxobenzothiazolin-3-ylacetic acid), a compound with hormonal growth regulatory activity, were first described in 1964.[1] Since that time formulations of its ester, benazolin-ethyl (4-chloro-2-oxobenzothiazolin-3-ylacetic acid ethyl ester) and various salts have continued to be developed for use on a wide range of weeds present in various crops.[2,3]

Despite the long usage of the herbicide little has been reported on its metabolism. Lewis[4] investigated the metabolism of the sodium salt of benazolin acid in barley, ryegrass, clover and chickweed, while Schafer and Stobbe[5] studied the metabolism of the dimethylamine salt in wild mustard and rape species. The degree of metabolism reported by both sets of authors varied markedly, but the major metabolite reported in each case was an acid labile conjugate of benazolin acid. No attempts however, were made to identify this conjugate.

The objective of the present study was to establish the route of benazolin-ethyl degradation in soybean. Excised leaflets incubated in a solution of [^{14}C]-benazolin-ethyl were used as a preliminary tool for rapidly producing sufficient quantities of metabolites to develop isolation and purification techniques. Attention was then concentrated on the metabolism in intact plants sprayed with a suspension concentrate formulation of [^{14}C]-benazolin-ethyl.

MATERIALS AND METHODS

Radiolabelled material

[^{14}C]-Benazolin-ethyl, universally labelled in the phenyl ring, with a specific activity of 2.1 μCi/mmol was used for the leaflet experiments and 2.8 μCi/mmol for the intact plant studies. The radiochemical purity of the material was >98%.

Plant treatment

The same variety of soybean (Glycine Max (L) cv. Amsoy 71) was used for both leaflet and intact plant studies.

Leaflets The first and second trifoliate leaves of young soybean plants (growth stage V3[6]) cultivated in vermiculite were excised under water and transferred to a 20 mg/l solution of [^{14}C]-benazolin-ethyl. Plants were maintained in a constant environment room at 25°C, under fluorescent lights with a photoperiod of 16 hours. The incubation solution was topped up as necessary from a [^{14}C]-benazolin-ethyl solution of the same specific activity and concentration. Leaflets were harvested after 90 hours and coarsely chopped.

Intact plants Soybean plants, three to a 9 cm square pot, were grown under glass in sterilised medium clay loam soil. Natural sunlight was augmented with 400 watt sodium lamps on a 16 hour photoperiod. The soil was top-watered.

Thirty six plants at growth stage V4 were sprayed with a 0.08% aqueous suspension of [^{14}C]-benazolin-ethyl using a glass chromatography spray unit at a rate approximate to 0.42 kg a.i./ha. Plants were harvested one month after spraying and divided into plant tissue present at treatment (i.e. up to the 5th node) and new growth. The former sample was coarsely chopped prior to extraction and analysis.

Extraction and isolation of metabolites

Samples of chopped tissue were processed according to the scheme shown in Figure 1.

Polar and lipophilic compounds were extracted and separated by the modified Bligh–Dyer[7] procedure of Still and Mansager[8] with the exception that two extractions were performed in each solvent system by homogenising for two minutes on a MSE overhead drive macerator and filtering through Whatman No. 4 paper. The methanol/water layer was reduced to aqueous and extracted with equal volumes of ethyl acetate (three times) at pH 9, then ethyl acetate (three times) at pH 2.

The pH 2 extract was purified by preparative TLC on a 1 mm thick Whatman PLK 5 silica plate with pre-absorbent layer. The chromatogram was developed for 15 cm in ethyl acetate-isopropanol-water (65:23:12). Radioactive areas were delineated by autoradiography (Osray M3 film, Agfa Gevaert) and eluted with acetone-water

