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Reaction of Dehydropyrrolizidine Alkaloids with Valine and Hemoglobin

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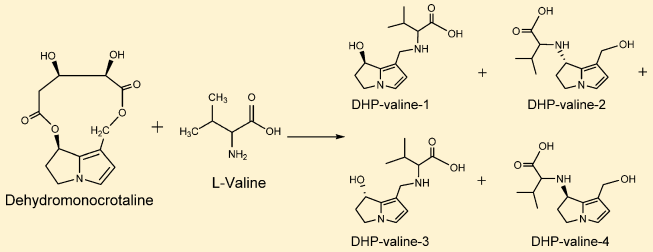
Supporting Information

ABSTRACT: Pyrrolizidine alkaloid-containing plants are probably the most common poisonous plants affecting livestock, wildlife, and humans. Pyrrolizidine alkaloids exert toxicity through metabolism to dehydropyrrolizidine alkaloids that bind to cellular protein and DNA, leading to hepatotoxicity, genotoxicity, and tumorigenicity. To date, it is not clear how dehydropyrrolizidine alkaloids bind to cellular constituents, including amino acids and proteins, resulting in toxicity. Metabolism of carcinogenic monocrotaline, riddelliine, and heliotrine produces dehydromonocrotaline, dehyroriddelliine, and dehydroheliotrine, respectively, as primary reactive metabolites. In this study, we report that reaction of dehydromonocrotaline with valine generated four highly unstable 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP)-derived valine (DHP−valine) adducts. For structural elucidation, DHP−valine adducts were derivatized with phenyl isothiocyanate (PITC) to DHP−valine−PITC products. After HPLC separation, their structures were characterized by mass spectrometry, UV−visible spectrophotometry, ^{1}H NMR, and ^{1}H−^{1}H COSY NMR spectral analysis. Two DHP−valine−PITC adducts, designated as DHP−valine−PITC-1 and DHP−valine−PITC-3, had the amino group of valine linked to the C7 position of the necine base, and the other two DHP−valine−PITC products, DHP−valine−PITC-2 and DHP−valine−PITC-4, linked to the C9 position of the necine base. DHP−valine−PITC-1 was interconvertible with DHP−valine−PITC-3, and DHP−valine−PITC-2 was interconvertible with DHP−valine−PITC-4. Reaction of dehydroriddelliine and dehydroheliotrine with valine provided similar results. However, reaction of valine and dehydroretronecine (DHR) under similar experimental conditions did not produce DHP−valine adducts. Reaction of dehydromonocrotaline with rat hemoglobin followed by derivatization with PITC also generated the same four DHP−valine−PITC adducts. This represents the first full structural elucidation of protein conjugated pyrrolic adducts formed from reaction of dehydropyrrolizidine alkaloids with an amino acid (valine). In addition, it was found that DHP−valine-2 and DHP−valine-4, with the valine amino group linked at the C7 position of the necine base, can lose the valine moiety to form DHP.



INTRODUCTION

Pyrrolizidine alkaloids and their N-oxide derivatives are common phytochemical constituents of hundreds of plant species of different botanical families widely distributed in many geographical regions of the world.[1−11] To date, at least 660 pyrrolizidine alkaloids and pyrrolizidine alkaloid N-oxides have been identified in over 6000 plants, and about half of them exhibit hepatotoxic activity.[1,7−9] It has been determined that about 3% of the world's flowering plants contain toxic pyrrolizidine alkaloids.[11] Humans are exposed to toxic pyrrolizidine alkaloids through staple foods, food contaminants, herbal teas, herbal medicines, and dietary supplements.[4,7,12−16] As early as 1920, it was reported that people in South Africa who ingested the pyrrolizidine alkaloid-containing plant *Senecio ilicifolius* were poisoned.[7] In 1954, retrorsine, a pyrrolizidine alkaloid, was determined to induce liver tumors in rats.[17] To date, it is believed that pyrrolizidine alkaloid-containing plants are possibly the most common poisonous plants affecting livestock, wildlife, and humans.[4,7]

Metabolism of toxic pyrrolizidine alkaloids generates dehydropyrrolizidine alkaloids as the primary pyrrolic metabolites that react with cellular protein and DNA *in vivo* to exert toxicity, including hepatotoxicity, mutagenicity, and tumorigenicity (Figure 1).[4,6,7,18−22]

Dehydropyrrolizidine alkaloids are highly unstable, with half-lives about 0.3−5.1 s[23] in aqueous medium, facilely hydrolyzed to the necine base, (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP), and easily dimerized and polymerized.[7] They also react with cellular proteins, DNA, and glutathione (GSH) to form protein−DHP,[4,7,24] DNA−DHP adducts,[4,25−28] and GSH−DHP adducts.[4,7,29] Similar to the primary dehydropyrrolizidine alkaloid metabolites, all secondary pyrrolic metabolites (protein−DHP, DNA−DHP, GSH−DHP, and DHP) containing a necine (DHP) moiety are also very unstable in aqueous medium, particularly under acidic

