IDENTIFICATION OF GLUTATHIONE-DERIVED METABOLITES FROM AN IP RECEPTOR ANTAGONIST

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ABSTRACT:

The metabolic fate of three aromatic carboxylic acid analogs under evaluation as prostaglandin I₂-prefering receptor antagonists was studied. The initial analog with unsubstituted phenyl groups was subject to a complex set of aromatic oxidative biotransformations. By introduction of one or two fluorines, these pathways were inhibited. All three analogs were metabolized to a wide variety of carboxylic acid conjugates. Among these were several conjugates formed via secondary metabolism and oxidation of acyl glutathione intermediates. Two of the structure classes, represented by the S-methyl-N-cysteinylglycine conjugate and the N-cysteinylglycine disulfide conjugates, have been described only rarely in the literature. The related S-oxide of the S-methyl-N-cysteinylglycine conjugate and the N,S-bis-acetyl derivative of cysteinylglycine are here described for the first time as conjugate metabolites of carboxylic drugs.

Materials and Methods

Materials. Except as otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All solvents used for HPLC and LC/MS were of chromatographic grade. Compounds 1 to 3 were synthesized as described (Cournoyer et al., 2001) using the standard coupling of a 5-phenylbenzofuranamylmethyl alcohol with an isocyanate.

Preparation of ¹⁴C-Labeled Compound 1. To a solution of sodium hydroxide (1.37 g, 33.9 mmol) in ethanol (100 ml) was added 5-phenylphenol (5 g, 34 mmol). The mixture was stirred at room temperature for 16 h and concentrated. Bromoacetalddehyde diethylacetal (6.9 g, 35.02 mmol) dissolved in dimethyl sulfoxide was added and the resulting solution was heated at 110°C for 24 h. The mixture was cooled, poured into aqueous sodium hydroxide, and extracted with ethyl acetate. The combined organic layers were dried and concentrated. Chromatography on silica gel with 5% ethyl acetate/hexane afforded 4.9 g of 4-(2,2-diethoxyethoxy)-biphenyl. MS (electron impact) m/z 286 (M⁺). ¹H NMR (CDCl₃) δ 1.25 (t, 6 H, J = 5), 3.6–3.85 (m, 4H), 4.05 (d, 2 H, J = 5), 4.85 (t, 1 H, J = 5.2), 7.0 (d, 2H, J = 10.5), 7.26–7.35 (m, 1 H), 7.24–7.6 (m, 6 H). A mixture of this acetal (2 g) and polyphosphoric acid (2 g) in benzene (45 ml) was heated at reflux for 2 h. Upon cooling to room temperature, the benzene layer was decanted through a cotton plug and concentrated. Purification on silica gel with 5% ethyl acetate/hexane gave 1.1 g of 5-phenylbenzofuran (79%). MS (electron impact) m/z 194 (M⁺). ¹H NMR (CDCl₃) δ 6.8 (d, 1 H, J = 2.8), 7.3–7.65 m, 8 H), 7.75 (s, 1 H). A solution of this ether (0.319 mg, 1.64 mmol) in tetrahydrofuran (6 ml) was cooled to −78°C and n-butyl lithium (0.94 ml, 1.5 mmol) was added dropwise via syringe. The resulting red solution was warmed to −15 to −5°C and stirred for 3 h. Upon cooling with liquid nitrogen, the flask was placed under high vacuum, and ¹⁴CO₂ (generated from Ba¹⁴CO₃/H₂SO₄, 80 mCi) was vacuum-transferred to the reaction. The flask was isolated, warmed to −70°C and stirred. No more dry ice was added to the Dewar and the mixture was allowed to warm to room temperature slowly and to stir overnight. The volatile components were vacuum-transferred into methanolic potassium hydroxide. The residue was diluted with aqueous HCl/brine/dichloromethane. The layers were separated, and the aqueous layer was extracted with 5% methanol/dichloromethane, dried over Na₂SO₄, and concentrated to give 44.2 mCi of...
Preparation of 14C-Labeled Compound 2. To a solution of crude 5-phenylbenzofuran-[14C]-2-carboxylic acid. Radio-HPLC analysis showed the compound to be 96% pure. A solution of this material in tetrahydrofuran (3 ml) was cooled to 0°C. Lithium aluminum hydride (43 mg, 1.14 mmol) was added and the mixture stirred at this temperature for 2 h. The solution was quenched with 4 M aqueous sodium hydroxide, filtered through a 0.45-μm nylon filter, and concentrated in vacuo and purified on silica gel with 30% ethyl acetate/hexane to give 28.2 mCi of crude 5-phenylbenzofuran-[14C]-2-carboxylic acid (11.2 mCi) in tetrahydrofuran (3 ml) was added freshly prepared propionic acid, [14C]-1. The compound was stored in 100 ml of ethanol. Specific activity: 59.5 mCi/mmol.

