Metabolism of Furans in Vitro: Ipomeanine and 4-Ipomeanol

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Ipomeanine (IPN), 4-ipomeanol (4-IPO), 1-ipomeanol (1-IPO), and 1,4-ipomeadiol (DIOL) are toxic 3-substituted furans found in mold-damaged sweet potatoes. IPN and 4-IPO are the most toxic, but all produce pulmonary toxicity in cattle and rodents, and 4-IPO induces hepatotoxicity in humans. These furans require metabolic activation to elicit toxicity, but the limited information obtained from previous metabolism studies prompted us to initiate the investigation reported here. Our initial studies of 4-IPO metabolism by rat liver microsomes demonstrated that the oxidation of 4-IPO to IPN and reduction to DIOL occurred and that more IPN was metabolized to a reactive species than 4-IPO or DIOL. Incubation of IPN and Gly produced a 2′-pyrroline-5′-one adduct establishing that IPN was metabolized to an enedial. N-Acetylcysteine reacted with the 5′-aldehyde of the enedial to give two 2′,5′-dihydro-2′-hydroxyfurans stabilized by H bonding between the 2′-OH and 3′-keto group. Reaction of the enedial metabolite of IPN with one GSH gave several adducts including a pyrrole derived from the 1,2-addition of GSH to the 5′-aldehyde as well as two tricyclic 2′-pyrrolines derived from the 1,4-addition of GSH at the 4′-position. The identities of the pyrrole and 2′-pyrroline GSH adducts were confirmed by observation of structurally similar adducts from Cys conjugation with the enedial metabolite of IPN. Several minor adducts from the conjugation of the enedial metabolite of IPN with two GSH were also detected. Mono-GSH and bis-GSH adducts were derived from both the 1,2- and 1,4-addition of GSH to the enedial metabolite of 4-IPO in rat liver microsomal incubations of 4-IPO and GSH. Sequential oxidation of 4-IPO to IPN and then to the enedial metabolite followed by GSH conjugation also occurred in the 4-IPO incubations. The complex structures of the reaction products of the enedial with biological nucleophiles may explain why the many attempts to identify 4-IPO adducts to protein have not been successful.

Introduction

Ipomeanine (IPN), 1-(3′-furyl)-1,4-pentanediol, 4-ipomeanol (4-IPO), 1-(3′-furyl)-4-hydroxy-1-pentanone, 1-ipomeanol (1-IPO), 1-(3′-furyl)-1-hydroxy-4-pentanone, and 1,4-ipomeadiol (DIOL) (Figure 1) are four 3-substituted furans responsible for the pulmonary toxicity that sometimes occurred when cattle were fed mold-damaged sweet potatoes (1, 2). These furans are degradation products of furanosesquiterpenoid metabolites that sweet potatoes produce in response to fungal infection or other stress (3). All four 3-substituted furans cause pulmonary toxicity in mice similar to that seen in cattle. The oral LD50 values in mice for IPN, 4-IPO, 1-IPO, and DIOL are 26 ± 1, 38 ± 3, 79 ± 9, 104 ± 12 mg/kg, respectively (2). Of the four furans, 4-IPO has received the most attention, including a clinical trial for the treatment of lung cancer. 4-IPO-treated patients developed severe hepatotoxicity with little toxicity to the lungs or the lung cancer (4).

4-IPO is bioactivated by lung or liver microsomes and NADPH to a metabolite that reacts with proteins and forms stable adducts with GSH (5, 6). Induction of cytochromes P450 by the pretreatment of rodents with 3-methylcholanthrene shifts toxicity to the liver, presumably by increasing metabolism in that organ (7). Only recently has an enedial metabolite from the bioactivation of 4-IPO been characterized. Alvarez-Diez and Zheng (8) reported an in vivo metabolism study of 4-IPO in bile-duct-cannulated rats. They detected four biliary metabolites that were characterized by MS monitoring at m/z 492 (M + H+), equivalent to adduction between the enedial metabolite (184) and one GSH (307). The proposed aldehyde-containing structures for these adducts, however, seemed improbable and prompted us to begin the studies reported here. Recently, Baer et al. (9) reported that in vitro metabolism of 4-IPO by rabbit CYP4B1, NADPH, and GSH gave two bis-GSH pyrrole adducts from conjugation of the enedial metabolite with two GSH following by the loss of two H2O molecules. Multiple human liver P450s that are capable of the 4-IPO bioactivation were also identified (9).

The metabolism of furans to a 1,4-dicarbonyl moiety is well established for furan and furans substituted with only alkyl groups. The microsomal metabolism of 2- and 3-methylfurans to acetylacrolein and 2-methylbutene-1,4-dial were the first examples (10). McClanahan et al. (11), subsequently, demon-

![Figure 1. Structures of ipomeanine (IPN), 4-ipomeanol (4-IPO), 1-ipomeanol (1-IPO), and 1,4-ipomeadiol (DIOL).]
strated that the metabolism of menthofuran resulted in the oxidation of the furan to a ketoal. The oxidation of furan to cis-2-butene-1,4-dial was demonstrated by Chen et al. (12). These studies relied on trapping the intermediates with semicarbazide. The products from the reaction of cis-2-butene-1,4-dial with amino acids and GSH have been extensively studied and hexahydro-3,6-dimethyl-1-(2-sulfoethyl)-2-idencyl methyl ether (2-ene) (11). The former is the taurine adduct of the ketoal.

