Preparative-Scale Synthesis and Physicochemical Properties of Cysteine and Glutathione Conjugates of Chloroacetamides

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The preparative-scale syntheses of cysteine and glutathione (GSH) conjugates (2,3) of the chloroacetanilide herbicide acetochlor (1a) and its plant degradation products (1b,c) were accomplished in high yield and in excellent purity by the reaction of chloroacetamides with stoichiometric GSH or cysteine in liquid ammonia in the presence of sodium amide. Cysteine conjugates of metabolites 1b,c without an *N*-alkoxyalkyl side chain could easily be prepared in alcoholic solutions of cysteine hydrochloride and sodium ethoxide by reactions with chloroacetamides. The physicochemical properties and ^{1}H NMR data of conjugates are also described.

INTRODUCTION

Glutathione (GSH) conjugation is a common route by which xenobiotics are detoxified in higher plants. GSH conjugates are transient, and the rate of peptidase enzymes in their catabolism to cysteine conjugates has been established in vivo (Lamoureux and Rusness, 1989). Partially because of difficulties in the synthesis of these conjugates in sufficient quantities for bioassays or biochemical studies, their exact biological significance is still unknown. Difficulties in the synthesis of these conjugates are due to low solubilities of GSH and cysteine in organic solvents and the usually low solubilities of xenobiotics in water.

Studies of chloroacetanilide herbicide acetochlor metabolism in plants have revealed that the herbicide is conjugated with glutathione irrespective of the sensitivity of the plant to the herbicide. However, differences in sensitivity can often be related to differences in rates of conjugation with GSH. After the GSH conjugates are formed, they are usually catabolized to cysteine conjugates (Jablonkai and Dutka, 1985; Breaux, 1986). During acetochlor metabolism by corn and wheat, other water-soluble metabolites could also be detected. These metabolites were identified as cysteine and GSH conjugates (2, 3b,c) of deethoxymethyl acetochlor (1b) and a chloroacetyl indoline derivative (1c) formed after cyclization of 1b (Jablonkai and Dutka, 1985). Previous methods for the preparation of GSH and cysteine conjugates of chloroacetamide herbicides have yielded only nanomole to micromole quantities of the conjugates at a great excess of GSH and cysteine using buffers as solvents (Lamoureux et al., 1971; Leavitt and Penner, 1979). Thus, a preparative-scale synthesis of these molecules is required (2, 3a–c, Figure 1) to facilitate studies of their biological significance. A two-step synthesis of gram quantities for the GSH conjugate of cyanazine [2-chloro-4-(ethylamino)-6-[1-cyano-(1-methylethyl)amino]-*s*-triazine] has been described by Crayford and Hutson (1972), who reacted a stable, water-soluble trimethylammonium salt of the parent cyanazine molecule with GSH in water. Also, the use of NMR in the characterization of xenobiotic conjugates is quite limited (Feil, 1986). In this project, our objectives were to prepare gram quantities of cysteine and GSH conjugates of 1a–c chloroacetamides by a convenient, one-step method and to establish the proton NMR and other physicochemical characteristics of these conjugates.

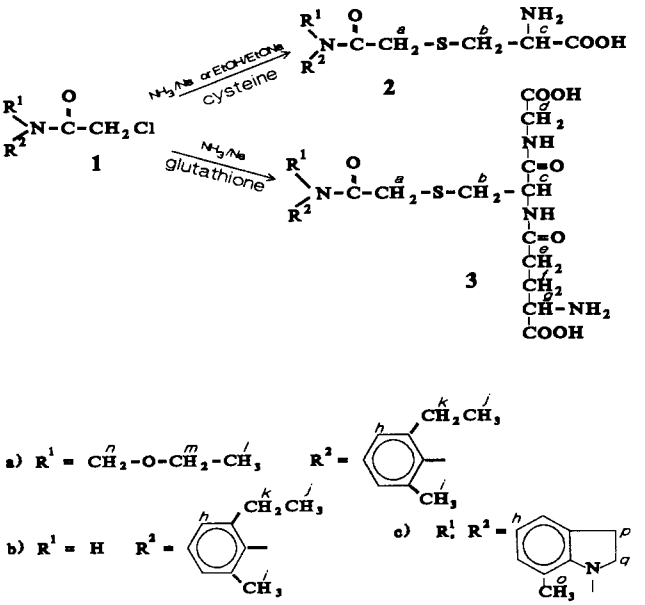

Figure 1. Synthesis of GSH and cysteine conjugates of acetochlor and its metabolites.