Preparation of 14C-Labeled Compound 3. A solution of 2-[14C]bromomethylmalonate (98 mCi, 1.81 mmol) and 2-hydroxy-5-(4-fluorophenyl)benzaldehyde (436.4 mg, 2 mmol) in 2-butane (22 ml) was treated with potassium carbonate (0.736 g, 5.33 mmol). The mixture was heated to reflux for 42 h, cooled, and then treated with 8 M potassium hydroxide (2 ml), water (50 ml), and ethyl acetate (50 ml). The organic layer was dried and concentrated. The residue was purified by column chromatography on silica gel using ethyl acetate/hexane (1:3) to yield 2-[14C]-2-hydroxyethyl-5-(4-fluorophenyl)benzofuran (75.8 mCi, 77.3%). A solution of the ester (48 mCi, 0.89 mmol) in tetrahydrofuran (9 ml) was cooled to 0°C, lithium aluminum hydride (63.9 mg, 1.68 mmol) was added, and the mixture was stirred at this temperature for 2.5 h. The solution was treated with ethyl acetate (10 ml) and stirred for 15 min. It was then treated with water (10 ml) and stirred for 10 min, followed by acidification with 1 M HCl (4 ml). The organic layer was removed and the aqueous layer extracted with ethyl acetate (1:3) to yield 2-[14C]-2-hydroxyethyl-5-(4-fluorophenyl)benzofuran (37.2 mCi, 77.5%). A solution of the alcohol (18.6 mCi, 0.34 mmol) in tetrahydrofuran (25 ml) was treated

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TABLE 1

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Source: a, drug candidate; b, metabolite or intermediate, structure proven by synthesis; c, observed in vitro metabolite; d, observed in vivo metabolite.
with freshly prepared p-fluoro-phenylalanine methyl ester isocyanate followed by triethylamine (100 μl, 72.6 mg, 0.72 mmol). The mixture was refluxed under nitrogen overnight, cooled to ambient temperature, and filtered through a 0.45-μm nylon filter. The filtrate was concentrated and the residue was purified by column chromatography on silica gel using ethyl acetate/hexane (1:3) to yield 3-phenyl-2-(5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxybenzyl)-3-phenyl-propionic acid methyl ester (14.48 mg, 77.8%). To a solution of this ester in tetrahydrofuran (5 ml) at 0°C was added a solution of lithium hydroxide (25 mg, 0.66 mmol) in water (4 ml) over 5 min. The mixture was stirred for 4 h and concentrated at ambient temperature. The residue was washed with a 1:1 mixture of ether/hexane, (twice, at 15 ml) and acidified with 1 M HCl (4 ml). The precipitated product was extracted into ethyl acetate (twice, at 20 ml). The solution was dried and concentrated to yield the product, \( \text{1H} \) NMR (400 MHz, DMSO-d6). M.p. 166.2–166°C. MS (ESI−) m/z 430 (M−H). \( \text{1H} \) NMR [dimethyl-d6-toluene (DMSO-d6)] 6.83 (dd, 1 H, J = 13.8, 10.6), 3.07 (dd, 1 H, J = 13.8, 4.4), 4.18 (m, 1 H), 5.07 (d, 1 H, J = 13.5), 5.13 (d, 1 H, J = 13.5), 6.85 (d, 2 H, J = 8.6), 6.89 (s, 1 H), 7.20 (m, 5H), 7.49 (J = 8.6), 5.74 (dd, 1 H, J = 9.6, 1.8), 7.58 (d, 1 H, J = 9.6), 7.72 (m, 1H), 7.97 (br s, 1H), 9.47 (s, 1H), 12.74 (s, 1 H).