The characterization of GSH adducts in recent studies of 4-IPO prompted a re-investigation of 4-IPO metabolism. This effort has demonstrated that the major metabolic processes in rat liver microsomal incubations include oxidation and reduction of 4-IPO to IPN and DIOL, respectively. The reduction of IPN to 4-IPO in microsomal incubations was also observed. The IPN to 4-IPO interconversion is likely to occur in vivo, and information gained from IPN studies is applicable to 4-IPO.

There was no published metabolism study for IPN, and therefore, the present study includes metabolism studies of both IPN and 4-IPO.

**Experimental Procedures**

**Materials.** IPN, 4-IPO, and DIOL were prepared as described previously (2). Trifluoroacetic acid, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP⁺, Gly, Cys, N-acetylcyesteine (AcCys), and GSH were obtained from Sigma-Aldrich Co. (St. Louis, MO). Male F344 rat liver microsomes (20 mg protein/mL) were purchased from In Vitro Technologies (Baltimore, MD).

**Instrumentation.** HPLC analyses were carried out on a Beckman System Gold module 126 solvent pump, a module 168 photodiode array detector. A gradient from 100% 0.1% trifluoroacetic acid in H₂O to 25% CH₂CN over 25 min, then held for 5 min at a flow rate of 1.5 mL/min on a Metachem (Torrance, CA) Inertsil C18 5 μm column (4.6 x 250 mm) was used for analysis and isolation of the products in the incubations.

Electrospray ionization mass spectra (ESI-MS) were obtained on a Thermo Finnigan LCQ DUO ion trap mass spectrometer (Riviera Beach, FL). Tandem mass spectra (ESI-MS/MS) were produced by collision-induced dissociation of the selected parent ions with the helium gas present in the mass analyzer. The heated capillary was maintained at 200 °C and the source voltage at 4.5 kV.

One-dimensional ¹H NMR spectra were acquired on Varian Gemini 300 MHz or INOVA 500 MHz NMR spectrometers (Palo Alto, CA). The chemical shifts are reported in ppm relative to solvents (D₂O at 4.80 ppm and acetone-d₆ at 2.05 ppm). Two-dimensional ¹H-¹H DQF COSY (17) and TROESY (18) experiments were acquired at 500 MHz. In these experiments, the ¹H sweep width was set at 12.8 ppm in each dimension, and 256 x 1024 complex points were acquired in the t₁ and t₂ dimensions, respectively. The TROESY experiment was obtained with a mixing time of 150 ms and a B1 field of 250 Hz, using 180°(x) 180°(x) pulses. A ¹H-¹³C HSQC (19, 20) experiment was obtained at 500 MHz. In this experiment, the ¹H sweep width was 12.8 ppm, and the ¹³C sweep width was 140 ppm, and 128 x 1024 complex points were acquired in the t₁ and t₂ dimensions, respectively.

**Oxidation of IPN by Dimethylidioxyanate-d₃.** Dimethylidioxyanate-d₃ was prepared as described (21). The reaction of IPN (7.1 mg, 0.043 mmol) with dimethylidioxyanate-d₃ in acetone-d₆ (0.05 M, 0.85 mM, 0.043 mmol) in a NMR tube was monitored by ¹H NMR spectroscopy (300 MHz) at 25 °C. The following signals, consistent with enedial 1, were observed in the spectrum: δ 10.25 (d, J = 5.8 Hz, 1H, 5'-H), 10.21 (d, J = 1.7 Hz, 1H, 2'-H), and 6.95 (dd, J = 6.1, 1.7 Hz, 1H, 4'-H); the other signals overlapped with the signals of IPN. A hydrate of 1 was also detected in the ¹H NMR spectrum. The hydrate existed as two isomers in a 1:7:1 ratio: the major isomer, δ 5.56 (s, 1H, 2'-H), 5.48 (d, J = 7.5 Hz, 1H, 5'-H), 5.00 (d, J = 7.7 Hz, 1H, 4'-H) and the minor isomer, δ 5.60 (s, 1H, 2'-H), 5.41 (d, J = 8.1 Hz, 1H, 5'-H), 5.02 (d, J = 7.4 Hz, 1H, 4'-H); the other signals overlapped with the signals of IPN.

**General Procedure for the Incubations of IPN with Rat Liver Microsomes and NADPH in the Presence of Gly, AcCys, Cys, or GSH.** Incubations of IPN (1 mM) with male F344 rat liver microsomes (2 mg protein/mL) were conducted in a 0.1 M potassium phosphate buffer (pH 7.4) in the presence of 3 mM MgCl₂, 25 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (2 units/mL), 4 mM NADP⁺, and 5 mM Gly (or AcCys, Cys or GSH). IPN was added as a CH₃CN solution (100 mM, 10 μL). The final volume was 1 mL, and the incubation took place at 37 °C in capped vials for 3 h (1 h for the AcCys experiment). The reactions were terminated by the addition of the 0.3 N Ba(OH)₂ (0.1 mL) and 0.3 N ZnSO₄ (0.1 mL). Control experiments omitting either NADP⁺ or Gly (or other trapping chemicals) were included so as to recognize the products. Following centrifugation, the supernatant was filtered through a Millex-HV syringe driven filter (Millipore Corporation, Bedford, MA: 0.45 μm, 13 mm), and the filtrate was analyzed by HPLC.

