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ACIDIC METABOLITES OF BENZYL ALCOHOL IN GREENBUG RESISTANT BARLEY*

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Abstract—(^{14}C-Carbinol)benzyl alcohol taken up through the roots of greenbug (*Schizaphis graminum*) resistant barley is metabolized into a large number of radioactive compounds which have been separated by ion exchange chromatography. Two of these acidic metabolites have been identified as *O*-benzoyl-L-malic acid and *N*-benzoylaspartic acid; these identifications were confirmed by synthesis.

INTRODUCTION

Barley varieties resistant to the greenbug *Schizaphis graminum* (Rondani) contain free benzyl alcohol while an isogenic barley line lacking the gene for greenbug resistance does not [1]. Benzyl alcohol was shown to depress greenbug reproduction on susceptible barley in the laboratory [1] and to partially protect greenbug-susceptible sorghum from the aphid when added to irrigation water at 100 ppm in field studies (R. L. Burton, K. J. Starks and E. A. Wood, unpublished observations). The major neutral metabolite of benzyl alcohol in greenbug resistant barley has been identified as β-benzyl-D-glucopyranoside and shown not to have antigreenbug activity in greenbug susceptible barley [2]. In the present paper a number of acidic metabolites formed from benzyl alcohol in resistant barley have been isolated. Two of these metabolites have been identified, synthesized chemically, and tested for antigreenbug activity in susceptible barley.

RESULTS AND DISCUSSION

The metabolites of benzyl alcohol in barley plants were separated by cation exchange into fractions A and B. Fraction B was identified as *O*-benzoyl-L-malic acid by direct comparison with an authentic sample. Fraction A was then submitted to anion exchange and in addition to several glycosidic metabolites, an acidic fraction I was separated. This was identified as *N*-benzoylaspartic acid by direct comparison with an authentic sample of the racemic acid. Since L-aspartic acid is the natural amino acid, we believe that the true metabolite is *N*-benzoyl-L-aspartic acid and that its racemization probably occurs during the isolation procedure.

Andreae and Good [5] reported that, in pea tissue, exogenously administered benzoic acid was conjugated with aspartic acid to yield *N*-benzoyl-L-aspartic acid. The identity of this metabolite was later questioned by Venis and Stoessl [6] who recharacterized the major metabolite as *O*-benzoyl-L-malic acid, the minor metabolite being identified as *N*-benzoyl-L-aspartic acid. Conversion of benzoic acid into its hydroxy and methoxy derivatives (ring substituted) in plants has been reported by several workers [7,8]. By contrast, very little information on the metabolism of benzyl alcohol, especially in plants, is available. Rapid oxidation of benzyl alcohol to benzoic acid followed by conjugation with glycine to form hippuric acid is known to occur in animals [9–11]. During the course of our investigation on the metabolism of benzyl alcohol in barley we have detected neither hippuric acid nor free or ring substituted benzoic acid. We have found that the major portion of benzyl alcohol given to the plant is transformed into several glycosides. Benzoic acid produced by the oxidation of benzyl alcohol must therefore be either rapidly degraded by plant enzymes or in its activated form, benzoyl CoA, conjugated with malic and aspartic acids. The presence of malic acid in barley was reported by Nelson and Mottern [12]. Chan [13] investigated the biochemical constituents of barley and found that aspartic acid was the major free amino acid in this plant. Therefore, it is not surprising that *O*-benzoyl-L-malic acid and *N*-benzoyl-L-aspartic acid are metabolites of benzyl alcohol in barley.

Synthetic *O*-benzoyl-L-malic acid and *N*-benzoyl-DL-aspartic acid were tested for antigreenbug activity in the susceptible barley test system previously used with benzyl alcohol [1]. Neither of these compounds at a level of 100 ppm had any effect on the rate of greenbug reproduction in this system. If these compounds are efficiently taken up through the roots it may be concluded that the antigreenbug activity of benzyl alcohol is not due to its metabolism to these products.

EXPERIMENTAL

Materials. Isogenic (Iso(R)) barley, (*Hordeum vulgare* L.) resistant to the greenbug *Schizaphis graminum* (Rondani) was

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developed by the Agronomy Department of Oklahoma State University [1]. The plants were grown from seed in a growth chamber under controlled conditions (humidity 40%, day temp. 29.4°, night temp. 18.8°, 16 hr light period, seedlings 8–10 days old and 5–6 inches tall were generally employed. (^{14}C-carbinol)-benzyl alcohol was purchased from Radiochemical Center, Amersham.