Preparation of Compound 5. To a solution of α-tyrosine methyl ester hydrochloride (1.0 g, 4.31 mmol) in acetonitrile (8 ml) were added to 0–5°C tetrahydrofuran (0.6 ml, 4.31 mmol), tert-butylmethylsilyl chloride (0.97 g, 6.47 mmol), and 1,8-diazabicyclo[5.4.0]undec-7-ene (1.08 ml, 7.7 mmol) under argon. The mixture was stirred at 25°C for 16 h. Water was added and the mixture was extracted with dichloromethane. The extract was dried over sodium sulfate and concentrated to dryness. Purification by flash column (SiO2, 95:5 dichloromethane/acetone) yielded 0.76 g of compound (R)-2-amino-3-[4-(tert-butyldimethylsiloxy)-phenyl]-propionic acid methyl ester (0.97 g, 72%). To the latter (0.41 g, 1.31 mmol) in acetonitrile (15 ml) were added 4-nitrophenyl 5-phenylbenzofuran-2-yl carbonate (0.51 g, 1.31 mmol), triethylamine (0.27 ml, 1.96 mmol), and 4-dimethylaminopyridine (0.18 g, 1.44 mmol) under argon. The mixture was heated at reflux for 5 h. Following addition of 10% aqueous KH2PO4/Na3PO4 to pH 2–3, the mixture was extracted with dichloromethane. The extract was washed extensively with 10% Na2CO3 and brine, dried on sodium sulfate, and concentrated to dryness. Purification by flash column (SiO2, 75:25 hexane/ethyl acetate) gave (R)-3-[3-(tert-butyldimethylsiloxy)-phenyl]-2-(5-phenylbenzofuran-2-ylmethoxybenzyl)-3-phenyl-propionic acid methyl ester (0.2 g, 22%) and (R)-3-(4-hydroxyphenyl)-2-(5-phenylbenzofuran-2-ylmethoxybenzyl)-3-phenyl-propionic acid methyl ester (0.18 g, 30%). The former product (0.2 g, 0.36 mmol) was transformed into the latter by treatment with 1 M tert-butyllamine hydrochloride (0.36 ml, 0.36 mmol) in tetrahydrofuran (3 ml) under argon. Following the addition of 1 N HCl to pH 1 to 2, the mixture was extracted with ether. The extract was washed with brine, dried on sodium sulfate, and concentrated to dryness. Purification by flash column (SiO2, 75:25 hexane/ethyl acetate) gave more of the above phenol (0.16 g, 99%). To a solution of phenol (0.35 g, 0.79 mmol) in tetrahydrofuran (6.4 ml) and water (1.6 ml) was added lithium hydroxide hydrate (0.07 g, 1.57 mmol) under argon. The mixture was stirred for 1 h. Following the addition of 1 N HCl to pH 1 to 2, the mixture was extracted with ethyl acetate. The extract was washed with brine, dried on sodium sulfate, and concentrated to dryness. Crystallization from dichloromethane gave compound 5 (0.18 g, 51%), m.p. 167.7–170.7°C. MS (ESI−) m/z 430 (M−H). \( \text{1H} \) NMR [DMSO-d6] 6.27 (dd, 1 H, J = 13.8, 10.3), 2.95 (dd, 1H, J = 13.8, 4.4), 4.10 (m, 1 H), 5.09 (d, 1 H, J = 13.5), 5.15 (d, 1 H, J = 13.5), 6.65 (d, 2 H, J = 8.4), 6.91 (s, 1 H), 7.04 (dd, 2 H, J = 8.4, 7.36 (m, 1H), 7.47 (t, 2 H, J = 7.5), 7.62 (m, 2 H), 7.69 (m, 3 H), 7.91 (br s, 1H), 9.21 (s, 1H), 12.7 (br, 1H).

Preparation of Compound 6. The same steps were followed as for Preparation of Compound 4 except that (R)-3-(4-fluorophenyl)-2-isocyanato-propionic acid methyl ester was used to obtain compound 6. m.p. 165.2–166°C. MS (ESI−) m/z 386 (M−H). \( \text{1H} \) NMR [DMSO-d6] 6.73 (dd, 1 H, J = 13.8, 9.9), 2.89 (d, 1 H, J = 13.8, 4.4), 4.10 (m, 1 H), 5.07 (d, 1 H, J = 13.5), 5.13 (d, 1 H, J = 13.5), 6.85 (d, 2 H, J = 8.5), 6.88 (s, 1 H), 7.07 (t, 2 H, J = 8.7), 7.28 (dd, 2 H, J = 8.7, 5.6), 7.49 (d, 2 H, J = 8.5), 7.56 (m, 2H), 7.75 (s, 1H), 7.77 (br s, 1H), 9.49 (br s, 1H).