The incubations were scaled up to 10 mL for the isolation of the products using HPLC and the solvents were evaporated by a Speed-Vac (ThermoSavant, Holbrook, NY) before analysis by MS and/or ¹H NMR spectroscopy. Samples were dissolved in CH₃OH-CH₂OH (1:1) and introduced to the mass spectrometer through direct infusion (2.5–25 μL/min) for negative ionization analysis (ESI(--)-MS or ESI(--)-MS/MS) or positive ionization analysis (ESI(+)--MS or ESI(+)--MS/MS). Acetic acid, equivalent to 2% by volume, was added to some of the samples in CH₃OH-CH₂OH (1:1) for positive ionization analysis. ¹H NMR analysis was performed on samples with sufficient abundance.

1. Gly. One major product (2) eluting at 14.5 min was observed by HPLC analysis. 2 had the following spectral properties: UV: λmax 223 and 295 (major) nm; ESI(--)-MS/MS: m/z 238 (M -- H⁺), 220 (M -- H⁺ -- H₂O), 194 (M -- H⁺ -- CO₂), ESI(+)--MS/MS: m/z 240 (M + H⁺), 222 (M + H⁺ -- H₂O), 142 (M + 2 H⁺ -- COCH₂CH₂COCH₃), 99 (COCH₂CH₂COCH₃)⁻; ¹H NMR (D₂O, 100 MHz): δ 7.93 (s, 1H, 2'-H), 4.18 (s, 2H, Gly α-CH₂), 3.73 (s, 2H, 4'-CH₂), 3.01 (t, J = 7.0 Hz, 2H, 2'-CH₂), 2.89 (t, J = 6.6 Hz, 2H, 3'-CH₂), 2.25 (s, 3H, 5'-CH₃).

2. AcCys. Two products (3a and 3b) eluting at 16.3 and 16.4 min were observed by HPLC. 3a and 3b had an identical UV absorption maximum (λmax 263 nm) and were collected from HPLC in one fraction for MS analysis: ESI(--)-MS/MS: m/z 346 (M -- H₂O), 232 (M -- H⁺), 155 (M -- H⁺ -- CO₂), 140 (M -- COCH₂CH₂), 133 (M -- H₂O, COCH₂CH₂), 119 (M -- 2H₂O, COCH₂CH₂), 99 (COCH₂CH₂COCH₃)⁻; ¹H NMR (D₂O, 100 MHz): δ 7.61 (s, 1H, 2'-H), 5.67 (d, J = 1.7 Hz, 1H, Cys α-CH), 4.02 (t, J = 5.5 Hz, 1H, Cys α-CH), 3.34 (dd, J = 11.3, 1.5 Hz, 1H, Cys β-CH), 3.24 (d, J = 5.8 Hz, 2H, Cys β-CH), 3.17 (t, J = 7.4 Hz, 2H, Cys β-CH), 2.94 (t, J = 6.3 Hz, 2H, 3'-CH₂), 2.93 (dd, J = 11.7, 7.4 Hz, 1H, Cys β-CH), 2.25 (s, 3H, 5'-CH₃). There was a trace amount of a rearranged product (6) present in the ¹H NMR spectrum of 4c: δ 7.92 (d, J = 2.1 Hz, 1H, 2'-H), 7.14 (d, J = 2.2 Hz, 1H, 3'-H).
Hz, 1H, 5’-H), 2.29 (s, 3H, 5-CH₃); the other signals overlapped with the signals of 4c.

The spectral properties of 5 were as follows: UV: λ max 231 (major) and 308 nm. ESI(−)-MS/MS: m/z 266 (M−H−), 222 (M−H−−CO₂), ESI(+)−MS/MS: m/z 268 (M−H+), 250 (M+H−H₂O), 170 (M+2H−CO₂CH₂COCH₃), 99 (COCH₂CH₂COCH₃). ¹H NMR (D₂O, 300 MHz): δ 7.77, 7.77 (s, 1H, 2’-H), 6.31 (s, 1H, 4’-H), 5.07 (dd, J = 7.7, 3.9 Hz, 1H, Cys α-CH), 4.12 (dd, J = 11.5, 7.7 Hz, 1H, Cys β-CH₂), 3.86 (dd, J = 11.5, 3.9 Hz, 1H, Cys β-CH₃), 3.13 (t, J = 6.3 Hz, 2H, 2-CH₂), 2.93 (t, J = 6.3 Hz, 2H, 3-CH₂), 2.27 (s, 3H, 5-CH₃).

4. GSH. Several peaks with a distinctive UV absorption at 260–320 nm (7a–b, 8a–b, 9, and 10a–b) were observed by HPLC reanalysis that most of 10a had rearranged to 10b after isolation.

10b. HPLC retention time 19.3 min; UV: λ max 245 and 277 nm. HPLC reanalysis showed that most of 10a had rearranged to 10b after isolation.
Metabolism of Ipomeanine and 4-Ipomeanol


Incubation of 4-IPO with Rat Liver Microsomes and NADPH in the Presence of GSH. Incubations of 4-IPO (1 mM) and GSH (5 mM) with male F344 rat liver microsomes (2 mg protein/mL) were conducted under the same conditions as IPN. The incubation took place at 37 °C in capped vials for 2 h. Control experiments omitting either NADP⁺ or GSH were included. HPLC-UV analysis of the incubation mixture revealed the formation of IPN, 4-IPO, and only GSH, the NADPH regenerating system, and the buffer and, therefore, is not a metabolite of IPN or 4-IPO.