MATERIALS AND METHODS

Chemicals. Acetochlor was purified on a silica gel column eluted with benzene–petroleum ether. 6-Ethyl-*o*-toluidine was redistilled before use. Both chemicals were from Nitrokemia (Fuzfogyartelep, Hungary). GSH and cysteine was purchased from Sigma Chemical Co. (St. Louis, MO). Ammonia was the product of Pet Nitrogen Works (Pet, Hungary). All other chemicals and solvents, purified by recrystallization and redistillation before use, were delivered by Reanal (Budapest, Hungary).

2-Chloro-*N*-(2-ethyl-6-methylphenyl)acetamide (1b). To a stirred mixture of 6-ethyl-*o*-toluidine (13.5 g, 0.1 mol) in 25 mL of chloroform and sodium carbonate (8.0 g) in 50 mL of water was slowly added chloroacetyl chloride (11.3 g, 0.1 mol) at 5 °C. After the addition was completed, stirring was continued for 1 h. The organic phase, which separated from the aqueous layer, was then washed with 5% sodium carbonate and dried over anhydrous magnesium sulfate. The solvent was evaporated, and the residue was recrystallized from benzene to give the product (19.8 g, 94%) as white crystals melting at 122–123 °C.

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Table I. Physicochemical Properties and ^{1}H NMR Data of Cysteine and GSH Conjugates

compd	mp, °C	$[\alpha]_D$*,[a] deg	R_f[b]	FAB-MS,[c] m/z, M + H	^{1}H NMR,[d] ppm (multiplicity)
2a	181–183 (dec)	0	0.51	355	a, 3.10 (s); b, 2.96, 3.22 (m); c, 3.69 (m); h, 7.20 (m); i, 2.27 (s); j, 1.18 (t); k, 2.60 (m); l, 1.24 (t); m, 3.64 (g); n, 4.98 (s)
2b	178–180 (dec)	+12.6	0.46	297	a, 3.60 (s); b, 3.18, 3.32 (m); c, 3.98 (m); h, 7.22 (m); i, 2.20 9s); j, 1.15 (t); k, 2.54 (g)
2c	183–186 (dec)	+2.7	0.41	295	a, 3.77 (s); b, 3.13, 3.28 (m); c, 3.94 (m); h, 7.14 (m); o, 2.20 (s); p, 3.06 (t); g, 4.15 (t)
3a	186–190	−11.9	0.36	541	a, 3.03 (s); b, 2.85, 3.07 (m); c, 4.66 (m); d, 3.87 (s); e, 2.53 (t); f, 2.14 (t); g, 3.67 (t); h, 7.20 (m); i, 2.26 (s); j, 1.16 (t); k, 2.62 (q); l, 1.24 (t); m, 3.64 (m); n, 4.98 (s)
3b	228–230 (dec)	−14.8	0.32	483	a, 3.54 (s); b, 3.04, 3.18 (m); c, 4.68 (t); d, 3.94 (s); e, 2.54 (t); f, 2.15 (t); g, 3.84 (t); h, 7.20 (m); i, 2.19 (s); j, 1.14 (t); k, 2.54 (m)
3c	212–214 (dec)	−16.8	0.28	481	a, 3.72 (s); b, 3.02, 3.18 (m); c, 4.63 (t); d, 3.95 (s); e, 2.54 (t); f, 2.16 (t); g, 3.77 (t); h, 7.18 (m); o, 2.20 (s); p, 3.08 (t); q, 4.16 (t)

[a] *c* 1 in 1 N HCl. [b] TLC retentions on silica gel (Merck, Kieselgel 60 F_{254}, 0.25-mm thickness); solvent, 1-butanol–acetic acid–water (12:3:5 v/v/v). [c] FAB mass spectra were recorded with an AEI-MS 902 mass spectrometer. Operating conditions: Xe, 9 keV. Matrix: 3-nitrobenzyl alcohol or phosphoric acid. [d] NMR spectra were run in a mixture of deuterated water and methanol at 50 °C using a Varian XL-400 instrument.

***N*-(2,6-Dimethylphenyl)formamide.** A mixture of 2,6-xylidine (24.2 g, 0.2 mol) and 90% formic acid was heated at 100 °C for 3 h. After the reaction mixture was allowed to stand overnight at room temperature, the product formed was filtered off and recrystallized from ethanol as white needles melting at 166–167 °C (25.9 g, 87%).