Preparation of Compound 20. Compound 2 (0.329 g, 0.759 mmol) was dissolved in tetrahydrofuran (35 ml) at room temperature. Triethylamine (0.106 ml, 0.759 mmol) and ethyl chloroformate (0.073 ml, 0.759 mmol) were added to the mixture. After 30 min, a precipitate had formed and was removed by filtration. The filtrate was added to a solution of glutathione (0.233 g, 0.759 mmol) and potassium bicarbonate (0.076 g, 0.759 mmol) in water (10 ml) and tetrahydrofuran (10 ml) and stirred for 3 h. The reaction was quenched with nine drops of concentrated hydrochloric acid, and then the tetrahydrofuran was removed in vacuo. The aqueous residue was extracted with diethyl ether, leaving a suspension of product in the aqueous layer which was filtered and dried. The solid was heated in ethyl acetate for recrystallization. The solid that would not dissolve was filtered off to give 0.056 g of a clean white powder. MS (ESI+) m/z 723.1 (M+H)+. \( \text{1H} \) NMR [DMSO-d6] \( \delta \) 1.66 (m, 2 H, 2.09 (br m, 2 H), 2.58 (dd, 1 H, J = 13.5, 11.3), 2.73 (dd, 1 H, J = 13.3, 9.9), 2.86 (dd, 1 H, J = 13.8, 8.2), 3.11 (m, 2 H), 3.58 (d, 1 H, J = 4.5), 3.48 (d, 2 H, J = 5.6), 4.14 (m, 2 H), 4.92 (s, 2 H), 6.6 (s, 1 H), 6.82 (t, 2 H, J = 8.8), 7.09 (m, 3 H), 7.25 (t, 2 H, J = 7.5), 7.41 (m, 5 H), 7.67 (s, 1 H), 8.90 (d, 1 H, J =
8.3, 8.25 (d, 1 H, J = 8.7), 8.50 (t, 1 H, J = 5.6); 13C NMR (DMSO-d6, δ 31.74, 36.03, 41.06, 51.84, 56.47, 58.51, 58.88, 62.97, 107.08, 111.80, 115.15, 119.82, 119.82, 124.38, 127.28, 127.45, 128.64, 129.31, 131.38, 136.02, 140.79, 153.64, 153.94, 154.39, 155.79, 159.80, 163.01, 170.46, 171.12, 171.95, 173.42, 200.92.

Synthesis of Glutathione-Related Metabolites. In vivo metabolism studies, microsomes. The metabolism and characterization of the rat microsomes used in these studies was similar to previously published methods (Swinney et al., 1994). Incubation mixtures (0.5 ml) contained compound 1, 2, or 3 (10 μM with a maximum of 1% dimethyl sulfoxide used as cosolvent), phosphate buffer (100 mM), NADPH (1 mM), magnesium chloride (5 mM) and microsomal protein (1 mg/ml) and were allowed to react at 37°C for 30 min.

Hepatocytes. Rat hepatocytes were isolated based on a two-step perfusion as previously described (Seglen, 1976). Briefly, the liver was perfused with Gibco Liver Perfusion Medium (Invitrogen, Carlsbad, CA) for 5 to 8 min, followed by Gibco Liver Digest Medium for 6 to 10 min. Dog hepatocytes were purchased from Invitrogen Technologies (Baltimore, MD). Hepatocytes were plated in 24-well collagen-coated cell culture plates (200,000 cells per well) and maintained in a humidified atmosphere of 5% carbon dioxide at 37°C. Incubation mixtures (0.5 ml) contained compound 1, 2, or 3 (10 μM with a maximum of 1% dimethyl sulfoxide used as cosolvent) in Gibco HepatoZyme media (Invitrogen) supplemented with 5% fetal bovine serum and 2 mM L-glutamine and were allowed to react for up to 24 h.

In Vivo Metabolism Studies. A total of five radiolabeled, in vivo studies were conducted. Doses for all animal studies were administered as solutions in 100 mM tris(hydroxymethyl)aminomethane (pH 7–9). Bile duct-cannulated male Han:Wistar rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Rats were administered 115.15, 119.82, 119.82, 124.38, 127.28, 127.45, 128.64, 129.31, 131.38, 136.02, 140.79, 153.64, 153.94, 154.39, 155.79, 159.80, 163.01, 170.46, 171.12, 171.95, 173.42, 200.92.