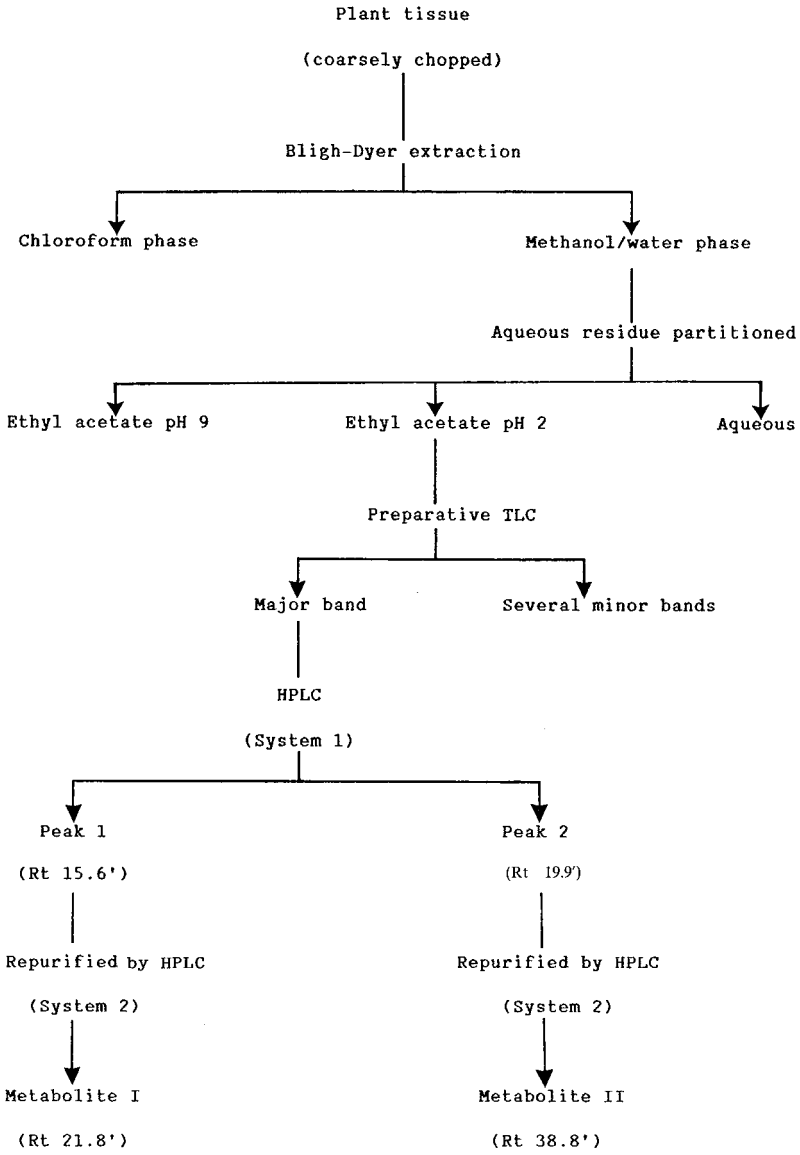


FIGURE 1 Extraction of major polar metabolites from soybean.

(1:1). The eluate from the major TLC band, resuspended in water, was further purified by high-performance liquid chromatography. Solvent flow rates of 2 ml/min (Waters 6000A pump) were used with a 5 μm Hypersil ODS column, 25 cm $\times$ 1 cm on two sequential solvent systems (System 1: methanol-water-formic acid (45:55:0.5); System 2: acetonitrile-water-formic acid (25:75:0.5)). Radioactive peaks were detected on a Berthold 503 HSD radiomonitor (solid scintillator: siliconised cerium activated glass) which controlled peak collection on a Gilson 201 fraction collector. The column eluent was additionally monitored by a Pye-Unicam LC-UV detector at 300 nm

Liquid scintillation counting

Quantitative [^{14}C]-measurements were made in PCS scintillation cocktail (Amersham International) in an Intertechnique SL 4200 scintillation spectrometer with automatic external standard channel ratio quench correction.

Mass spectroscopy

Positive and negative ion fast atom bombardment mass spectra (FAB/MS) were recorded on a ZAB mass spectrometer (VG Analytical Ltd.) with xenon as the ionising gas and an accelerating voltage of 8 kV. Samples were dissolved in a glycerol matrix. Accurate mass determinations were run in negative ion mode against PEG 400 as the calibration standard.

Nuclear magnetic resonance spectrometry (NMR)

300 MHz proton NMR spectra were recorded on a Bruker WM 300 spectrometer with pulse programming and an Aspect 2000 data collection system.