7-Methylindole. In a 250-mL three-neck round-bottom flask equipped with a reflux condenser and a gas inlet tube which contained 120 mL of *tert*-butyl alcohol, potassium (5.8 g, 0.15 mol) was added in small pieces to the alcohol in a dry nitrogen stream. The addition should be done *cautiously* because potassium has been known to ignite in air. The mixture was heated on 100 °C oil bath until all of the potassium disappeared, and then *N*-(2,6-dimethylphenyl)formamide (14.9 g, 0.1 mol) was added and brought into solution. The condenser was replaced with a distillation apparatus, and the alcohol was removed under in vacuum. The reaction flask was immersed into a 350–360 °C metal bath for 30 min, and the 2,6-xylidine was removed by distillation and collected in a receiver. The residue was allowed to cool in a stream of nitrogen, and then 60 mL of water was added; the product was extracted twice with 100 mL of ether. The combined extract was washed with 5% hydrochloric acid, water, and 5% sodium carbonate. After drying, the ether was removed by distillation. The product was obtained after recrystallization of the residue from benzene–petroleum ether as pale yellow crystals melting at 77–85 °C (5.1 g, 78% based on 2,6-xylidine).

7-Methyl-2,3-dihydro-1*H*-indole. 7-Methylindole (5.0 g, 3.8 mmol) was dissolved in 80 mL of glacial acetic acid at 15–20 °C, and sodium cyanoborohydride (18.3 g, 30 mmol) was slowly added with stirring. After the addition was completed, stirring was continued for 3 h at room temperature. Acetic acid was removed under vacuum (<40 °C). The residue was washed with 10% sodium carbonate, and the indoline was extracted with ether. The ether extract was dried, and the solvent was removed by distillation. The product was obtained by distillation as colorless oil boiling at 111–113 °C/12 mmHg (3.4 g, 68%).

1-(Chloroacetyl)-7-methyl-2,3-dihydro-1*H*-indole (2c). 7-Methyl-2,3-dihydro-1*H*-indole (3.4 g, 2.6 mmol) was chloroacetylated in a manner similar to that used in the preparation of **1b**. The product is a white crystalline material melting at 66–67 °C (4.9 g, 92%).

General Procedure for Preparing Cysteine (2) and GSH (3) Conjugates in Liquid Ammonia. Sodium (0.13 g, 5 mmol) in small pieces was dissolved in 100 mL of liquid ammonia under magnetic stirring in a 250-mL round-bottom flask, and reduced L-glutathione (1.52 g, 5 mmol) or L-cysteine (0.61 g, 5 mmol) was added. After the GSH or cysteine was dissolved, the blue color of the solution disappeared. The corresponding chloroacetamide (5 mmol) was added, and stirring was continued for 1 h. The solution was poured into a beaker under a hood, and the ammonia was allowed to evaporate at room temperature. In the case of **1b** and **1c** derivatives, residues were dissolved in 10 mL of water and extracted with 25 mL of ether for removing unreacted chloroacetamides. Conjugates were precipitated from the aqueous solution by adjusting to pH 2 (GSH conjugates) or pH 5 (cysteine conjugates) with 2 N hydrochloric acid. Conjugates were purified by recrystallization from 50% aqueous ethanol. In the preparation of conjugates of acetochlor (**2a** and **3a**) after ether extraction aqueous solutions were concentrated to near dryness and products were obtained by precipitation from 2-propanol with ether. All conjugates were isolated as white crystals in 80–90% yield. Acetochlor cysteine and GSH conjugates (**2a** and **3a**) were purified from contaminating **2b** and **3b** by reverse-phase preparative HPLC using 30% aqueous acetonitrile containing 1% acetic acid as eluent.

All products were subjected to ^{1}H NMR and FAB mass spectrometry (Table I) and elemental analyses, which confirmed the structure of the corresponding conjugate. Elemental analyses were as follows.

2a. Found: C, 57.08; H, 7.11; N, 7.63; S, 8.82. Calcd for $C_{17}H_{26}N_2O_4S$: C, 57.55; H, 7.34; N, 7.90; S, 9.03.

2b. Found: C, 56.21; H, 6.58; N, 9.17; S, 10.52. Calcd for $C_{14}H_{20}N_2O_3S$: C, 56.73; H, 6.80; N, 9.45; S, 10.82.

2c. Found: C, 56.93; H, 6.07; N, 9.37; S, 10.38. Calcd for $C_{14}H_{18}N_2O_3S$: C, 57.12; H, 6.16; N, 9.52; S, 10.89.

3a. Found: C, 52.94; H, 6.84; N, 10.16; S, 5.74. Calcd for $C_{24}H_{36}N_4O_8S$: C, 53.27; H, 6.66; N, 10.36; S, 5.92.

3b. Found: C, 52.09; H, 6.18; N, 11.36; S, 6.38. Calcd for $C_{21}H_{30}N_4O_7S$: C, 52.27; H, 6.27; N, 11.61; S, 6.64.

3c. Found: C, 52.17; H, 5.76; N, 11.74; S, 6.34. Calcd for $C_{21}H_{28}N_4O_7S$: C, 52.49; H, 5.87; N, 11.66; S, 6.67.