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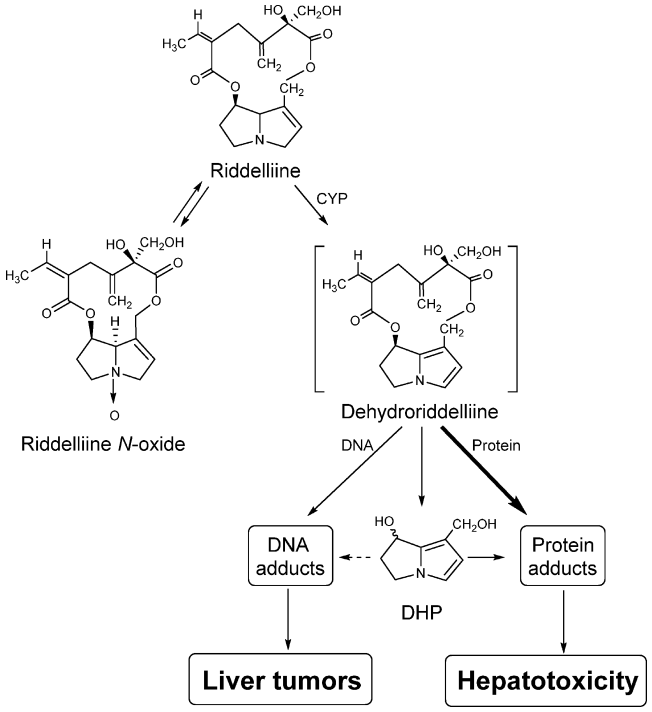

Figure 1. Metabolic activation of pyrrolizidine alkaloids leading to toxicity.

conditions. Consequently, mechanistic studies of pyrrolizidine alkaloid-induced hepatotoxicity and tumorigenicity are difficult to conduct at the molecular level. We recently determined that riddelliine, a tumorigenic pyrrolizidiene alkaloid, induces liver tumors through a DNA adduct-mediated genotoxic mechanism.[1,30−35] The DNA adducts are a pair of epimers of 7-hydroxy-9-(deoxyguanosin-N^2-yl) dehydrosupinidine (DHP−dG-3 and DHP−dG-4) and a pair of epimers of 7-hydroxy-9-(deoxyadenosin-N^6-yl)dehydrosupinidine (DHP−dA-3 and DHP−dA-4).[35] Further studies demonstrated that DHP−dG-3, DHP−dG-4, DHP−dA-3, and DHP−dA-4 were also formed in the liver of rats treated with seven hepatocarcinogenic pyrrolizidine alkaloids and riddelliine N-oxide.[36] These results indicate that this set of DNA adducts is a common biological biomarker of pyrrolizidine alkaloid-induced liver tumor formation.[36]

Although a role for DHP−protein adducts formed *in vivo* in the liver has been proposed for pyrrolizidine alkaloid-induced hepatotoxicity,[4,7,37,38] the structures of these protein-DHP adducts have not been characterized and it is not clear how these protein−DHP adducts can affect hepatotoxicity. Our success in determining the structures of DHP−DNA adducts *in vivo* suggested that the structures of protein−DHP adducts could also be characterized. Following our strategy on the structural determination of DHP−dG and DHP−dA adducts, we determined the products formed from the reaction of valine with dehydroriddelliine, dehydromonocrotaline, and dehydroheliotrine, which are metabolites of monocrotaline, riddelliine, and heliotrine, respectively (Figure 2). Both monocrotaline and riddelliine are retronecine-type pyrrolizidine alkaloids, heliotrine is a heliotridine-type tumorigenic pyrrolizidine alkaloid, and all are among the most studied pyrrolizidine alkaloids regarding cytotoxicity, genotoxicity, and tumorigenicity.[7] Valine, an aliphatic branched-chain amino acid involved in the promotion of cell normal growth, tissue repair, and providing the body with energy, is a neutral and extremely hydrophobic essential amino acid. In this study, we report the characterization of the main products derived from the *in vitro* reaction of valine with dehydroriddelliine, dehydromonocrotaline, and dehydroheliotrine and the formation of these adducts in the N-terminal valine of hemoglobin of rats treated with dehydromonocrotaline.

EXPERIMENTAL PROCEDURES

Caution: *These chemicals are dangerous. Monocrotaline, riddelliine, heliotrine, dehydrmonocrotaline, dehydroriddelliine, dehydroheliotrine, and DHR are carcinogenic in laboratory animals. They should be handled with extreme care, using proper personal protective equipment and a well-ventilated hood.*

Chemicals. Monocrotaline, *o*-chloranil, valine, rat hemoglobin, acetonitrile, potassium carbonate, chloroform, and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO). Dimethylformamide (DMF) and phenyl isothiocyanante (PITC) were obtained from Fisher Scientific (Pittsburgh, PA). AX 1-X8 anion exchange resin was purchased from Bio-Rad (Hercules, CA). Heliotrine was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY).

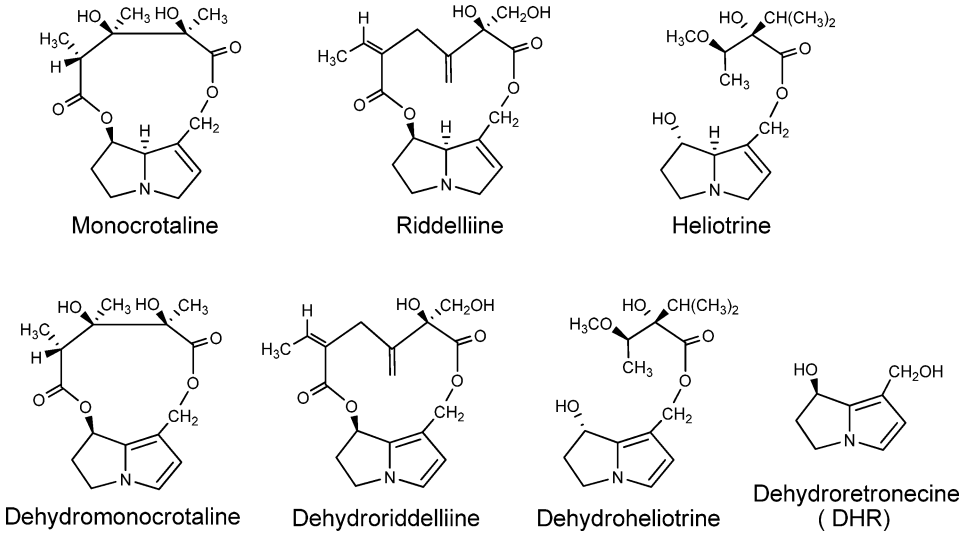

Figure 2. Names and structures of pyrrolizidine alkaloids.