Hydrolysis of conjugates

To aid structural elucidation sub-samples of purified metabolites were hydrolysed in 1 ml of hydrochloric acid (2 M) for 2 hours at 100°C. Enzyme hydrolyses were carried out in 2 ml sodium acetate buffer (50 mM) at pH 5 by incubating with 1 mg of β-glucosidase

(type II from Almonds, Sigma Chemical Co.) for 16 hours at 37°C. Hydrolysed extracts were adjusted to pH 2 and extracted into ethyl acetate (three times). Ethyl acetate extracts were admixed with unlabelled, authentic benazolin acid and chromatographed on silica TLC plates (0.25 mm thick; Machery-Nagel) in two systems (System A: chloroform-acetic acid (95:5); System B: toluene-methanol-acetone-acetic acid (80:15:5:1)). Plates were developed for 15 cm in lined tanks at room temperature. Reference standards were visualised under UV light (254 nm). Radioactivity was detected by autoradiography, as before, and quantified by liquid scintillation counting following plate scraping.

Synthesis of aspartic acid conjugate of benazolin acid

The initial step in the preparation of the amino acid conjugate was the synthesis of the acid chloride of benazolin acid. Benazolin acid was heated at reflux for 2 hours under anhydrous conditions with thionyl chloride. The majority of the thionyl chloride was removed by distillation. Trituration of the residual oil with ice-cold diethyl ether afforded benazolin acid chloride as pale yellow crystals. The amino acid conjugate was prepared according to the method described by Wood and Fontaine.[9] The crude conjugate obtained from this reaction was purified by column chromatography on silica using toluene-ethanol-ethyl acetate-acetic acid (80:10:5:1) as the solvent. The pure aspartic acid conjugate was obtained in 45% yield from the acid chloride and its identity was confirmed by FAB/mass spectral and H^1NMR analysis (Figure 8).

m/z (positive ion FAB) 359 M+H, (negative ion FAB) 357 M−H; δ_H {MeOD} 7.40 (1H, q, J=7.8, 1.1 Hz, aromatic C−*H*a or *H*c), 7.22 (1H, q, J=8.1, 1.2 Hz, aromatic C−*H*a or *H*c), 7.05 (1H, t, J=8.0 Hz, aromatic C−*H*b), 5.02 (2H, 2 doublets, N−C−*H*d, *H*e), 4.81 (2H, s, $2CO_2$*H*j), 4.67 (1H, t, J=5.8 Hz, N−C*H*g), 2.76 (2H, 2 doublets, CH−C−*H*h, *H*i), N−*H*f not apparent due to deuterium exchange.

RESULTS AND DISCUSSION

Metabolite characterisation in excised leaflets

Thirty-eight leaflets were extracted and purified by the procedure

outlined in Figure 1. The majority of radioactivity (61%) was detected in the chloroform layer from the Bligh–Dyer extraction but this was shown to be unchanged benazolin-ethyl by co-chromatography with authentic standards on TLC (Systems A and B). The bulk of radioactivity in the methanol/water layer showed acidic properties, partitioning from aqueous into ethyl acetate at pH 2. Preparative TLC of this extract gave one major band (Rf 0.53–0.63) and several minor bands. The radioactivity in the major band accounted for approximately 60% of the methanol/water extract, with individual minor metabolites in all other fractions each accounting for less than 15%. The major TLC band gave two well resolved peaks on HPLC System 1 (Figure 2). Peak 1 (Rt 15.6

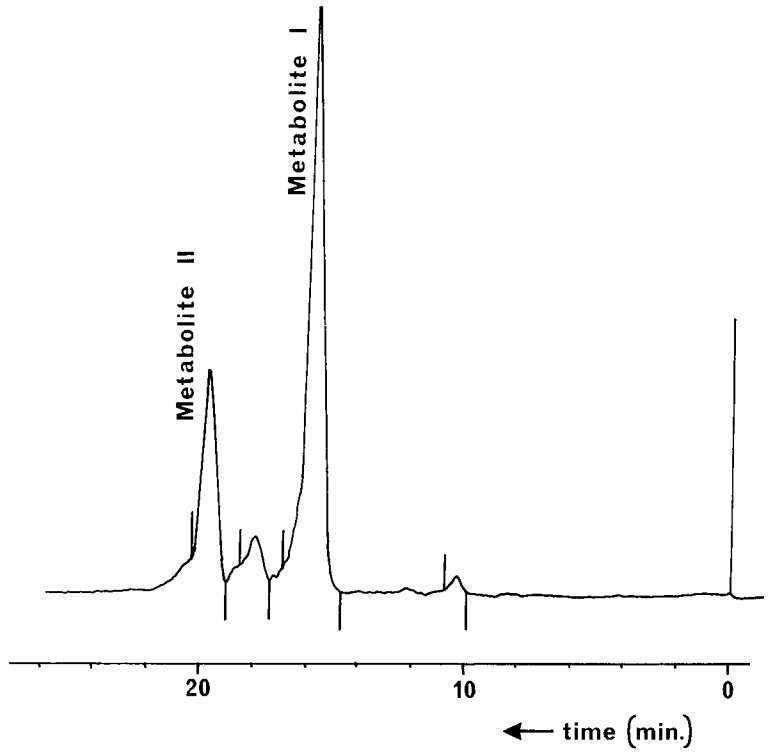


FIGURE 2 Radiochromatogram from purification of major polar metabolites in excised leaflets on HPLC System 1.