Metabolite Quantification. Total radioactivity in urine and bile samples was determined using a model TR1900 (PerkinElmer Life and Analytical Sciences) liquid scintillation counter with external standardization. Each sample was dissolved in an 8-ml aliquot of β-Blend (MP Biomedicals) scintillation fluid. These results were used to adjust percentage chromatographic peak area into percentage of applied dose.

Results

The structures of title compounds and important metabolites are shown in Table 1. Full synthetic details for the preparation of the 14C-labeled versions of the title compounds, 1 to 3, as well as the hydroxymethylmetabolites, 4 to 6, are included under Materials and Methods. Since mass spectrometry was used for all structure determinations, Table 1 also shows the negative ion M–H molecular ion and major MS/MS fragments for each compound. The negative ion spectrum of compound 1 was dominated by the molecular ion (M–H) at m/z 414 and an in-source fragment ion of m/z 190. The positive ion mass spectra of 1, analogs, and metabolites taken under conditions of method A show weak sodium adduct parent ions and are not useful. Thermally induced in the source or in the ion trap by CID during MS/MS, the central carbamate bond (shown in Table 1) is cleaved to yield a 5-phenylbenzofuranyl methyl alcohol and an isocyanate. The alcohol is termed the “left side.” It is not observable in negative ion unless a negative stabilizing substituent such as sulfate is present. The isocyanate is termed the “right side” and is always observable in negative ion spectra. A secondary loss of HNCO is often observed from the right-side fragment. For compound 1, the primary and secondary right-side ions are observed at m/z 190 and 147, respectively.

Oxidative Metabolism of 1 to 3: Compounds 4 to 6 and Their Conjugates. The structures of the metabolites, along with their MS data, and whether they were observed in vitro, in vivo, and/or synthesized, are shown in Table 1. Rat liver microsomal incubations showed that 1 and 14C-labeled 1 are converted readily to the monooxidized derivatives 4 and 5. The MS/MS-observed right-side ion for compound 4 is the same as that seen for 1. For compound 5, the right-side ion is displaced 16 amu. These identifications were confirmed by comparison with authentic samples. When compound 1 was
administered orally to monkey, the sulfate of 4 was a major metabolite observed in urine and bile. The MS/MS of 4S showed a strong left-side fragment at m/z 319 due to the negative charge-stabilizing sulfate group. This identification was confirmed after sulfatase treatment (Schaber et al., 2001) to give 4. This sulfate and other sulfate conjugates tailed badly under acidic HPLC conditions, such as method A. These molecules behaved much better when the chromatography was conducted at pH 10 with method B. The Polaris columns are polar-modified and stable for use (but not extended storage) at this pH.

When the right-side oxidation is blocked by fluorine in molecule 2, the left-side oxidation to give 6 is a major pathway. Compound 6 and its sulfate were also identified by their characteristic fragmentation, sulfatase treatment, and synthesis. The oxidative metabolism of 1 is complicated by additional sites of oxidation on each ring. Additional left-side oxidation is also seen with compound 2. Compounds 1 and 2 also form dihydrodiol and glutathione adduct metabolites from aromatic oxidative pathways. Several of these were observed as minor metabolites in vivo but were not characterized by isolation; the regiochemistry of these metabolites is not known.

Figure 1 shows the base peak, negative ion electrospray mass (top), UV (middle), and radio (bottom) chromatograms (method B) for a sample of bile from a dog treated with compound 2. The MS data are convoluted by the presence of the large peak due to bile acid. The UV and radiochromatograms are in remarkable concordance. The oxidative metabolism of 1 is isomerized by several minor isomers and hydrolyze back to their parent molecules. Collection of bile onto dry ice provided a stabilized sample for quantitative analysis of 7 to 9. Upon CID, the acyl glucuronides yield four major ions: the right-side ions displaced by 176 amu, the loss of 176 from the M – H, the loss of 176 from the right-side ion, and the m/z 175 glucuronide anion.

**Compounds 7 to 9.** The acyl glucuronides of 1 to 3 were observed in animal and human hepatocyte incubations and in vivo samples. These metabolites are unstable (Shipkova et al., 2003) and isomerize to several minor isomers and hydrolyze back to their parent molecules. Confirmation of the structures was obtained by running LC/MS/MS on the Sciex 4000 triple quadrupole where the diagnostic (Shirley et al., 1997) taurine conjugate ions at m/z 124 (NH2-CH2-CH2-SO3-) and 80 (SO3-) were observed for compound 10. The taurine conjugate of 1 was not observed.