14. HPLC retention time 15.1 min; UV: λmax 312 nm; ESI(−)-MS/MS: m/z 454 (M−H⁺), 436 (M−H⁺−H₂O), 420 (M−H⁺−H₂S), 410 (M−H⁺−CO₂), 392 (M−H⁺−H₂O−CO₂), 376 (M−H⁺−H₂S−CO₂), 358 (M−H⁺−H₂O−H₂S−CO₂); ESI(+)-MS/MS: m/z 456 (M+H⁺), 438 (M+H⁺−H₂O), 420 (M+H⁺−H₂O−H₂S), 381 (M+H⁺−Gly), 363 (M+H⁺−Gly−H₂O), 278 (M+H⁺−Gly−Cys−Gly).

The formation of 15 required 4-IPO, rat liver microsomes, and the NADPH regenerating system but not GSH. Its properties were as follows: HPLC retention time 15.4 min; UV: λmax 214 and 257 nm; ESI(−)-MS/MS: m/z 788 (M−H⁺∗), 770 (M−H⁺−H₂O), 690 (M−H⁺−H₂O−PO₄), 653 (M−H⁺−adenine), 620 (M−H⁺−4-IPomeanol), 441 (M−H⁺−adenosine 2′-monophosphate), 426 (adenosine 2′, 5′-diphosphate), 408 (adenosine 2′, 5′-diphosphate−H₂O).

16. HPLC retention time was 17.5 min; UV: λmax 250 and 262 nm; ESI(−)-MS/MS: m/z 454 (M−H⁺), 436 (M−H⁺−H₂O), 410 (M−H⁺−CO₂), 392 (M−H⁺−H₂O−CO₂), 366 (M−H⁺−2CO₂), 335 (M−H⁺−CO₂−Gly), 185 (γ-Glu-Cys−CH₂NO₂).

17. HPLC retention time was 18.7 min; UV: λmax 242 and 277 nm. This product was isolated from HPLC along with DIOL (retention time at 19.0 min, UV: λmax 212 nm). The other spectral properties of 17 were as follows: ESI(−)-MS/MS: m/z 454 (M−H⁺), 436 (M−H⁺−H₂O), 410 (M−H⁺−CO₂), 310 (M−CO₂−COCH₂CH₂CH(OH)CH₂CH₂CH₂CH₂−), ESI(+)-MS/MS: m/z 438 (M+H⁺−H₂O), 420 (M+H⁺−2H₂O), 363 (M+H⁺−H₂O−Gly), 356 (M+H⁺−COCH₂CH₂CH(OH)CH₂CH₂CH₂−), 335 (M+H⁺−H₂O−HCONHCH₂COOH), 281 (M+H⁺−Gly−COCH₂−CH₂CHOHCH₂−); 'H NMR (D₂O, 300 MHz): δ 7.85 (s, 1H, 2′-H), 7.00 (s, 1H, 4′'-H), 5.19 (dd, J = 11.7, 4.2 Hz, 1H, Glu−CH), 1.19 (d, J = 6.3 Hz, 3H, 5-CH₃); the other signals were not resolved or overlapped with the signals of DIOL.

18. Approximate HPLC retention time was 17.3 min; ESI(−)-MS/MS: m/z 761 (M−H⁺), 743 (M−H⁺−H₂O), 709 (M−H⁺−H₂O−H₂S), 632 (M−H⁺−129 (Glu)), 488 (M−H⁺−273 (GSH−H₂S)).

19. Approximate HPLC retention time was 17.9 min; ESI(−)-MS/MS: m/z 761 (M−H⁺), 743 (M−H⁺−H₂O), 709 (M−H⁺−H₂O−H₂S), 632 (M−H⁺−129 (Glu)), 488 (M−H⁺−273 (GSH−H₂S)), 470 (M−H⁺−273 (GSH−H₂S−H₂O), Compd 20 was isolated along with 17. ESI(−)-MS/MS of 20: m/z 733 (M−H⁺), 715 (M−H⁺−H₂O), 681 (M−H⁺−H₂O−H₂S), 604 (M−H⁺−129 (Glu)), 460 (M−H⁺−273 (GSH−H₂S)).

Incubation of DIOL with Rat Liver Microsomes and NADPH in the Presence of Gly, AcCys, Cys, or GSH. All incubation mixtures were analyzed by HPLC with UV detection from 190 to 350 nm. IPN eluted at 23.7 min. 4-IPO (retention...
time at 22.6 min), generated from the reduction of IPN, was observed in all incubations. The other products were isolated by HPLC and characterized by MS and/or $^1$H NMR spectroscopy. The spectral data of these products are listed in Experimental Procedures, and the structural assignments are described below.

1. Gly. One product eluting at 14.5 min (2) had a UV maximum at 295 nm and a molecular weight (MW) of 239. The MS is equivalent to IPN + [O] + Gly minus one H$_2$O. The MS and $^1$H NMR spectrum indicate that the side chain of IPN remains intact in the product; therefore, the microsomal oxidation occurred in the furan moiety of IPN. The intense UV absorption maximum at 295 nm in the metabolite can only be due to a $\beta$-aminoenone (COC=CHNR$_2$) from the reaction of the enedial with Gly. Using Woodward’s Rules to estimate the UV absorption maxima of enones, only $\beta$-amino and $\beta$-thioenones are predicted to have maxima in the 290+ nm range. NADPH, for example, contains a similar $\beta$-amino-$\alpha$, $\beta$-unsaturated amide that accounts for the observed UV maximum at 340 nm. One $^1$H NMR signal at 7.93 ppm as a singlet confirms the presence of the olefin proton 2'-H in the product. The chemical shift of 2'-H in 2 is upfield compared with that of 2'-H in IPN (8.38 ppm in D$_2$O), consistent with the replacement of the furyl O with an N. Product 2 is assigned as a 2'-pyrrolin-5'-one (Scheme 1). The 4'-CH$_2$ resonance at 3.37 ppm as a singlet is consistent with the assigned structure.