General methods. Mp's are uncorrected. PC on Whatman No. 1 was in EtoAc–HoAc–H_2O (5:3:2). Glycosides and sugars were detected with $AgNO_3$–NaOH reagent [4]. Analytical TLC was performed on Sil gel sheets in I, $CHCl_3$–cyclohexane–HOAL (8:2:1); II, EtoH–H_2O–25% NH_4OH (6:1:1); III, *n*-BuOH–HoAc–H_2o (12:3:5, upper phase); IV, EtoAc–HoAc–H_2O (5:3:2). Amino acid analyses were carried out on a Technicon amino acid analyzer. Low resolution MS were determined with the prototype [14] of the LKB–9000 GC–MS with the use of the direct inlet probe. High resolution mass spectra were determined with a CEC–21–110B DuPont instrument. IR spectra were determined in Nujol. NMR spectra were obtained in acetone-d6. Elemental analyses were performed by Galbraith Labs, Inc. Radioactivity measurements were made in a toluene–ethanol cocktail (3:2) containing 4 g PPO and 200 mg POPOP per l. using a Packard Tri-Carb Liquid Scintillation Spectrometer.

Administration of (^{14}C)-benzyl alcohol. After removing plants from soil, roots were thoroughly washed, blotted dry and the plants transferred into small glass bottles (5.1 cm in diameter and 7.6 cm in height). The soln of benzyl alcohol (1%) containing some ^{14}C tracer, was slowly pipetted onto the roots (generally 1 ml of soln per 20–25 seedlings was used). The bottles were wrapped in aluminium foil and the plants left until nearly all of the solution was taken up into the roots (3–4 hr). The roots and the lower portion of the stem were then covered with a nutrient soln and the plants were transferred to the growth chamber where they were kept until harvest.

Extraction and partial purification of the metabolites. 48 hr after treatment, the plants were removed and the nutrient soln from the stem and roots was washed off. Plants were then severed at about 1/2 cm above the seed attachment. Vegetative tissue (leaves and stems) was ground with liquid N_2 to a fine powder. H_2O (5 ml/g fr. wt) was added and the slurry stirred vigorously at 2–3° for 2 hr. The brei was taken to dryness in order to remove the volatile components. The plant residue was again stirred with H_2O and filtered through 8 layers of cheesecloth. This treatment was repeated once more. The total filtrate was evaporated to a yellow viscous residue, mixed with H_2O and centrifuged at 20000 g for 20 min. The product obtained on evaporation of the dark yellow supernatant was thoroughly mixed with MeOH. The pptd material was removed by filtration. The residue obtained upon removal of MeOH was dissolved in a minimum amount of H_2O. The resulting soln contained the metabolites of benzyl alcohol and was fractionated by ion exchange chromatography on Bio-Rad AG-50W-X8 and Bio-Rad AG1-X8.