**Compounds 10 to 11.** Compounds 10 and 11, which coelute with the corresponding acyl glucuronides, are the taurine conjugates of 2 and 3, respectively. They were observed only in samples of monkey or dog bile. In CID on the ion trap, each gives a strong right-side ion at m/z 315 with subsequent loss of vinylsulfonic acid to give m/z 207. Confirmation of the structures was obtained by running LC/MS/MS on the Polaris columns at pH 10 with method B. The Polaris columns are polar-modified and stable for use (but not extended storage) at this pH.

**Compounds 12 to 15.** The remaining major metabolite peaks, labeled 12, 14, 16, and 18 in Fig. 1, were not readily assignable. Moreover, whereas peaks 16 and 18 gave strong radio and UV signals, no signal was observed in negative ion ESIMS. The key to the structure of these metabolites was a careful analysis of their mass spectral fragmentation, especially for 12 and its congener 13, a metabolite of 3. Compounds 12 and 13 are unusual metabolites of molecular weight 174 more than each parent. The MS/MS spectra of
12 and 13 are identical due to the collision-induced loss of the unfluorinated left side from 12 and the loss of the fluorinated left side from 13 to each give a right-side ion at \( m/z \) 382; apparently, the incremental 174 amu are due to changes on the right side. MS\(^n\) experiments on 13 showed the sequence 624→382→334→290→246. A loss of 48 amu (382–334) is quite unusual and could only be explained as CH\(_3\)SH (Davis et al., 2002). The accurate masses of the molecular ion of 13 and its three key fragments were determined as shown in Table 2. The data are consistent with a formula of C\(_{19}\)H\(_{33}\)F\(_2\)N\(_2\)O\(_2\)S and the losses being C\(_{19}\)H\(_2\)NO\(_2\) (the left side), followed by CH\(_3\)SH, followed by CO\(_2\). These data reveal that the conjugating increment in 13 has the formula C\(_{19}\)H\(_{33}\)N\(_2\)O\(_2\)S and is best explained as the S-methylcysteinylglycine conjugate formed by secondary metabolism of a glutathione conjugate (Grillo and Benet, 2001); see Fig. 2 for the conjugating structures of 12 and 13.

Peak 14 of Fig. 1 has a molecular weight 190 amu greater than that of the corresponding parent and a fragmentation pattern much like that of 12. The 190 mass units are on the right side because the MS/MS of the M – H ion at \( m/z \) 622 shows a right side ion at \( m/z \) 398. The 398 ion further fragments to give 334, 290, and 246 ions identical to those for 12. This is consistent with 14 being the S-oxidized analog to 12, a loss of 64 amu being known as diagnostic for oxidized S-methylcysteine (Davis et al., 2002). The analogous metabolite, 15, was observed for 3, again yielding the same MS/MS right-side fragments.

**Compounds 16 to 19.** With the structures of 12 to 15 in hand, it became clear why no MS signal was observed for peaks 16 and 18 from Fig. 1. These must be related to the dimers that Grillo and Benet (2001) described in their paper. The dimer structure 16 would have a molecular weight of 1184, well outside the scan range of our standard ESI MS methods. Method C was thus used to obtain a molecular ion of 1183 for 16. In MS/MS, 16 loses two consecutive left-side fragments to give ions at \( m/z \) 959 and 735.

With a larger mass range scanned, peak 18 of Fig. 1 was found to have a negative molecular ion at \( m/z \) 1007. In MS/MS, this metabolite lost a left side to give an ion at \( m/z \) 783 or lost an entire acyl group to give an ion at \( m/z \) 592. This behavior is explained by the structure being the N,S-bis-acetyl derivative of cys-gly as drawn in Fig. 2.

Fully analogous metabolites 17 and 19 were observed in bile when compound 3 was administered to dogs and monkeys. The symmetric dimer 17 has an M – H of 1219 and consecutive losses of 242 amu to give product ions at 977 and 735. The N,S-diacyl peptide 19 has an M – H = 1043, a loss of 242 to give \( m/z \) 801 or an acyl loss to give \( m/z \) 610. Metabolite 19 is a major metabolite of the difluorinated compound 3, which does not undergo oxidative biotransformation.