minutes) was repurified by HPLC using System 2. The single radio-peak (Rt 21.8 minutes) eluted was concentrated and labelled Metabolite I. Peak 2 (Rt 19.9 minutes) from HPLC System 1 was repurified twice on HPLC System 2. The single radio-peak (Rt 38.8 minutes) eluted from the final column was concentrated and labelled Metabolite II.

Characterisation of Metabolite I

Metabolite I was not hydrolysed by β-glucosidase but gave partial hydrolysis in 2 M hydrochloric acid (2 h, 100°C) to benazolin acid (62%) as characterised by TLC (Systems A and B).

The existence of the benazolin acid moeity in Metabolite I indicated that the mass spectrum should contain a molecular ion with a mono-chlorinated fragmentation pattern. FAB/MS in both negative and positive ion mode (Figures 3, 4) suggested a molecular species with mass 358, by assignment of the highest mono-chlorinated ions at m/z 357 to M−H (negative ion spectrum) and m/z 359 to M+H (positive ion spectrum). Several ions were observed in the positive ion spectrum at higher m/z ratios but these

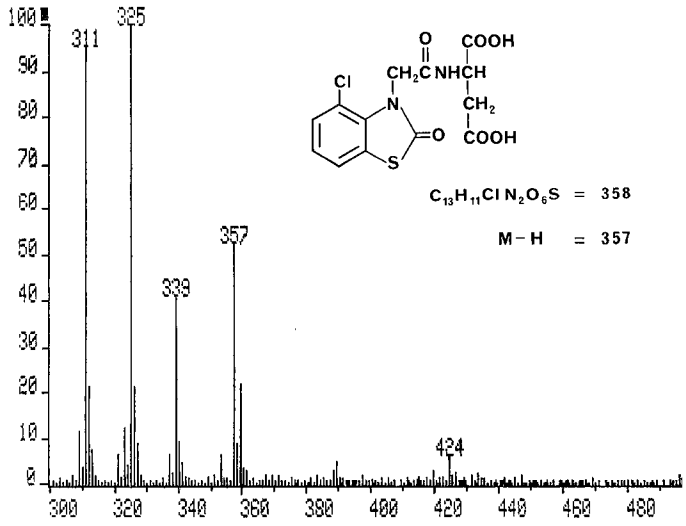
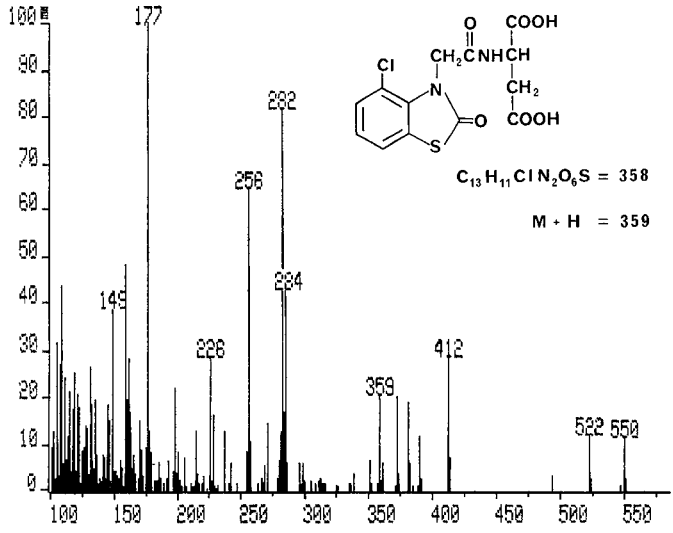


FIGURE 3 Negative ion FAB mass spectrum of Metabolite I.