2. AcCys. Two products (3a and 3b) eluting at 16.3 and 16.4 min were observed. They had a similar UV maximum at 263 nm. An analysis of the MS of 3a and 3b indicated that they had a MW of 345, corresponding to a 1:1 adduct of enedial 1 and AcCys. Characterization of the adducts from the conjugation of 1 with Cys or GSH indicated that the addition of thiols to 1 occurred at the 4'- or 5'- but not the 2'-positions (vide infra). AcCys is expected to add to 1 at either the 4'- or the 5'-position, but only the addition at the 5'-aldehyde would give an enone product with a UV maximum at 263 nm. The adducts 3a--b are tentatively assigned as two diastereomeric 2',5'-dihydro-2'-hydroxyfurans (Scheme 1).

3. Cys. Two minor products eluting at 15.7 min (4a) and 16.3 min (4b) and two major products eluting at 16.7 min (4c) and 27.6 min (5) were observed. The UV spectra of 4a--c were similar, with a single maximum around 300 nm. An analysis of the MS indicated that 4a--c all had a MW of 388 with similar MS/MS fragmentation patterns corresponding to an adduct between enedial 1 and two Cys molecules minus two H$_2$O molecules. Only 4c was abundant enough for a $^1$H NMR spectrum. The NMR spectrum contained two one-proton singlets at 7.61 ppm (2'-H) and 5.35 ppm (5'-H). Previous studies with furan and 4-IPO have shown that the enedial metabolites form pyrrole adducts with reactants containing a primary amine and a thiol such as GSH (9, 13, 14). Compd 1 incorporated two Cys molecules to give 4a--c, but the UV absorption and the NMR spectrum of 4c suggest that the structures of 4a--c are similar to 2 rather than those of the expected pyrylones. The single UV maximum of 4c at 304 nm and the olefin proton at 7.61 ppm are in agreement with an enone substituted with a $\beta$-amino group (COC=CHNR$_2$), but the 5.35 ppm singlet is not what would be expected for a pyrrole proton. The chemical shift is consistent with a proton on a carbon substituted with one N and one S, as the proposed structure for 4c shows in Scheme 1. The signal corresponding to 4'-H is likely obscured by the D$_2$O peak. The structure consists of a bicyclic 2-pyrrole with the thiols of two Cys molecules substituted at the 4'- and 5'-positions to form two chiral centers. The 5.35 ppm singlet assigned to the 5'-H of 4c indicates that coupling between 4'-H and 5'-H is near zero. GMMX-calculated coupling constants of vicinal protons in a saturated cyclopentane ring have been published: J cis is up to 11.5 Hz and never below 5 Hz, whereas J trans varies from 0.3 to 13 Hz (22). The experimental data from more than 30 saturated cyclopentanes generally follow this trend (22). The 4'-H and 5'-H in 4c are assigned as trans to each other on the basis of this observation. 4a and 4b were not abundant enough to obtain $^1$H NMR spectra, and they are tentatively assigned as diastereomers of 4c (Scheme 1).

An analysis of the MS of 5 indicated a MW of 267, corresponding to a 1:1 adduct between enedial 1 and Cys followed by loss of two H$_2$O molecules. Compd 5 had UV maxima at 231 (major) and 308 nm. The two maxima are consistent with the UV absorption of a pyrrole molecule in contrast to the single maximum near 300 nm for the $\beta$-aminoenones. The $^1$H NMR spectrum of 5 contained two one-proton singlets at 7.77 ppm and at 6.31 ppm. The singlet at 7.77 ppm is slightly more downfield than that of the olefin proton (2'-H) in 4c (7.61 ppm), as expected for a pyrrole 2'-CH. The singlet at 6.31 ppm is upfield compared to that of the furyl proton (4'-H) in IPN (6.80 ppm). Compd 5 is assigned as a mono-Cys 5'-S-pyrrole with the singlets at 6.31 and 7.77 ppm assigned to 4'-CH and 2'-CH, respectively, as shown in Scheme 1.

4. GSH. Numerous products with distinctive UV absorption maxima were observed by HPLC (Figure 2A and B). The products can be divided into two groups: one with UV maxima around 250 nm, which includes 7a--b, 9, 10a--b (Figure 2A), and the other with UV maxima around 300 nm, which includes 8a--b (Figure 2B). The two most polar products (7a and 7b), eluting at 13.6 and 13.7 min, had a similar UV maximum at 262 nm. An analysis of the MS of the mixture of 7a and 7b indicates that they had a MW of 489, corresponding to a 1:1 adduct between enedial 1 and GSH. The UV absorption and MS of 7a and 7b are similar to those of 3a and 3b, indicating adducts of similar structure. Compds 7a and 7b are assigned as diastereomeric 2',5'-dihydro-2'-hydroxyfurans (Scheme 2). After 7a and 7b were stored at −20 °C for some time, HPLC reanalysis showed that they had converted to 10a and 10b in a 1:1 ratio.