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Riddelliine was obtained from Dr. Po-Chuen Chan, National Toxicology Program (NTP). Dehydromonocrotaline, dehydroriddelliine, dehydroheliotrine, and DHR were synthesized by dehydrogenation of monocrotaline, riddelliine, heliotrine, and retronecine, respectively, in chloroform with *o*-chloranil as previously described.[34,39,40] H_2O^{18} (isotopic purity 97%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Blood Collection from Rats Treated with Pyrrolizidine Alkaloids. Female F344 rats were treated by gavage with riddelliine or monocrotaline at daily doses of 24 μmol/kg body weight in 0.5 mL of 10% DMSO in water for 3 consecutive days and sacrificed by CO_2 inhalation 24 h after the last dose, as reported previously.[36] Blood samples from each animal were collected by cardiac puncture into heparinized tubes, and red blood cells were collected by centrifugation and stored at −80 °C before use.

Reaction of Valine with Dehydropyrrolizidine Alkaloids. A mixture of 2 mg of valine (2.8 equiv to dehydromonocrotaline), 2 mg of K_2CO_3, 80 μL of distilled water, and 160 μL of DMF was sonicated until the solution turned clear. The valine solution was added dropwise into a solution of 2 mg of dehydromonocrotaline in 240 μL of DMF. The reaction was monitored at different time intervals by HPLC monitored at 218 nm. After reaction for 48 h, the resulting reaction mixture was filtered through a 0.4 μm RC membrane, and the reaction products were separated by HPLC with a 250 × 10 mm Prodigy column (Phenomenex, Torrance, CA) using the following chromatographic conditions: 0−5 min, 2% acetonitrile in water; 5−45 min, 2−8% acetonitrile in water; flow rate: 1 mL/min. The eluate was monitored by UV absorbance at 218 nm.

The material eluted from 2−8% acetonitrile in water was further purified by HPLC with a Phenomenex C18 Luna (2) column (250 × 4.6 mm). The chromatographic conditions were as follows: 0−10 min, 100% water; 10−13 min, 0−2% acetonitrile in water; 13−15 min, 2−3% acetonitrile in water; 15−18 min, 3−4% acetonitrile in water; 18−50 min, 4−8% acetonitrile in water. The flow rate was 1 mL/min.

To obtain sufficient amount of valine−DHP adducts for further experiments, reactions were repeated with a larger scale (up to 50 mg of dehydromonocrotaline).

Reactions of valine with dehydroriddelliine, dehydroheliotrine, and DHR were conducted similarly.

Reaction of DHP−Valine Adducts Mixture with Phenyl Isothiocyanate (PITC). To the DHP−valine adducts mixture described above, dissolved in 1 mL of acetonitrile, was added 2 μL of phenyl isothiocyanate (PITC). The reaction was kept under shaking in a water bath at 45 °C for 2 h. The resulting isothiohydantion adducts (DHP−valine−PITC) were isolated by HPLC, using a Phenomenex C18 Luna (2) column (250 × 4.6 mm) eluted at a flow rate of 1 mL/min with a mobile phase of 43% acetonitrile in water and monitored at 268 nm. The resulting isomeric DHP−valine−PITC adducts were characterized by HPLC-ES-MS/MS and NMR analysis.

The full ^{1}H NMR spectral assignments of DHP−valine−PITC adducts follow.

DHP−Valine−PITC-1. ^{1}H NMR (acetonitrile-d_3) δ 0.74 (3H, d, H11′/H10′, *J* = 7 Hz), 1.11 (3H, d, H10′/H11′, *J* = 7 Hz), 1.38−1.45 (1H, m, H7′), 2.72−2.82 (2H, m, 9-OH, H6b), 3.02−3.10 (1H, m, H6a), 4.04−4.09 (1H, m, H5b), 4.12−4.18 (1H, m, H5a), 4.30 (1H, d, H5′, *J* = 3 Hz), 4.43 (1H, two sets of d, H9b, *J* = 5.5 Hz), 4.55 (1H, two sets of d, H9a, *J* = 4 Hz), 6.20 (H, d, H2, *J* = 2.5 Hz), 6.41−6.44 (1H, m, H7), 6.67 (1H, d, H3, *J* = 2.5 Hz), 7.24−7.32 (2H, m, H8′, H9′), 7.49−7.55(3H, m, H12′, H13′, H14′).

DHP−Valine−PITC-2. ^{1}H NMR (acetonitrile-d_3) δ 0.94 (3H, d, H11′/H10′, *J* = 7 Hz), 1.20 (3H, d, H10′/H11′, *J* = 7 Hz), 2.29−2.37 (1H, m, H6b), 2.62−2.65 (1H, m, H7′), 2.73−2.82 (1H, m, H6a), 3.41 (1H, w, 7-OH), 3.88−3.93 (1H, m, H5b), 4.09−4.14 (1H, m, H5a), 4.20 (1H, d, H5′, *J* = 3.5 Hz), 4.40 (1H, d, H9b, *J* = 15 Hz), 5.19−5.20 (1H, m, H7), 5.62 (1H, d, H9a, *J* = 15 Hz), 6.28 (1H, d, H2, *J* = 2.5 Hz), 6.62 (1H, d, H3, *J* = 2.5 Hz), 7.26−7.30 (2H, m, H8′, H9′), 7.46−7.55 (3H, m, H12′, H13′, H14′).