**Synthesis of 16 and 18.** The identifications of 16 and 18 were confirmed by synthesis. Compound 20, the glutathione conjugate of 2, was prepared by coupling 2 and glutathione. Compound 20 has an M – H = 721; in MS/MS it shows loss of the left side to \( m/z \) 497 and a GS\(^-\) fragment at \( m/z \) 306. (These peptidic conjugates can also be analyzed in positive ion ESI, as shown in the experimental description of their synthesis.) When 20 was treated with \( \gamma \)-glutamyltranspeptidase, it was quickly consumed, and a mixture was obtained. Figure 3a shows the LC/UV chromatogram obtained after a 2-h incubation. A small amount of starting material 20 remains, along with the hydrolysis product 2 and a new peak, labeled B, of M – H = 592, which must be the monomer structure B (Fig. 2). In MS/MS, peak B loses a left side to give \( m/z \) 368, followed by loss of H\(_2\)S to give \( m/z \) 334. Peak B disappeared when this reaction was worked up.

The late-eluting peaks of Fig. 3 are the symmetric dimer 16 and the N,S-diacyl peptide 18. Figure 3b shows the UV chromatogram for the dog bile sample, rechromatographed using method C and demonstrating the retention time comparison between the synthetic and in vivo 16 and 18. The synthetic 16 is identical by LC retention time using both methods B and C as well as by MS and MS/MS to the metabolite observed in the dog after administration of 2. When this reaction was scaled up for isolation of the dimers, less of the symmetric S,S dimer was obtained. The structure elucidation of 16 is based solely on its MS behavior.

The latest-eluting component of the \( \gamma \)-glutamyltranspeptidase reaction was isolated by preparative HPLC. Its retention time in two methods and its MS and MS/MS behavior are identical to those of the metabolite labeled peak 18 in Fig. 1. The \( ^1\)H NMR of the synthetic 18 is listed in Table 3, compared with data for 2 and 20. The assignments were confirmed by heteronuclear multiple quantum coherence spectroscopy and correlation spectroscopy experiments. Integration of the

**TABLE 2**

<table>
<thead>
<tr>
<th>Accurate masses and formulas of ions in mass spectrum of 13</th>
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<tr>
<td><strong>Data</strong></td>
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![Fig. 2. Scheme for chemical synthesis or biosynthesis of the glutathione-derived metabolites.](image-url)
resonances in the $^1$H spectrum of 18 was telling: there was 1 cysteine $\alpha,\beta$ pattern and one glycine pattern but two (mostly overlapping) resonances for all of the protons of the benzofuran and phenylalanine portions of the molecule.

Metabolite Quantification. When compound 1 was administered to monkeys, 64.3% and 4.3% of the dose was excreted, respectively, in bile and urine within 24 h. The main urinary and biliary radioactive components were parent, 7, 4, and 4S. The glutathione-derived metabolites were minor for 1.

When compound 2 was administered to rats, 37.5% and 3.5% of the dose was excreted, respectively, in bile and urine within 24 h. No urinary metabolites were identified. In the case of bile, the major radioactive components were parent, 8, and $6^S$. Compounds 12, 14, 16, and 18 added together constituted 6.1% of the radioactivity observed in bile.

When compound 3 was administered to rats, 43.7% and 1% of the dose was excreted, respectively, in bile and urine within 24 h. No urinary metabolites were identified. In the case of bile, the major radioactive components were parent, 9, and 19. Compounds 13, 15, 17, and 19 added together constituted 25.7% of the radioactivity observed in bile.

When compound 3 was administered to dogs, >95% and <1% of the dose was excreted, respectively, in bile and urine within 24 h. No urinary metabolites were identified. In the case of bile, the major radioactive components were parent, 9, and 19. Compounds 13, 15,
of both aromatic cores. In vivo, the products of these oxidations are seen mainly as sulfate conjugates. Evidence was also obtained for the presence of dihydrodiol and glutathione metabolites of both aromatic cores. These metabolites are formed via arene oxide intermediates, which have been implicated in covalent binding to biological macromolecules. The acyl conjugation reactions are relatively less important for compound 1.

The introduction of a fluorine (compound 2) has the classic effect (Park et al., 2001) of preventing the oxidative metabolism of the right-side ring. Compound 2 still shows extensive oxidation of the left side, including the formation of dihydrodiols and glutathione-derived conjugates. Compound 2 shows relatively more of the acyl conjugates.