Two major products (8a and 8b), eluting at 16.6 and 17.2 min, had distinctive single UV maxima at 313 and 303 nm, respectively. An analysis of the MS indicated both 8a and 8b had MWs of 453 with a similar MS/MS fragmentation, corresponding to a 1:1 adduct between enedial 1 and GSH minus two H$_2$O molecules. The $^1$H NMR spectrum of 8a contained a one-proton singlet at 7.77 ppm and a one-proton doublet at 6.26 ppm (J = 10.9 Hz). A 2D $^1$H--$^1$H DQF COSY NMR experiment indicated that the 6.26 ppm proton coupled with a peak obscured by the D$_2$O peak. The $^1$H NMR spectrum of 8b contained a one-proton singlet (2'-H) at 7.61 ppm and a one-proton doublet (J = 9.3 Hz, 5'-H) at 5.97 ppm. The single UV maximum at 313 or 303 nm and the olefin proton singlet (2'-H) observed at 7.77 or 7.61 ppm are in agreement with an enone substituted with a $\beta$-amino group (COC=CHNR$_2$). The chemical shifts of 5'-H in 8a and 8b are consistent with the protons on sp$^3$ carbons substituted with one N and one O, and 4'-H and 5'-H are coupled to each other with the coupling constant around 10 Hz. The UV and NMR spectra demonstrate that 8a and 8b are structurally similar to the bicyclic 2'-pyrrole adducts 4a--c except that the Cys of GSH is substituted at the 4'-position, and the Glu $\alpha$-carboxylic acid of GSH is substituted at the 5'-position to form tricyclic 2'-pyrrolines, as shown Scheme 2. There are two chiral centers at 4'-C and 5'-C, and in theory, there should
be four diastereomers. Two isomers, 8a and 8b, were observed, but the cis-trans relationship of 4'-H and 5'-H cannot be decided on the basis of the coupling constants of 5'-H. As stated earlier, both vicinal cis and trans protons in a saturated cyclopentane ring can have coupling constants up to 11.5–13 Hz (22).

A portion of 8a and 8b rearranged to 9 eluting at 18.7 min upon storage. Compd 9 had two UV maxima at 248 and 263 nm and a MW of 453, the same MW as that of 8a. After collection from HPLC, the UV absorption of 9 was not successful because it partially degraded during HPLC isolation. Compd 9 is tentatively assigned as a 4'-S-pyrrole adduct shown in Scheme 2.

The UV absorption of one minor product (10a, UV: \( \lambda_{max} \) 245 and 277 nm) eluting at 19.3 min and one major product (10b, UV: \( \lambda_{max} \) 240 and 277 nm) eluting at 19.9 min indicates that they are likely to be pyrroles. Most of 10a rearranged to 10b after collection from HPLC. The MS showed that 10b had a MW of 453, the same MW as that of 8a and 8b. The UV absorption of 9 indicates that it is likely a pyrrole. An attempt to obtain a 1H NMR spectrum of 9 was not successful because it partially degraded during HPLC isolation. Compd 9 is tentatively assigned as a 4'-S-pyrrole adduct shown in Scheme 2.

Incubation of 4-IPO with Rat Liver Microsomes and NADPH in the Presence of GSH. HPLC-UV analysis of the incubation mixture revealed the formation of IPN, 8a–b, and 10b along with new products (14–17) (Figure 2C and D). These products can be divided into two groups: one with a UV maximum around 250 nm, which includes 15–17 and 10b (Figure 2C), and the other with a UV maximum around 300 nm, which includes 14 and 8a–b (Figure 2D). There was a peak eluting at 19.0 min with a UV maximum at 212 nm. The product was characterized as a DIOL on the basis of a comparison of its HPLC retention time, UV maximum, and 1H NMR data with those of synthetic DIOL diastereomers (HPLC). One minor product (11) and a MW of 732, 28 Da, a C5 molecule. The UV maxima of 15 at 214 and 257 nm are similar to those of 4-IPO and adenine. The formation of 15 required 4-IPO, rat liver microsomes, and the NADPH regenerating system.
system but not GSH. The displacement of the nicotinamide (121) of NADP$^+$ (743) by 4-IPO (167) would give 15 (Scheme 3). Both the MS/MS fragmentation and the UV absorption support the assigned structure.

Both 16 and 17 have a MW of 455, corresponding to a 1:1 adduct between the enedial metabolite of 4-IPO and GSH minus two H$_2$O molecules. UV absorption of 16 (UV: $\lambda_{\text{max}}$ 250 and 262 nm) and 17 (UV: $\lambda_{\text{max}}$ 242 and 277 nm) indicates that they are likely pyrroles. The $^1$H NMR spectrum of 17 clearly showed two pyrrole protons at 7.85 ppm ($2^{\prime}$-CH) as a singlet and at 7.00 ppm ($4^{\prime}$-CH) as a singlet. Compds 16 and 17 had UV absorptions similar to those of 9 and 10b, respectively, and MWs 2 Da higher than those of 9 and 10b. The chemical shifts of $2^{\prime}$-H and $4^{\prime}$-H in 17 are 0.06 and 0.04 ppm, respectively, upfield than those in 10b. Compds 16 and 17 were assigned as mono-GSH 4$^{\prime}$-S- and 5$^{\prime}$-S-pyrroles (Scheme 3). Both 18 and 19 had a MW of 762, consistent with the conjugation of the enedial metabolite of 4-IPO with two GSH molecules minus 2 H$_2$O molecules. The MWs are 2 Da higher than those of 11 and 12. The UV absorption of 18 and 19 were not distinctive. Compds 18 and 19 are tentatively assigned as bis-GSH 4$^{\prime}$-S- and 5$^{\prime}$-S-pyrroles (Scheme 3).