Major fragment ions (mono-chlorinated):-

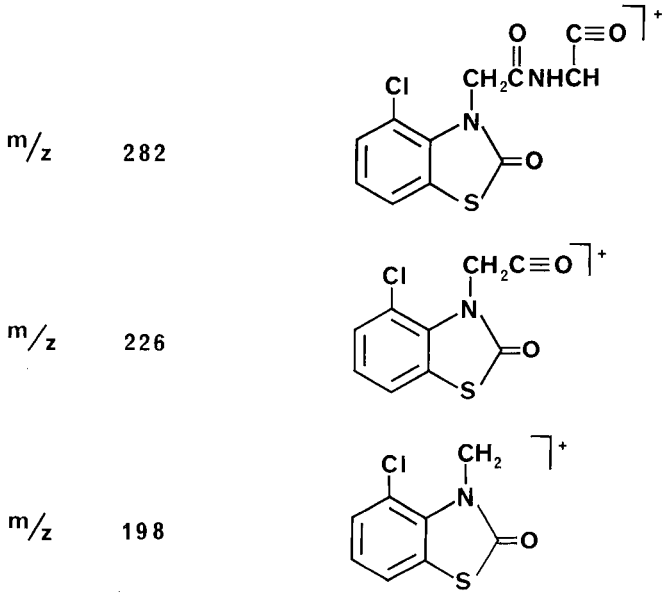


FIGURE 4 Positive ion FAB mass spectrum of Metabolite I.

were clearly non-chlorinated. The structure assigned to Metabolite I on the basis of a molecular weight of 358 was the aspartic acid conjugate of benazolin acid (Figure 5).

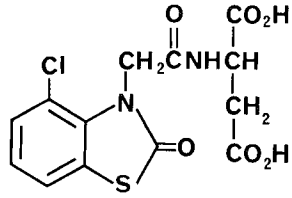


FIGURE 5 Structure of Metabolite I.

Accurate mass determination on the pseudo-molecular negative ion gave a mass of 356.9927, which is in good agreement ($\Delta M =$ 2.1 m.m.u.) with the value calculated for the ascribed structure, 356.9948. Futher evidence for this structure is provided by the assignment of the mono-chlorinated species at m/z 198, 226 and 282 to probable fragment ions (Figure 4).

Characterisation of Metabolite II

Metabolite II was completely hydrolysed by both β-glucosidase and acid hydrolysis to benazolin acid as characterised by TLC (Systems A and B), indicating that benazolin acid was most likely conjugated to sugar moeity. FAB/MS in the positive ion mode yielded no mono-chlorinated ions, but negative ion FAB/MS (Figure 6) gave a spectrum with the highest mono-chlorinated species at m/z 490 assigned to the pseudo-molecular ion M−H. The ion at m/z 526 has a characteristic di-chlorinated pattern and may result from an M+Cl adduct. In a subsequent experiment to determine the accurate mass of the species at m/z 490 and 529 the latter ion was not observed. Assigment of a simple sugar conjugate of benazolin acid to a mass of 491 was not possible, but the partitioning properties of the conjugate and the likelihood of the fragment ion at m/z 446 arising from M−COOH (Figure 6) suggested the presence of an acid function on the sugar. Metabolite II was tentatively assigned the structure of a malonyl-β-glucose ester of benazolin acid (Figure 7).

Substantiating evidence for this structure was provided by accurate mass determination and the MS fragmentation pattern. The

