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MONURON METABOLISM IN EXCISED *GOSSYPIUM HIRSUTUM* LEAVES: ARYL HYDROXYLATION AND CONJUGATION OF 4-CHLOROPHENYLUREA*

D. STUART FREAR and HARLEY R. SWANSON

North Central Region, Agricultural Research Service, U.S. Department of Agriculture, Metabolism and Radiation Research Laboratory, Fargo, ND 58102, U.S.A.

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Abstract—A new glycoside was isolated as a minor metabolite in excised cotton leaves treated with either [carbonyl-^{14}C] or [ring-^{14}C] 3-(4-chlorophenyl)-1-methylurea and 4-chlorophenylurea. The aglycone from β-glucosidase or hesperidinase hydrolysis was identified as 4-chloro-2-hydroxyphenylurea by TLC, radioisotope dilution and MS.

INTRODUCTION

IN PART II of this series[1] we reported two new major polar metabolites of 3-(4-chlorophenyl)-1,1-dimethylurea (monuron) in excised cotton leaves. These metabolites were identified as the *O*-glucosides of *N*-hydroxymethyl intermediates in the oxidative *N*-methylation of monuron and monomethylmonuron [3-(4-chlorophenyl)-1-methylurea]. Additional minor polar metabolites were also noted, but their isolation and characterization were not attempted. The present study reports the isolation and identification of one of these minor polar metabolites as an *O*-glycoside of 4-chloro-2-hydroxyphenylurea.

RESULTS AND DISCUSSION

A close examination of previously reported[1] monuron metabolites in cotton leaves revealed that one minor polar metabolite (<2% of the methanol-soluble fraction 4 days after treatment) was stable to mild acid hydrolysis, was hydrolyzed by either β-glucosidase or hesperidinase, and yielded an ether-soluble aglycone that was more polar than 4-chlorophenylurea. Furthermore, this metabolite was formed in leaf tissues when excised cotton leaves or roots of cotton plants were treated with either [carbonyl-^{14}C] or [ring-^{14}C] monomethylmonuron or 4-chlorophenylurea. Thus, it appeared that this metabolite was an *O*-glycoside of an aryl-hydroxylated 4-chlorophenylurea. The glycoside was purified by adsorption, ion exchange, gel permeation, and TLC. The aglycone was isolated after enzyme hydrolysis of the purified glycoside and identified by TLC, MS, and radioisotope dilution.

* Part V in the series "*N*-Demethylation of Substituted 3-(Phenyl)-1-methylureas". For Part IV see *Phytochemistry* **11**, 2709 (1972).

[1] FREAR, D. S. and SWANSON, H. R. (1972) *Phytochemistry* **11**, 1919.

A comparison of published and experimental R_f values for several substituted phenylureas is shown in Table 1. The isolated aglycone cochromatographed with known 4-chloro-2-hydroxyphenylurea and 4,5-dichloro-2-hydroxyphenylurea in solvents I and II. Previous studies[2,3] showed that 4,5-dichloro-2-hydroxyphenylurea and 4-chloro-2-hydroxyphenylurea chromatographed at approximately the same R_f whereas 4-chloro-3-hydroxyphenylurea had a lower R_f. Hydrogen bonding between the 2-hydroxy group and the ureido carbonyl group was probably responsible for the higher R_f values.[2,3] Possible chlorine migration as the result of an oxidation NIH shift reaction would also be expected to result in a compound with a lower R_f. These data indicated that the aglycone of the metabolite was hydroxylated at the 2-position rather than the 3- or 4-positions and was probably 4-chloro-2-hydroxyphenylurea. Purified [^{14}C]aglycone was also recrystallized to constant specific activity with authentic material as further evidence for hydroxylation at the 2 position. Evidence for aryl hydroxylation was also obtained by comparing the MS of the purified aglycone with those of known substituted ureas. It had a similar fragmentation pattern to other 2-hydroxyphenylureas.