Compd 14 had a UV maximum at 312 nm and a MW of 455, the same as that of 16 and 17. The structure of 14 is likely similar to those of 8a$-$b, except for the side chain having a 4-OH instead of a 4-C$\equiv$O (Scheme 3). Compd 20 was isolated along with 17 and, subsequently, detected by mass spectrometry. Compd 20 had a MW of 734, 28 Da, a C$\equiv$O group, less than those of the bis-GSH pyrrole adducts 18 and 19 and 2 Da higher than that of 13. Compd 20 is tentatively assigned as the bis-GSH pyrrole adduct (Scheme 3).

Discussion

While the metabolic activation of 4-IPO by cytochromes P450 seems well established, only recently have metabolites derived from the reactive intermediate been characterized. However, the multicenter reactivity of the enedial intermediate has not been evident in metabolites identified in previous studies (8, 9). These recent studies focused on 4-IPO metabolism as did our initial studies. The yield of products from microsomal incubations of 4-IPO was limited, apparently because oxidizing equivalents were being consumed by oxidation of the alcohol moiety in 4-IPO to a ketone. By starting with IPN, this pathway was not available, and higher yields of metabolites were produced. The chemical oxidation of 4-IPO by dimethyldioxirane also led to oxidation of the alcohol and reduced the yield of oxidized furan products (data not shown).

The chemical oxidation of IPN by dimethyldioxirane to enedial 1 and subsequent reaction with H$_2$O to form a hydrate, was observed using NMR spectroscopy, cis-2-Butene-1,4-dial from the oxidation of furan forms a stable hydrate (12), and likewise, the hydrate of enedial 1 is more stable than 1. Although metabolic schemes for furans often include epoxides, furan epoxides have been detected only at 0 °C or lower temperatures (23–25). In several studies, only endiels were observed at room
temperature from the chemical oxidation of furans (12, 24, 25), and the present study confirms these results. Furans substituted with an electron-withdrawing group in the 3-position have generally been resistant to oxidation by reagents that are electrophilic in nature, for example, peracids (26). Dimethyl-dioxirane is considered to be nucleophilic (21). If that is true, then the keto functionality at the 3′-position reduces electron density at 2′-C and may actually facilitate oxidation by this reagent. However, compared to the complete oxidation of furan by dimethyldioxirane to cis-2-butene-1,4-dial within 30 min (12), the oxidation of IPN by dimethyldioxirane to enedial 1 was incomplete, suggesting that the electron-withdrawing carbonyl group may still slow the oxidation even though the oxidant has some nucleophilic properties. The nucleophilic attack at 2′-C may be slowed by steric interference, the reaction may be inherently slower, or the double bonds in furan are identical, and therefore, a statistical factor may also be involved there. Demonstration of adduct formation was not possible in the chemical oxidation studies because the usual nucleophiles were destroyed by the excess oxidizing equivalents.

Investigation of microsomal oxidation of IPN began with simple nucleophiles and progressed to more complex ones. The assignment of structure at each level of complexity was aided by the information provided by earlier, simpler products. 2′-Pyrrolin-5′-one adduct 2 was identified in the rat liver microsomal incubation of IPN containing NADPH and Gly. The characterization of this 2′-pyrrolin-5′-one adduct establishes that IPN is metabolized by rat liver microsomes to enedial 1. Primary amines are known to conjugate with cis-2-butene-1,4-dial to give a mixture of 3-pyrrolin-2-one and 4-pyrrolin-2-one or with the ketoenal metabolite of menthofuran to give a 3-pyrrolin-2-one (13, 16).

The 1,2-addition of AcCys or GSH to the 5′-aldehyde of the enedial 1 gave isolable dihydrohydroxyfuran adducts 3a–b and 7a–b. The intramolecular H bonding between the 2′-OH group and 3′-keto group probably stabilizes these adducts. Four biliary metabolites of 4-IPO with MWs 2 Da higher than those of 7a–b were detected in rats (8). Several aldehyde-containing structures were proposed in ref 8, but we suspect that two metabolites are likely analogues to 7a–b as the structures show in Scheme 3.

Alvarez-Diez and Zheng (8) and the present work demonstrate that both enedial metabolites of IPN and 4-IPO conjugate with thiols to give persistent dihydrohydroxyfuran adducts. These dihydrohydroxyfuran adducts are capable of reacting with an amine to form cross-linking pyrrole adducts and, therefore, might play roles related to the toxicity of IPN and 4-IPO. Metabolism of menthofuran in rats gave a biliary metabolite, 2-(glutathion-S-yl)menthofuran, from the reaction of the ketoenal metabolite with GSH followed by dehydration of the hemiketal intermediate (15). The hemiacetal groups in 3a–b and 7a–b do not dehydrate to form furans. Instead, if the trapping agents also contained amino groups, then the dihydrohydroxyfuran metabolites would be subject to the addition of the amine followed by dehydration to give 5′-S-pyrrole adducts. That is, cyclization of 7a–b gave 10a and 10b, and the addition of a second GSH to 7a–b formed 12. The 5′-S-pyrrole adduct 5 was a major product in the rat liver microsomal incubation of IPN, NADPH, and Cys. The dihydrohydroxyfuran intermediates from the conjugation of the Cys thiol with the 5′-aldehyde of 1 were too short-lived for observation.