DHP−Valine−PITC-3. ^{1}H NMR (acetonitrile-d_3) δ 0.91 (3H, d, H11′, *J* = 7 Hz), 0.96 (3H, d, H10′, *J* = 7 Hz), 1.40−1.44 (1H, w, H7′), 2.70−2.73 (1H, m, H6b), 2.94−2.96 (1H, m, 9-OH), 3.12−3.17 (1H, m, H6a), 4.03−4.06 (2H, m, H5a, H5b), 4.39 (1H, d, H5′, *J* = 3 Hz), 4.45−4.50 (2H, m, H9a, H9b), 6.08 (1H, m, H7), 6.26 (1H, d, H2, *J* = 2.5 Hz), 6.74 (1H, d, H3, *J* = 2.5 Hz), 7.30−7.32 (2H, m, H8′, H9′), 7.49−7.55 (3H, m, H12′, H13′, H14′).

DHP−Valine−PITC-4. ^{1}H NMR (acetonitrile-d_3) δ 0.97 (3H, d, H11′/H10′, *J* = 7 Hz), 1.17 (3H, d, H10′/H11′, *J* = 7 Hz), 2.32−2.38 (1H, m, H6b, overlapped with solvent peak), 2.52−2.57 (1H, m, H7′), 2.73−2.81 (1H, m, H6a), 3.30 (1H, w, 7-OH), 3.91−3.95 (1H, m, H5b), 4.08−4.13 (2H, m, H5a, H5′), 4.39 (1H, d, H9b, *J* = 15 Hz), 5.11−5.12 (1H, m, H7), 5.72 (1H, d, H9a, *J* = 15 Hz), 6.22 (1H, d, H2, *J* = 2.5 Hz), 6.64 (1H, d, H3, *J* = 2.5 Hz), 7.27−7.30 (2H, m, H8′, H9′), 7.48−7.54 (3H, m, H12′, H13′, H14′).

Stability of DHP−Valine Adducts. Each DHP−valine adduct was dissolved in acetonitrile/distilled water (v/v 1:20) to afford a solution with an absorbance of approximately 0.5 absorbance units at 268 nm. The solution was incubated at room temperature, and aliquots were taken at different time intervals for analysis by HPLC. For the mechanistic study, pure DHP−valine-2 and DHP−valine-4 adducts in acetonitrile/H_2O^{18} (v/v, 1:20) were incubated separately at room temp for 24 h. The resulting DHP−valine-2 and DHP−valine-4 adducts were collected by HPLC for LC/MS analysis.

Interconversion between DHP−Valine−PITC-1 and DHP−Valine−PITC-3 Adducts. Four DHP−valine−PITC adducts, designated as DHP−valine−PITC-1, DHP−valine−PITC-2, DHP−valine−PITC-3, and DHP−valine−PITC-4, were identified from the reaction. To determine the possible interconversion among these adducts, a 0.5 mL solution of DHP−valine−PITC-1 in acetonitrile/H_2O (v/v, 1:1) at room temp was monitored by HPLC at 0, 1, 2, and 3 days. The HPLC analytical conditions were as follows: ACE C18 AR column (4.6 × 250 mm, 5 μ, from Mac-Mod Analytical, Inc., Chadds Ford, PA), monitored at 268 nm, flow rate, 1 mL/min; gradient program: 0−5 min, 10% acetonitrile in water; 5−50 min, 10−18% acetonitrile in water. The percentage of DHP−valine−PITC-1 and the resulting DHP−valine−PITC-3 was calculated on the basis of the ratio of their HPLC peak areas. The sum of peak areas was stable (±3%) over the incubation period. No additional new peaks were found during the HPLC analysis, indicating that under these conditions both the DHP−valine−PITC-1 and DHP−valine−PITC-3 adducts were stable.

An experiment with pure DHP−valine−PITC-3 in acetonitrile/H_2O (v/v, 1:1) was similarly conducted and monitored by HPLC daily up to 6 days. The percentage of DHP−valine−PITC-1 and DHP−valine−PITC-3 was similarly calculated.

Interconversion between DHP−Valine−PITC-2 and DHP−Valine−PITC-4. The study of interconversion between DHP−valine−PITC-2 and DHP−valine−PITC-4 was conducted under the conditions described above for DHP−valine−PITC-1 and DHP−valine−PITC-3, with the exception that the reactions were monitored by HPLC at 0, 1, 2, and 3 h (or up to 7 h) respectively.

Reaction of Dehydromonocrotaline and DHR with Hemoglobin Followed by Reaction with Phenyl Isothiocyanate. A solution of 20 mg of rat hemoglobin, 350 μL of deionized water, and 100 μL of 0.5 M K_2CO_3 was added dropwise into a 50 μL solution of 2 mg of dehydromonocrotaline in DMF and kept with shaking at 37 °C for 3 h, followed by the addition of 2 equiv of PITC (in relation to the dehydromonocrotaline). The reaction continued at 37 °C with shaking for 16 h. After 3 mL of acetonitrile was added, the reaction solution was centrifuged (15 000*g*, 5 min), the supernatant was collected, and solvent was removed in a rotary evaporator under reduced pressure. The resulting DHP−valine−PITC adducts were purified by HPLC (following the conditions previously described for separation of DHP−valine−PITC) and analyzed by LC/MS/MS.

Reaction of DHR with rat hemoglobin was conducted under similar experimental conditions.