General Procedure for Preparation of Cysteine Conjugates of 1b and 1c Chloroacetamides in Alcohol with Sodium Ethoxide. To a stirred suspension of L-cysteine hydrochloride monohydrate (0.88 g, 5 mmol) in 25 mL of absolute ethanol was added sodium (0.46 g, 20 mmol) in small pieces at room temperature. After the last piece of sodium disappeared, 5 mmol of **1b** or **1c** chloroacetamide was added. After 20 min, the reaction mixture was poured into 50 mL of water and the pH of the solution was adjusted to 5.0–5.5 with 2 N hydrochloric acid. The conjugate which precipitated from acidic solution was filtered off, washed with ether, and recrystallized from 50% aqueous ethanol. Yields were 70–82%.

RESULTS AND DISCUSSION

The intermediate deethoxymethyl acetochlor (**1b**) was prepared by chloroacetylation of 2,6-disubstituted aniline. The 7-substituted indoline derivative (**1c**) was obtained by a sequence of reactions starting with the formylation of 2,6-dimethylaniline. After the cyclization of the formanilide intermediate (Tyson, 1955), the product was selectively reduced by sodium cyanoborohydride to give 7-methyl-2,3-dihydro-1*H*-indole (Gribble et al., 1974). Finally, **1c** was obtained by chloroacetylation of this indole derivative.

The cysteine conjugates of **1b** and **1c** were easily prepared by treatment of cysteine hydrochloride in

absolute ethanol with sodium followed by addition of the appropriate chloroacetamide (Figure 1). The higher yield (82%) of **2c** to **2b** (70%) can be attributed to the higher alkylating activity of the chloroacetyl indoline derivative (Jablonkai and Dutka, 1989). Similarly excellent yields were reported in the synthesis of certain S-substituted cysteines with the more reactive alkyl and benzyl halogenides (Theodoropoulos, 1959). In the reaction of acetochlor with cysteine hydrochloride, besides the formation of **2a** 20–30% of **2b** could be detected as a byproduct. This is due to the hydrolytic sensitivity of the *N*-ethoxymethyl group in acetochlor (Jablonkai et al., 1982). The preparation of GSH conjugates according to this method provided desired products in poor yield such as in the synthesis of some aryl-substituted *S*-benzylglutathiones (Cohen and Smith, 1964). This is probably because of the lower solubility of GSH in absolute ethanol.

The syntheses of cysteine and glutathione conjugates (**2**, **3a–c**) of chloroacetamides were alternatively accomplished in higher yields and with greater purities by the reaction of chloroacetamides with stoichiometric cysteine and GSH in liquid ammonia in the presence of sodium (Figure 1). Liquid ammonia excellently solves both GSH and cysteine. The reaction of sodium amide with the SH group of these nucleophiles provides their sodium salts (RS^-Na^+), which can readily react with the chloroacetamides. In the reaction of acetochlor with both GSH and cysteine many fewer (about 5%) contaminating deethoxymethylated conjugates were formed at liquid ammonia temperature (−35 °C). Since the p*K* values of thiol groups in GSH and cysteine have been reported as 8.5–8.9 (Jocelyn, 1972; Friedman, 1973), under physiological conditions the mercaptide (RS^-) anion is undoubtedly the reactive species of these thiols. The pH dependence of the alkylating reactivity of haloacetates and haloacetamides with cysteine and GSH (Jocelyn, 1972; Friedman, 1973) as well as of the in vitro conjugation of alachlor with GSH (Leavitt and Penner, 1979) confirmed that the rates of reaction increased with increasing pH and reached a maximum at pH 8.6.

In Table I the chemical shifts of hydrogen atoms in molecules taken at 400 MHz and the physicochemical properties of conjugates are listed. The cysteine conjugate (**2a**) of acetochlor became optically inactive during the synthesis. It is also remarkable that the GSH conjugate (**3a**) of acetochlor melts without decomposition as compared with other conjugates. Depending on the matrix [3-nitrobenzyl alcohol or phosphoric acid (P)], the FAB-MS of these compounds were characterized by $(M + H)^+$ or $(M + H)^+$, $(M + H + P)^+$, and $(M + H + 2P)^+$ quasi-molecular ions (in Table I only M + H values are shown). The assignments of proton shifts of conjugates provided values similar to those reported for cysteine and GSH (Feil, 1986). Because of a chiral center in cysteine, the protons on the b carbon are magnetically nonequivalent. Protons of both cysteine and the glutamyl moieties yielded ABC coupling patterns.

ABBREVIATIONS USED

Acetochlor, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(ethoxymethyl)acetamide; cyanazine, 2-chloro-4-(ethylamino)-6-[1-cyano-(1-methylethyl)amino]-*s*-triazine; deethoxymethyl acetochlor, 2-chloro-*N*-(2-ethyl-6-methylphenyl)acetamide; GSH, reduced glutathione.

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