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The 1,4-addition (Michael addition) of Cys or GSH to 1 at the 4-position gave 2-pyrorlines, 4a–c and 8a–b, instead of the expected 4′-S-pyroroles. The proposed mechanisms for their formation are shown in Scheme 4. The GSH Michael adduct of 1 yields an imine upon cyclization, which could tautomerize to the corresponding enamine. The enamine aldehyde would be in equilibrium with the corresponding iminium ion, as shown by Castagnoli et al. (27) in their study of cyclic tertiary amine oxidation. The addition of Glu α-carboxylic acid to the iminium ion gives 8a–b. The mechanism for the formation of 4a–c is similar. Starting from the Cys Michael adduct of 1, the intermediate reacts with the amino group in a second Cys molecule to form an imine. Tautomerization of the imine followed by dehydration gives an iminium ion. Addition of the second Cys thiol to the iminium ion gives the products (Scheme 4). The GSH or Cys Michael adducts of 1 shown in Scheme 4 might exist in the incubations, but unlike 3a–b and 7a–b, these Michael adducts could not be observed because of a lack of UV absorption. Similarly, the AcCys Michael adduct of 1, if it exists, could not be observed because of a lack of UV absorption and a lack of further reactions to give products with UV chromophores. A portion of 8a–b rearranged to the 4′-S-pyrorole 9 during storage. The addition of a second GSH to the GSH Michael adduct of 1 and subsequent loss of two H2O molecules would give 11. Loss of C=O, as formate, via a retro-aldo condensation followed by the addition of a second GSH and dehydration would give the pyrrole adduct 13 (Scheme 2). No adducts from the conjugation of thiols with the 2′-aldehyde in 1 were observed. Baer et al. (9) and this study demonstrate that addition of thiols to the enedial metabolites of 4-IPO and IPN occurred only at the 4′- or 5′- but not 2′-positions, probably due to the fact that 4′-C is β to both a ketone and an aldehyde, which facilitates Michael addition, and 5′-C may be more accessible than 2′-C. The mono-GSH adducts 7a–b, 8a–b, 9 and 10b were also observed in the rat lung microsomal incubations of IPN (data not shown). The major difference between rat liver and lung microsomal incubations of IPN is that a smaller amount of IPN was reduced to 4-IPO in the lung preparation. The metabolites with the distinct UV maximum around 300 nm such as 2, 4a–c and 8a–b or their analogues would be good biomarkers for studying the in vivo metabolism of IPN.

GSH conjugation with the enedial metabolite of 4-IPO gave adducts with structures similar to those from the GSH conjugation with 1, except that the microsomal metabolism of 4-IPO to the enedial was to a lesser extent than the metabolism of IPN to 1. The adducts characterized include the tricyclic 2′-pyrorline adduct 14, two mono-GSH pyrrole adducts 16 and 17, and two bis-GSH pyrrole adducts 18 and 19 (Scheme 3). The bis-GSH pyrrole adducts have been identified by Baer et al. (9). Another bis-GSH pyrrole adduct 20 was also detected. In addition to the enedial adducts, an additional metabolite 15 was present in all incubations that contained 4-IPO and NADPH, with or without added nucleophiles. The MS of this product was consistent with an adduct of 4-IPO with NADP. This product is undoubtedly the unknown, non-GSH conjugated, metabolite observed by Buckpitt and Boyd (6) in their study of 4-IPO metabolism.

Other products present in the incubation mixture of 4-IPO included 8a–b, 9, 10b, 11, and 12, derived from sequential oxidation of 4-IPO to IPN then to 1 followed by GSH conjugation. Conversely, GSH adducts derived from the enedial metabolite of 4-IPO were not present in the incubations of IPN. Sequential oxidation of 4-IPO to IPN then to the enedial 1 is likely responsible for some of the toxicity from 4-IPO. The intraperitoneal LD50 of 4-IPO in rats increased from 24 mg/kg to 120 mg/kg when the rats were pretreated with pyrazole, an inhibitor of alcohol dehydrogenase, and might be due to the inhibition of the conversion of 4-IPO to IPN (7). Interconversion of the four 3-substituted furans (Figure 1) found in mold-damaged sweet potatoes can be achieved chemically (2). Similar interconversions of 4-IPO, IPN, and DIOL were observed in the microsomal incubations. Interconversion is likely to occur in vivo where additional enzymes, e.g., alcohol dehydrogenases, are available. However, the three 3-substituted furans studied may not be equally oxidized to enedial metabolites in the microsomal incubations. GSH trapping of the reactive metabolites generated in the incubations indicated that IPN was metabolized to the enedial to the greatest extent, DIOL the least, and 4-IPO fell in between (Figure 2). Only a trace amount of one product was observed in the rat liver microsomal incubation of DIOL, NADPH, and GSH (data not shown).

In conclusion, it is clear that the reactivity of the enedial metabolites of IPN and 4-IPO with thiols and/or amines is responsible for their toxicity. The in vivo metabolism of IPN and 4-IPO is worthy of reinvestigation so as to determine the occurrence of the IPN and 4-IPO interconversion and the predominant scavengers for these reactive enedials in vivo.

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References