Analysis of DHP−Valine−PITC Adducts in Hemoglobin of Rats Treated with Monocrotaline and Riddelliine. To identify DHP−hemoglobin adducts formed in blood of rats treated with monocrotaline or riddelliine, blood samples were treated with PITC to convert DHP−hemoglobin adducts to phenylthiohydantoin (DHP−

valine−PITC) products through the Edman rearrangement reaction.[41,42]

In brief, red blood cells (100 μL) collected from rats treated with monocrotaline or riddelliine were lysed by adding 120 μL of dH_2O and reacted with 5 μL of PITC in 20 μL of 0.5 M K_2CO_3 with shaking at 37 °C for 16 h. The reaction was stopped by adding 1.5 mL of acetonitrile. After centrifugation and concentration, the resulting sample was analyzed by LC/MS/MS under similar conditions as those described for the identification of DHP−valine−PITC adducts.

LC/MS/MS Analysis of DHP−Valine and DHP−Valine−PITC Adducts. DHP−valine and DHP−valine−PITC adducts were dissolved in water for LC/MS/MS analysis. The liquid chromatography system consisted of a Shimadzu Prominence HPLC system, including a CBM-20A system controller, two LC-20AD pumps, a SIL-20AC HT autosampler, a SPD-20A UV/vis detector (Shimadzu Scientific Instrument, Columbia, MD 21046), and an automated switching valve (TPMV, Rheodyne, Cotati, CA). The switching valve was used to divert the column effluent to either waste or to MS instrument. The Shimadzu Prominence HPLC system was used for sample injection and separation. Each sample (10−30 μL) was loaded onto a reverse-phase column (ACE 3 C18, 2.1 mm × 100 mm, 3 μm, Mac-Mod Analytical, Inc., Chadds Ford, PA) with a water/acetonitrile gradient at 0.2 mL/min, and the sample components were eluted into the mass spectrometer. The column chamber's temperature was set to 45 °C. The mobile phases were water and acetonitrile. The initial gradient consisted of 0% acetonitrile for 0.5 min followed by a linear gradient up to 15% acetonitrile over 50 min; acetonitrile was increased to 95% in 1.5 min. After holding 95% acetonitrile for 5 min, the instrument was reset to the initial conditions in 1 min. The analytical column was equilibrated with the starting mobile phase for 12 min. The total run time for analysis was 70 min.

The HPLC eluate was coupled with an AB Sciex 4000 QTrap LC/MS/MS system (AB Sciex, Foster City, CA) equipped with a Turbo V ion source and a desolvation temperature of 500 °C. Nitrogen was used as curtain, nebulizer, heater, and collision gases. The samples were acquired in positive ion spray mode using MS full scan and enhanced product ion scan (EPI) as well as using multiple reaction monitoring methods (MRM). The ion transitions of the MRM method for specific detection of DHP−valine and DHP−valine−PITC adducts in the samples were m/z 253 → 136 and m/z 392 → 136, respectively. The ion spray voltage was 4500 V; ion source gases 1 and 2 were set to 60 and 50, respectively. The declustering potential was 50 V, and the collision energy was 25 eV for all transitions.

Instrumentation. For HPLC analysis, a Waters (Milford, MA) HPLC system, consisting of a model 600 controller, a model 996 photodiode array detector, and a 600 pump, was used for separation and purification of the DHP-derived DNA adducts. ^{1}H nuclear magnetic resonance (NMR) experiments were carried out at 301 K on a Bruker Avance III spectrometer equipped with a Bruker BBFO Plus Smart Probe (Bruker Instruments, Billerica, MA) operating at 500 MHz. Samples were dissolved in acetonitrile-d_3 or acetonitrile-d_3 with a trace of D_2O. Chemical shifts are reported in parts per million downfield from tetramethylsilane, and coupling constants are reported in hertz. Two-dimensional NMR homonuclear decoupling (COSY) experiments were conducted to assist in assigning proton resonances.

RESULTS

Reaction of Valine with Dehydropyrrolizidine Alkaloids. The difficulty of the synthesis is that, while dehydropyrrolizidine alkaloids are highly unstable in water-containing solvent system, the reaction has to be conducted in water-containing medium because valine is soluble only in water. Therefore, to synthesize DHP−valine adducts, reaction of dehydromonocrotaline and valine was repeated under several different experimental conditions. The optimal conditions used are described in the Experimental Procedures.

After reaction of valine with dehydromonocrotaline for 48 h, the resulting reaction mixture was filtered through a 0.4 μm RC membrane, and products were separated by HPLC with a Prodigy column (250 × 10 mm). The prepurified material was further purified by HPLC with a Phenomenex C18 Luna (2) column (250 × 4.6 mm). An initial elution with water removed the unreacted valine and necic acid formed from hydrolysis of dehydromonocrotaline; further elution with acetonitrile in water afforded a mixture of reaction products (Figure 3A).