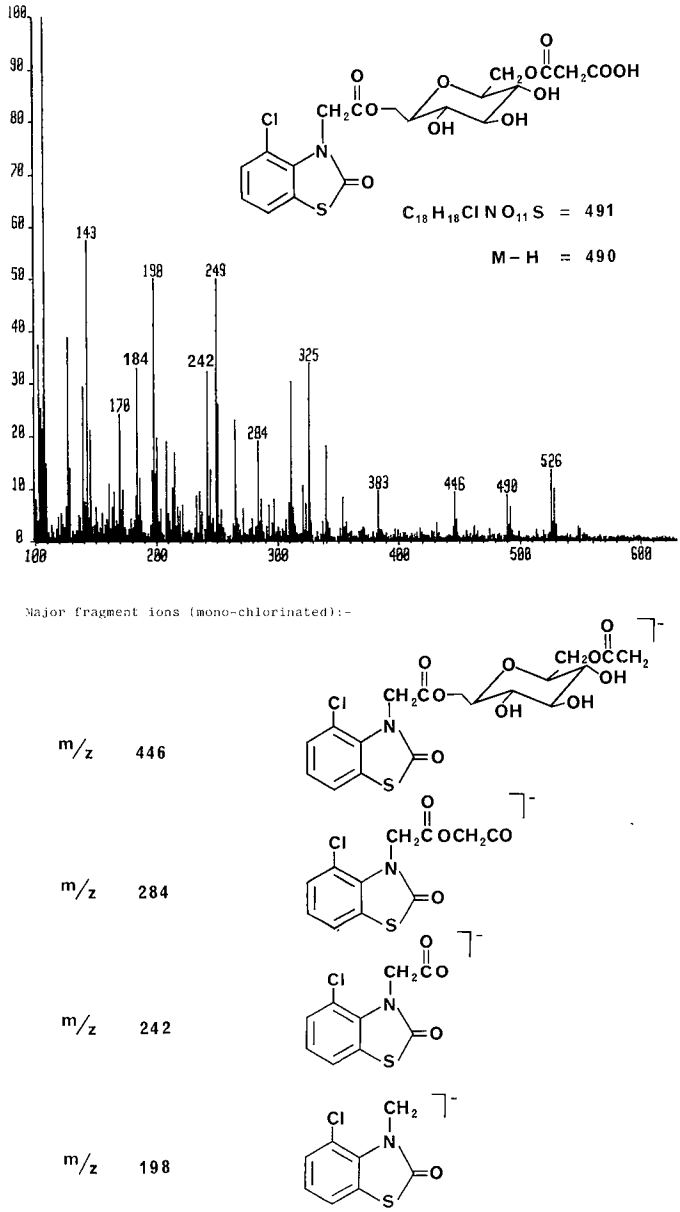


FIGURE 6 Negative ion FAB mass spectrum of Metabolite II.

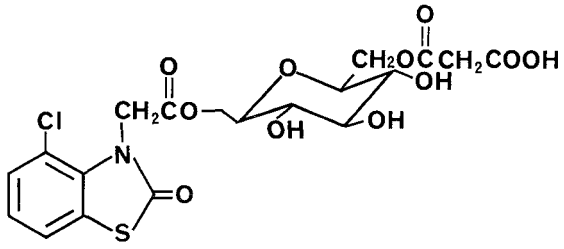


FIGURE 7 Proposed structure of Metabolite II.

observed accurate mass of the pseudo-molecular ion M−H, 490.0232, was in good agreement ($\Delta M = 2.1$ m.m.u.) with the calculated mass, 490.0211. Mono-chlorinated ions at m/z 446, 284, 242, 198, 184, 170 and 143 can be assigned to probable fragment ions of the proposed structure, as illustrated for the first four in Figure 6. Non-chlorinated ions at m/z 265 and 249 are also notable as they may be ascribed to the malonyl-β-glucose moiety less H and OH, respectively.

The presence of malonyl-β-glycosides of xenobiotics and natural constituents in plants is now well established.[10–13] In conjugates which have been completely identified, the malonate has been shown to be conjugated to the 6-position of the sugar.[10,12] Malonyl-glycosides, however, have shown themselves to be resistant to β-glucosidase hydrolysis.[10,12,13] Reports of malonyl-glucose ester have been less frequent[14,15] and their reactions with β-glucosidase has not been reported. Other explanations for Metabolite II being susceptible to β-glucosidase hydrolysis are that the malonyl group may not be at the 6-position as in the previously documented glucosides, or alternatively a secondary enzyme may have been present in the β-glucosidase preparation used.

Metabolite I identification (intact plants)

The treated areas of the [^{14}C]-benazolin-ethyl sprayed plants were subjected to the extraction/purification procedure described for leaflets (Figure 1).

The bulk of the radioactivity extracted by the modified Bligh–Dyer procedure was accounted for by unchanged benazolin-ethyl

(27%), polar metabolities extracting into ethyl acetate, pH 2 (41%) and highly polar metabolites not extracting into ethyl acetate (25%).