The introduction of a second fluorine (compound 3) essentially blocks all aromatic oxidations. The in vivo metabolism of 3 is thus dominated by the conjugation of the carboxylic acid moiety to yield taurine and acyl glucuronide conjugates and, most interestingly, a set of glutathione-derived metabolites. Benet and his colleagues have characterized this metabolic pathway for clofibric acid (Stogner and Fenselau, 1982; Shore et al., 1995; Grillo and Benet, 2001). Clofibric acid metabolites analogous to 12 and 16 were observed in urine of rats treated intravenously with the glutathione conjugate of clofibric acid, but intraperitoneal administration of the free acid yielded only small amounts of the disulfide (Grillo and Benet, 2001). Figure 2 shows the corresponding pathway for metabolic conversion of compound 2 into 12, 14, 16, and 18. The acyl-glutathione conjugate, 20, is probably derived via intermediacy of an acyl-CoA derivative or the acyl glucuronide, 8 (Grillo and Benet, 2002; Li et al., 2002). We have not observed the relatively unstable acyl-CoA metabolite or the acyl glutathione metabolite in hepatocyte incubations or in vivo, but others are reporting these with careful study (Grillo et al., 2003; Grillo and Hua, 2003). When synthetic 20 is exposed to γ-glutamyltransferase, it is converted to two even more unstable intermediates. The primary product, labeled A in Fig. 2, undergoes an S,N intramolecular acyl transfer to form product B. Product B is unstable in relation to dimerization. In the in vitro reaction, it is converted to the S,S dimer, 16, or to the N,S-diacyl peptide 18. In vivo, an alternative stabilization of B is found through S-methylation to give 12. Further oxidation of 12 gives the stable sulfoxide 13.

The enzymes responsible for the steps of this pathway after the γ-glutamyltransferase are not clear. The S to N rearrangements of A to give B and 18 are probably nonenzymatic; in our hands, they occurred spontaneously, as did the thiol dimerization of B to 16. Dimerization would certainly be concentration-dependent. We cannot say whether the dimers observed in bile samples were true metabolites or artifacts formed after sample collection. S-Methylation with S-adenosylmethionine requires a thiol methyltransferase, a widely distributed enzyme (Stevens and Bakke, 1990). Oxidation of thiouethers is a common nonenzymatic (Davis et al., 2002) or enzymatic (Davies et al., 1995) reaction.

The N,S-diacyl peptide, 18, and the corresponding 3-derived conjugate, 19, are novel phase 2 metabolites. Compounds 18 and 19 retain the thioester bond, which is expected to be unstable to hydrolysis. That these compounds are biologically and chemically stable and isolable from in vivo samples can be attributed to their extreme hydrophobicity. The large aromatic groups must exclude water from the vicinity of their thioesters.

Compounds 12 to 19 were not the only glutathione-derived metabolites observed in the metabolism of 1 to 3. Analogs of 16 and 18 were observed as minor monkey metabolites of 1. In the same sample of monkey bile, a minor peak was found to have an LC/MS/MS behavior (M – H = 547; right-side ions at 323→259→214) consistent with an oxidized S-methyl, N-cysteinyl acyl derivative of 1. Several other metabolites of 2 seemed to have left-side oxidation and sulfation combined with the acyl S-methyl cyst-gly dipeptide, as does 12. Interestingly, these metabolites were found only in bile. Biliary excretion might be expected for metabolites of such high molecular weight (Levine, 1981) and may explain the paucity of literature descriptions of this pathway. The glutathione-derived metabolites were not observed in animal or human hepatocyte incubations. Their toxicological implications are unknown. In this study, the glutathione-derived metabolites were not observed in circulation or in urine, so there may not be a large exposure to them. The fact that they are not described in the literature means these pathways are probably not major pathways in any case.

This novel glutathione-based metabolism can lead to a very complex metabolic pattern. The overall metabolism of 1 to 3 is summarized in Fig. 4. Preclinical evaluation of the metabolism of carboxylic acid therapeutic agents should include consideration of these glutathione-derived conjugates. We suggest that databases of metabolic pathways should include the 174- and 190-amu transformations among the list of possible metabolites. The dimers are more difficult to add to a single listing of molecular weight ranges. Dimer 16 has a molecular weight 2 times the carboxylic acid weight plus 318. Dimer 18 has a molecular weight 2 times the carboxylic acid weight plus 142. We suggest that consideration be given to scanning larger than normal mass ranges when studying the metabolism of carboxylic acids susceptible to the formation of dimeric structures.

References


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