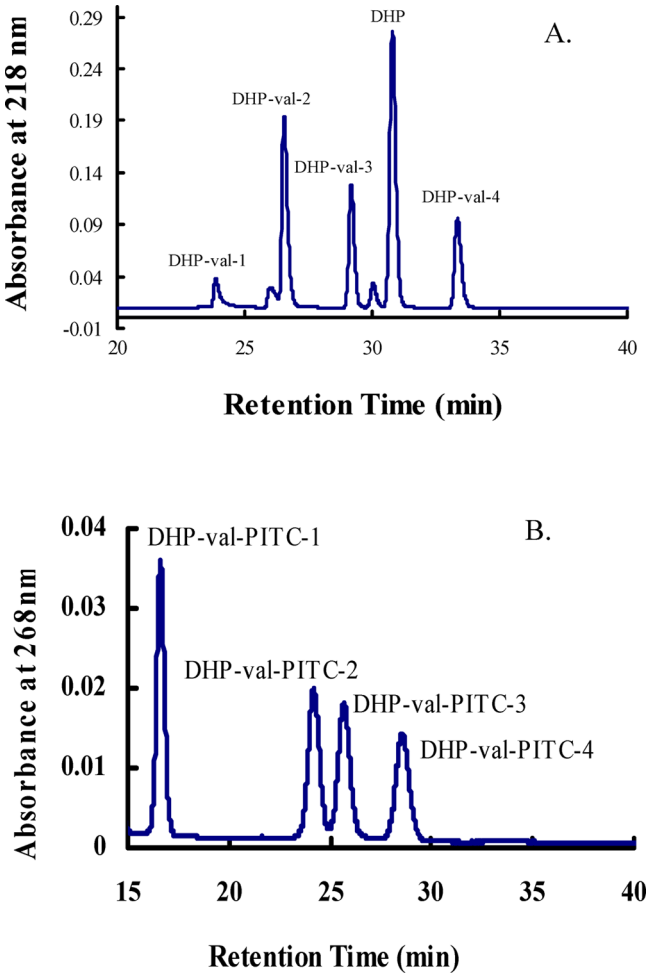

Figure 3. Reversed-phase HPLC profiles of (A) four DHP−valine adducts formed from the reaction of dehydromonocrotaline and valine and (B) four DHP−valine−PITC adducts formed from reaction of DHP−valine mixture with phenyl isothiocycnate. HPLC conditions: Phenomenex C18 Luna (2) column (250 × 4.6 mm); flow rate: 1 mL/min; mobile phase (linear gradient): (A) 0−10 min, 100% water; 10−13 min, 0−2% acetonitrile in water; 13−15 min, 2−3% acetonitrile in water; 15−18 min, 3−4% acetonitrile in water; 18−50 min, 4−8% acetonitrile in water; (B) 43% acetonitrile in water at flow rate 1 mL/min.

LC/MS analysis of the reaction mixture showed that there were four products, each having a mass spectrum with protonated molecule ions $(M + H)^+$ at m/z 253. These results suggested that the reaction mixture contained four isomeric DHP−valine adducts (m/z 253, designated as DHP−valine-1, DHP−valine-2, DHP−valine-3, and DHP−valine-4, respectively). Because these adducts are unstable and decomposed during HPLC purification, we were unable to obtain these products in a sufficient quantity for structural identification by ^{1}H NMR spectral analysis. Thus, the product mixture was treated with phenyl isothiocyanate (PITC) to convert DHP−

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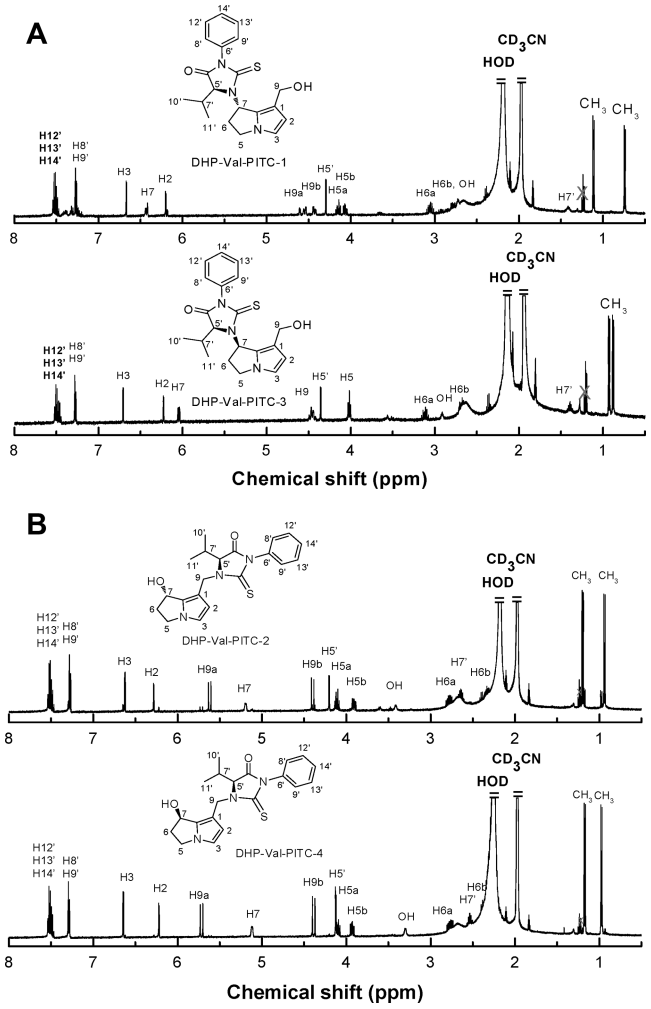

Figure 4. ^{1}H NMR spectra of products identified as (A) DHP–valine–PITC-1 and DHP–valine–PITC-3 adducts and (B) DHP–valine–PITC-2 and DHP–valine–PITC-4 adducts.

valine adducts to the phenylthiohydantoin (DHP–valine–PITC) products through Edman rearrangement reaction.[42]

Reaction of DHP–Valine Adducts with Phenyl Isothiocyanate (PITC). After reaction of DHP–valine adducts with PITC, the resulting phenylthiohydantoin adducts (DHP–valine–PITC adducts) were separated by reversed-phase HPLC (Figure 3B). Chromatographic peaks eluted at 16.9, 23.9, 26, and 28.1 min had similar UV absorption spectra (Figure S1 in the Supporting Information) and mass spectra with preponderant sodiated ions at m/z 392 and ions at m/z 352 corresponding to the loss of water by the protonated adduct (Figure S2 in the Supporting Information), which suggested that they were four isomeric DHP–valine–PITC adducts.