Identification of metabolite B. The aq plant extract was fractionated on a cation exchange column into 2 components, a large peak A and a relatively smaller peak B. The fractions under peak B (77–110) were pooled and the soln (176 ml) was concentrated to 9.5 ml. This soln was then placed on a column of AG1-X8 (formate, 200–400 mesh) anion exchange resin. The column was eluted successively with water, 2N HCO_2H and 4N ammonium formate. The last eluant gave a single sharp peak containing labeled material. The soln (130 ml) obtained by pooling fractions comprising this peak was reduced to a small vol and allowed to stand at −15° overnight. This treatment ppd most of the ammonium formate. The aq layer was lyophilized. The fine powder thus obtained was extracted with MeOH–toluene (1:1) and the insoluble ammonium formate removed by centrifugation. The supernatant was evaporated to dryness and the residue dissolved in 1.5 ml H_2O. After addition of cold 1N HCl (4 ml), the soln was extracted with Et_2O (3 × 10 ml). Removal of the solvent under red pres gave a white shiny product. It was dissolved in dil Na_2CO_3 and the soln extracted several times with Et_2O which was discarded. The metabolite was re-extracted into ether after acidification of the aq layer. The final product obtained by evaporation of ether melted at 141–2°. It appeared as a single UV quenching spot when examined by analytical TLC; R_f values in solvents I–IV were 0.43, 0.57, 0.67 and 0.73 respectively. This metabolite was not hydrolyzed when heated for 15 min. with 1 N HCl at 100°. Basic hydrolysis with NaOH, however, yielded benzoic acid, identified by TLC and MS. Both low and high resolution mass spectra of the metabolite gave fragment ions (*m/e* 122, 105, 77) characteristic of benzoic acid. This information coupled with the hydrolytic behaviour indicated that the molecule was a benzoic acid ester. High resolution mass spectral analysis of the metabolite gave ions such as $C_{11}H_8O_5$ (found 220.036094 requ 220.037168) $C_{10}H_{10}O_4$, $C_9H_{10}O_3$, $C_7H_6O_2$ and C_7H_5O (see ref [33]). Additional ions at *m/e* 148, 89, 77, 55, 50, 45, 44 and 43 were observed. The PMR spectrum (100 MC) gave chemical shifts of δ = 8.09–7.3 (*m*, 5H), δ = 5.68 (*m*, 1H) and δ = 3.06 (*m*, 2H). On this basis, the metabolite was assigned the structure *O*-benzoyl-L-malic acid. This was substantiated by elemental analysis: Calc for $C_{11}H_{10}O_6$, C = 55.47%, H = 4.23%. Found C = 55.89%, H = 4.31%. Final confirmation was achieved by IR, m.n., MS and R_f comparison with an authentic sample.

Separation of acidic metabolites by anion exchange. Fraction A (150 ml) from the cation exchange column was reduced in vol to 11.7 ml and resolved into several metabolites on an anion exchange column which was eluted with water, 2N HCO_2H and 4N ammonium formate. Peaks C, D, E, F and G contain glycosides on the basis of color reactions. Peak C, which eluted with water, has previously been identified as β-benzyl-D-glucopyranoside [2]. Peak F, the quantitatively major acidic metabolite in barley (previously referred to as metabolite I [2]) was shown to be formed from β-benzyl-D-glucopyranoside [2]. It is a highly labile compound and decomposes rapidly on heating or slowly on storage even at −20° to form a compound which migrates like β-benzyl-glucoside on TLC. Peaks D and E contain weakly acidic compounds which are probably phenolic derivatives of benzyl glycosides.

Identification of metabolite I. This compound which eluted with ammonium formate was purified in the same manner as described for *O*-benzoyl-L-malic acid. Migration on TLC in solvents III and IV (R_f 0.67 and 0.64 resp), and the mp (163–4°) were identical with an authentic sample of racemic *N*-benzoylaspartic acid. Metabolite I was hydrolysed by heating 0.5 mg with 0.4 ml of 6N HCl for 4 hr at 100° to give benzoic acid as the only radioactive component. Aspartic acid was the only amino acid detected in the aqueous layer.

Synthesis of O-*benzoyl*-L-*malic acid.* This was prepared by a modification of an existing procedure [15]. L-malic acid (5 g) and benzoyl chloride (12.5 g) were heated at 75° for 2 hr. The solid was stirred with CCl_4 (30 ml) for 30 min. and filtered. The insoluble material was stirred with $CHCl_3$ (20 ml) and filtered again. The product thus formed was thoroughly mixed with H_2O (20 ml), filtered and air dried. The product was the anhydride, yield 4.3 g. mp 154–5°, NMR δ = 8.12–7.48 (*m*, 5H), δ = 6.08 (*m*, 1H) and δ = 3.55 (*m*, 2H). Found C = 60.25%, H = 3.84% calc for $C_{11}H_8O_5$ C = 60.00%, H = 3.66%). The anhydride ring was opened by dissolving the product in hot (85–90°) 1N HCl. After the soln cooled, *O*-benzoyl-L-malic acid was extracted with ET_2O. Removal of solvent from the dried ether extract gave 4.35 g (yield 49%). Elemental analysis found C = 55.49%, H = 4.18%; Calc for $C_{11}H_{10}O_6$, C = 55.47%, H = 4.23%.

Synthesis of N-*benzoylaspartic acid.* Authentic *N*-benzoyl-aspartic acid was prepared from benzoyl chloride and L-aspartic acid according the the method of Fischer [16]. The product gave a mp of 164–5° indicating that the compound was a racemic mixture (mp 164–5°) (16–18), rather than *N*-benzoyl-L-aspartic acid (mp 184–5°) [16].

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