TABLE 1. TLC OF SUBSTITUTED PHENYLUREA ANALOGS*

Compound	R_f (× 100) in:†	
	Solvent I	Solvent II
3-(4-Chlorophenyl)-1-methylurea	52 (59)‡	58 (47)
3-(4-Chloro-2-hydroxyphenyl)-1-methylurea	— (51)	— (42)
3-(3,4-Dichlorophenyl)-1-methylurea	52 [76]§	58 [57]
4-Chlorophenylurea	41 (49)	31 (31)
3,4-Dichlorophenylurea	41 [57]	30 [32]
4-Chloro-3-hydroxyphenylurea	— (23)	— (14)
4-Chloro-2-hydroxyphenylurea	25 (35)	23 (23)
4,5-Dichloro-2-hydroxyphenylurea	25 [32]	23 [24]
3,4-Dichloro-2-hydroxyphenylurea	— [37]	— [21]
5-Trifluoromethyl-2-hydroxyphenylurea	23 —	27 —
Aglycone	25 —	23 —
Glycoside‖	0	0

* Silica gel HF (250 μ).
† Solvent I = $CHCl_3$–EtOH–HOAc (18:1:1); solvent II = $CHCl_3$–EtOH (9:1).
‡ Ref. 2 R_fs ( ).
§ Ref. 3 R_fs [ ].
‖ This glycoside had R_fs of 32 and 37 in $CHCl_3$–MeOH–H_2O (65:25:4) and n-BuOH–EtOH NH_4OH (2:1:1) respectively.

Thus, the conjugate is an *O*-glucoside of 4-chloro-2-hydroxyphenylurea. This is the first definitive report of oxidative aryl hydroxylation in the metabolism of substituted phenylurea herbicides by higher plants. Previous studies with metobromuron in tobacco[4] and fluometuron in wheat and corn[5] have suggested that both of these substituted phenylurea herbicides were ring-hydroxylated, but specific metabolites and aglycones were not iso-

[2] ERNST, W. and BÖHME, C. (1965) *Food Cosmet. Toxicol.* **3**, 789.
[3] BÖHME, C. and ERNST, W. (1965) *Food Cosmet. Toxicol.* **3**, 797.
[4] GEISSBÜHLER, H. and VOSS, G. (1971) in *Pesticide Terminal Residues*, pp. 305–322, Butterworths, London.
[5] NEPTUNE, M. D. (1970) Ph.D. Thesis, Auburn University.

lated and identified. A recent report[6] on ring-hydroxylated monuron metabolites in excised corn and bean leaves also failed to isolate and identify specific metabolites or aglycones. Indeed, previous short-term studies with excised cotton leaves,[1] and recent repeated studies with [methyl-^{14}C] and [carbonyl-^{14}C] monomethylmonuron-treated corn and bean leaves have shown that the major portion of the initial polar metabolites in these tissues result from a rapid oxidative *N*-demethylation rather than an aryl hydroxylation.

Ring hydroxylation and conjugation of substituted phenylurea herbicides have been reported in animals.[2,3] Monuron metabolism studies in rats[2] showed the presence of trace quantities of both free and conjugated forms of 4-chloro-2-hydroxyphenylurea, 4-chloro-3-hydroxyphenylurea, and 3-(4-chloro-2-hydroxyphenyl)-1-methylurea in urine. In the present study, however, the latter two compounds or their derivatives were not detected.

Previous studies have suggested that the amide bond of phenylurea herbicides is not readily cleaved and that substituted phenylureas accumulate as 'terminal' metabolic products in higher plants.[4,7] The present study indicates that these 'terminal' residues are subject to additional biotransformations by slow oxidative aryl hydroxylation and subsequent conjugation. The failure to detect 3-(4-chloro-2-hydroxyphenyl)-1-methylurea suggests that monomethylmonuron was probably *N*-demethylated before ring hydroxylation. However, the possibility of an initial ring hydroxylation followed by *N*-demethylation cannot be ruled out.

EXPERIMENTAL

Radiolabeled compounds. [Carbonyl-^{14}C] 3-(4-chlorophenyl)-1-methylurea (5·85 μCi/μmol); [carbonyl-^{14}C] 4-chlorophenylurea (5·85 μCi/μmol); [ring-^{14}C] 3-(4-chlorophenyl)-1-methylurea (1·0 μCi/μmol); and [ring-^{14}C] 4-chlorophenylurea (0·21 μCi/μmol) were synthesized as described previously, or isolated and purified as metabolites from excised cotton leaves treated either with [carbonyl-^{14}C] 3-(4-chlorophenyl)-1-methylurea (5·85 μCi/μmol) or [ring-^{14}C] 3-(4-chlorophenyl)-1-methylurea (0·21 μCi/μmol).[1]

Instrumentation. MS were obtained at 70 eV by direct solid sample insertion into a Varian M-66 spectrometer with the ion source at 180°. Quantitative [^{14}C] measurements were made with a Packard 3375 liquid scintillation spectrometer.[1] A Packard 7201 radiochromatogram scanner was used for the location of [^{14}C] on TLCs. Eluates from column chromatography were monitored for [^{14}C] with a Picker Nuclear Scinti/Flow system.