These adducts were further purified by HPLC to obtain sufficient quantity for structural identification by ^{1}H NMR (Figure 4). The four chromatographic peaks eluted at 16.9, 23.9, 26, and 28.1 min are designated as DHP–valine–PITC-1, DHP–valine–PITC-2, DHP–valine–PITC-3, and DHP–valine–PITC-4 adducts, respectively.

The ^{1}H NMR data of these four DHP–valine–PITC adducts were compared with ^{1}H NMR data of phenylthiohydantoin adducts[42,43] and DHR, the necine base (present study, assignments shown in Table 1). In accordance with the reported data of a phenylthiohydantoin moiety, these adducts had five phenyl protons at 7.2–7.6 ppm and six valine methyl protons at 0.94–1.17 ppm. These adducts had the H5′ proton as a doublet at 4.15–4.40 ppm. While DHP–valine–PITC-1 and DHP–valine–PITC-3 had the H7′ proton at 1.38–1.45 and 1.40–1.44 ppm, respectively, DHP–valine–PITC-2 and DHP–valine–PITC-4 had the H7′ proton at 2.52–2.57 ppm. Their NMR resonances of H2 and H3 were nearly identical to those of the DHR moiety. Both DHP–valine–PITC-2 and DHP–valine–PITC-4 had the pairs of geminal H5 and H6 protons almost unaffected by adduct formation. DHP–valine–PITC-1 and DHP–valine–PITC-3 had their H7 proton resonance at 6.41–6.44 and 6.08 ppm, respectively, whereas DHP–valine–PITC-2 and DHP–valine–PITC-4 had their H7 proton resonance at 5.19–5.20 ppm and 5.11–5.12 ppm, respectively (Table 1). The H7 of DHR resonates at 4.99 ppm. Taken together, these data indicate that the DHP–valine–PITC-1 and DHP–valine–PITC-3 adducts had their phenylthiohydantoin group located at the C7 position of the DHP moiety (the necine base) and that they were a pair of epimers. Similar comparison of the CH_2 (C9 position) group of the DHP moiety of these adducts with that of DHP (DHR) indicated that DHP–valine–PITC-2 and DHP–valine–PITC-4 adducts had their phenylthiohydantoin group located at the C9 position of the DHP moiety and that they were a pair of epimers. Thus, comparison of the chemical shifts of the necine base of these four DHP–valine–PITC adducts with those of DHR (Table 1) helped to determine the structures of these phenylthiohydantoin adducts. The NMR assignments were further confirmed by COSY NMR analysis (Figures S3–S4 in the Supporting Information).

The NMR data indicated that DHP–valine–PITC-3 and DHP–valine–PITC-1 are a pair of epimers and that DHP–valine–PITC-2 and DHP–valine–PITC-4 are another pair of

Table 1. Proton NMR Spectroscopic Data (500 MHz) of the Necine Base in the Synthetically Prepared DHP–Valine–PITC Adducts Measured in Acetonitrile-d_3

assignment	DHR	DHP–val–PITC-1	DHP–val–PITC-2	DHP–val–PITC-3	DHP–val–PITC-4
$H6_b$	2.15–2.20 (1H, m)	2.75–2.82 (1H, m)	2.29–2.41 (1H, m)	2.70–2.73 (1H, m)	Overlapped with solvent peak (HOD)
$H6_a$	2.57–2.65 (1H, m)	3.02–3.10 (1H, m)	2.73–2.82 (1H, m)	3.12–3.17 (1H, m)	2.73–2.81 (1H, m)
$H5_b$	3.75–3.79 (1H, m)	4.04–4.09 (1H, m)	3.88–3.93 (1H, m)	4.03–4.06 (2H, m)	3.91–3.95 (1H, m)
$H5_a$	3.94–3.98 (1H, m)	4.12–4.16 (1H, m)	4.09–4.14 (1H, m)		4.08–4.13 (1H, m)
$H9_b$	4.27 (1H, d, J = 12)	4.43 (1H, dd, J = 5.5)	4.40 (1H, d, J = 15)	4.45–4.50 (2H, m)	4.39 (1H, d, J = 15)
$H9_a$	4.33 (1H, d, J = 12)	4.63 (1H, dd, J = 4)	5.62 (1H, d, J = 15)		5.72 (1H, d, J = 15)
H2	6.02 (1H, d, J = 2.5)	6.20 (H, d, J = 2.5)	6.28 (1H, d, J = 2.5)	6.26 (1H, d, J = 2.5)	6.22 (1H, d, J = 2.5)
H7	4.99 (1H, dd, J = 6.5, 2.5)	6.41–6.44 (1H, m)	5.19–5.20 (1H, w)	6.08 (1H, m)	5.11–5.12 (1H, w)
H3	6.54 (1H, d, J = 2.6)	6.67 (1H, d, J = 2.5)	6.30 (1H, d, J = 2.5)	6.74 (1H, d, J = 2.5)	6.64 (1H, d, J = 2.5)