Purification of the acidic fraction gave one major peak on HPLC which co-chromatographed with Metabolite I from the leaflets. A sufficient quantity of the conjugate was obtained to allow H^1NMR analysis to be conducted and comparison of the spectrum of the metabolite with that of the authentic synthesised material (Figure 8),

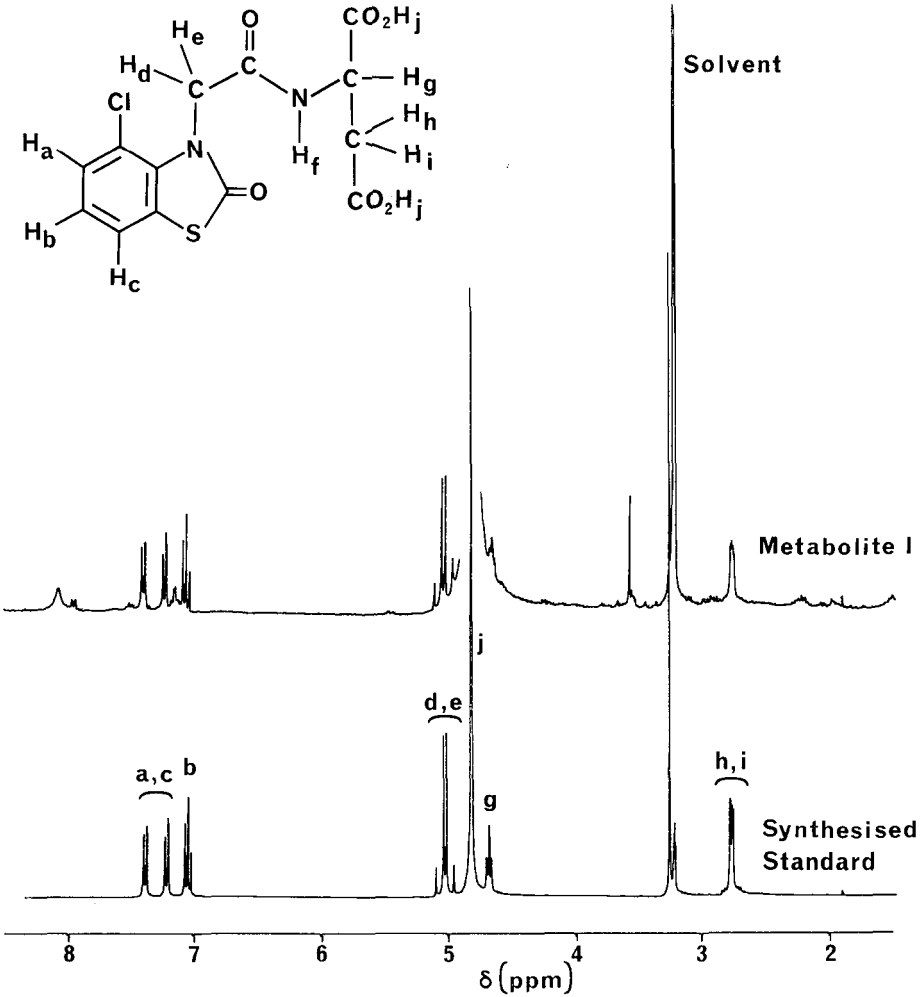


FIGURE 8 Comparison of 300 MHz H^1NMR of Metabolite I and synthesised aspartic acid conjugate of benazolin acid.

established conclusively that Metabolite I was the aspartic acid conjugate of benazolin acid.

Several minor metabolites were also present in the acidic fraction but no compound co-chromatographing with Metabolite II was observed. This does not rule out the possibility that Metabolite II is an earlier intermediate, incorporated perhaps into complex highly polar sugar conjugates.

CONCLUSIONS

Excised soybean leaflets produced two major metabolites of benazolin-ethyl, a malonyl-glucose ester and an aspartic acid conjugate of benazolin acid, as characterised by FAB/MS. The aspartic acid conjugate was unequivocally identified as the major metabolite in intact plants, one month after treatment, by comparison of its NMR spectrum with that of a synthesised standard. The malonyl-glucose ester was not observed in intact plants but the possibility exists that it was produced as an earlier intermediate. These results are consistent with previous reports[4,5] that the major metabolic reaction of benazolin acid was direct conjugation, although no attempts were made to identify the conjugate.

Characterisation of pesticide conjugates by mass spectrometry generally requires derivatisation of the sample before electron impact or chemical ionisation techniques can be used. This often complicates interpretation and may introduce artefacts. In this study fast atom bombardment mass spectrometry gave both molecular ion data and several useful fragment ions which aided characterisation, illustrating the usefulness of FAB/MS as an alternative to field desorption mass spectrometry for studying polar metabolites.

Acknowledgements

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