Plant materials and treatment. Fully expanded, greenhouse grown[8] cotton (*Gossypium hirsutum* L.) leaves, including petioles, were excised under dist. H_2O. Each petiole was placed in a 15 × 75 mm test tube containing 1–5 ml of aq. [^{14}C] substrate. In preparative metabolic isolation experiments, 1·2 × 10^6 dpm of the appropriate ^{14}C-phenylurea was supplied to each of several leaves at concentrations approaching 5 × 10^{-4} M. Rapid pulsed uptake (<3 hr) of the [^{14}C] treating soln was followed by treatment with an additional 2–3 ml of dist. H_2O. After the uptake of the dist. H_2O, the tubes were filled with dist. H_2O and maintained at 21° and 61% RH with a 12-hr photoperiod. More than 90% of the [^{14}C] was absorbed by the excised leaves under these conditions. Metabolism was terminated after 3–4 days by extraction with MeOH.

Metabolite isolation and purification. Treated cotton leaves were homogenized in an Omnimixer with 5 parts of MeOH (v/fr. wt). The homogenate was filtered on a 10μ Teflon filter and the insoluble residue was exhaustively washed with MeOH. The combined MeOH filtrates were concentrated almost to dryness at room temp. and taken up in 25 ml dist. H_2O. Nonpolar [^{14}C] compounds were extracted with Et_2O (4 × 50 ml). The aqueous phase was concentrated to 5 ml at 30°, adsorbed on a 2·5 × 25 cm column of Amberlite XAD-2, and washed with dist. H_2O. Adsorbed [^{14}C] polar metabolites were eluted with MeOH, concentrated to 5 ml and chromatographed on a 2·5 × 25 cm column of Sephadex LH20. The major broad peak was eluted from the LH20 column at a flow rate of 1·0 ml/min with 2–3 void vols. of MeOH. Peak fractions were pooled, evaporated to dryness, and dissolved in 1–2 ml MeOH. The MeOH concentrate was further purified by two preparative TLC separations on 500 μ silica gel plates. The first separation was achieved with $CHCl_3$–MeOH–H_2O (65:25:4) and the second with BuOH–EtOH–NH_4OH (2:1:1). The partially purified metabolite was scraped off the preparative plates from the second TLC separation, eluted with MeOH, taken to dryness and dissolved in 1 ml dist. H_2O.

[6] LEE, S. S. and FANG, S. C. (1973) *Weed Res.* **13,** 59.
[7] GEISSBÜHLER, H. (1969) in *Degradation of Herbicides* (KEARNEY, P. C. and KAUFMAN, D. D., eds.), pp. 77–111, Marcel Dekker, New York.
[8] FREAR, D. S., SWANSON, H. R. and TANAKA, F. S. (1969) *Phytochemistry* **8,** 2157.

The metabolite was further purified by anion exchange chromatography on a 1·5 × 15 cm DEAE–cellulose column that had been previously equilibrated with 2M Tris-HCl buffer at pH 8·0 and exhaustively washed with dist. H_2O. A single sharp peak was eluted from the DEAE column in less than 2 void vols. with dist. H_2O at a flow rate of 1·0 ml/min. This peak fraction was lyophilized and used for enzyme hydrolysis and the identification of the aglycone moiety. Recoveries of [^{14}C] were consistently better than 80% and generally better than 90% for each step in metabolite purification.

Enzyme hydrolysis of the purified metabolite. The purified metabolite was hydrolyzed with 1 mg/ml of either β-D-glucosidase (emulsion) or hesperidinase (*Aspergillus niger*) in 0·2–0·5 ml 0·01 NaOAc buffer (pH 5·3) for 16 hr at 30°. Hydrolysis was generally greater than 70% with β-glucosidase and usually better than 90% with hesperidinase.

Isolation and purification of aglycone. The enzyme hydrolysates were adjusted to 2 ml with dist. H_2O and the [^{14}C]aglycone was extracted with Et_2O (4 × 3 ml). The combined Et_2O extracts were evaporated to dryness and dissolved in 0·2–0·5 ml of MeOH for purification on 500 μ silica gel HF TLC plates with C_6H_6–Me_2CO (2:1)[1] and with $CHCl_3$–EtOH–HOAc (18:1:1). The separated aglycone was scraped off the TLC plates, eluted with MeOH, concentrated, and chromatographed on a Sephadex LH20 column (1·5 × 25 cm). The aglycone was eluted from the LH20 column with MeOH as a single sharp peak at a flow rate of 1 ml/min. Peak fractions were pooled, evaporated to dryness, and used for MS analysis.

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