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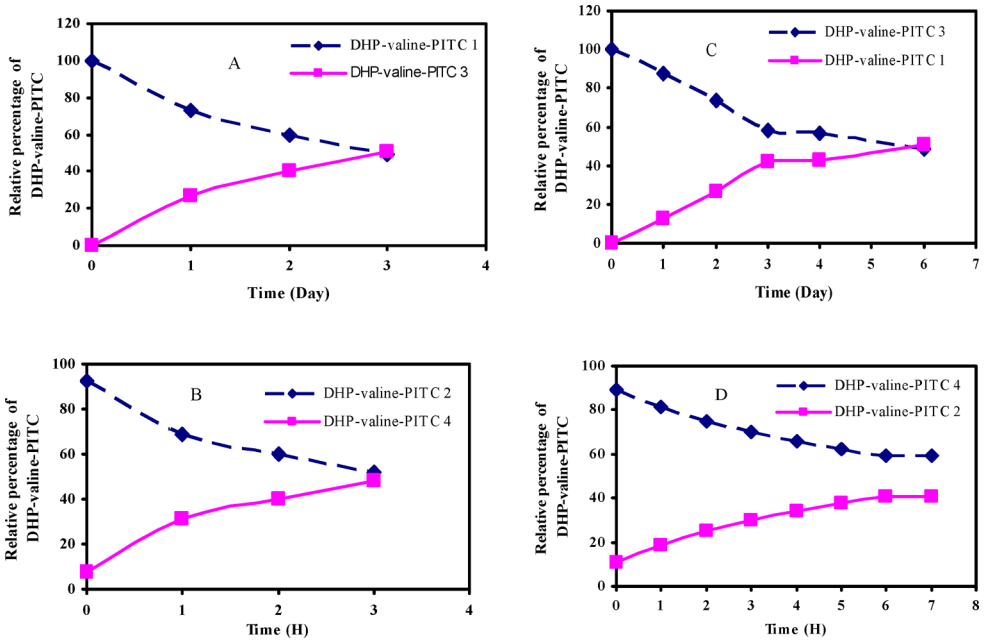

Figure 5. Interconversion between DHP−valine−PITC-1 and DHP−valine−PITC-3 and between DHP−valine−PITC-2 and DHP−valine−PITC-4 in acetontrile/water (v/v, 1:1): (A) starting from pure DHP−valine−PITC-1, (B) starting from pure DHP−valine−PITC-2, (C) starting from pure DHP−valine−PITC-3, and (D) starting from pure DHP−valine−PITC-4. The relative percentages were calculated on the basis of their HPLC peak area ratios.

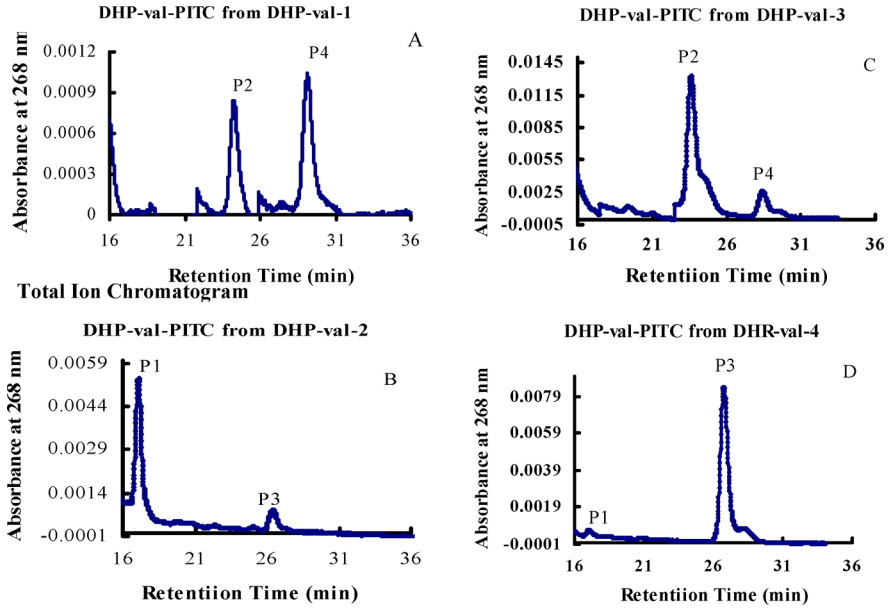

Figure 6. HPLC chromatogram of derivatives formed from the reaction of each DHR−valine adduct with PITC: (A) from DHP−valine-1, (B) from DHP−valine-2, (C) from DHP−valine-3, and (D) from DHP−valine-4. In the figure, P1, P2, P3, and P4 designate DHP−valine−PITC-1, DHP−valine−PITC-2, DHP−valine−PITC-3, and DHP−valine−PITC-4, respectively. HPLC conditions: Phenomenex C18 Luna (2) column (250 × 4.6 mm); flow rate: 1 mL/min; mobile phase: 43% acetonitrile in water.

epimers. As expected, the experimentally determined UV molar extinction coefficients of DHP−valine−PITC-1 and DHP−valine−PITC-3 were identical, and the UV molar extinction coefficients of DHP−valine−PITC-2 and DHP−valine−PITC-4 were also identical. On the basis of ^{1}H NMR spectroscopic analysis in the presence of a known quantity of *tert*-butanol as an internal standard, the UV molar extinction coefficients of DHP−valine−PITC-1 and DHP−valine−PITC-3 were determined to be 1.66×10^4 M^{-1} cm^{-1} (at 268 nm) in acetonitrile, and those of DHP−valine−PITC-2 and DHP−valine−PITC-4, 1.46×10^4 M^{-1} cm^{-1} (at 270 nm) in acetonitrile. Accordingly, the reaction yields of DHP−valine−PITC-1, DHP−valine−PITC-2, DHP−valine−PITC-3, and DHP−valine−PITC-4 were estimated to be 1.2, 1.4, 1.8, and 1.6%, respectively. These results indicate that reaction of valine and dehydromonocrotaline was nonregioselective, with the reaction at the C7 and C9 positions of the necine base of dehydromonocrotamine proceeding at a near equal ratio.

Interconversion between DHP−Valine−PITC-1 and DHP−Valine−PITC-3 Adducts. We determined that at

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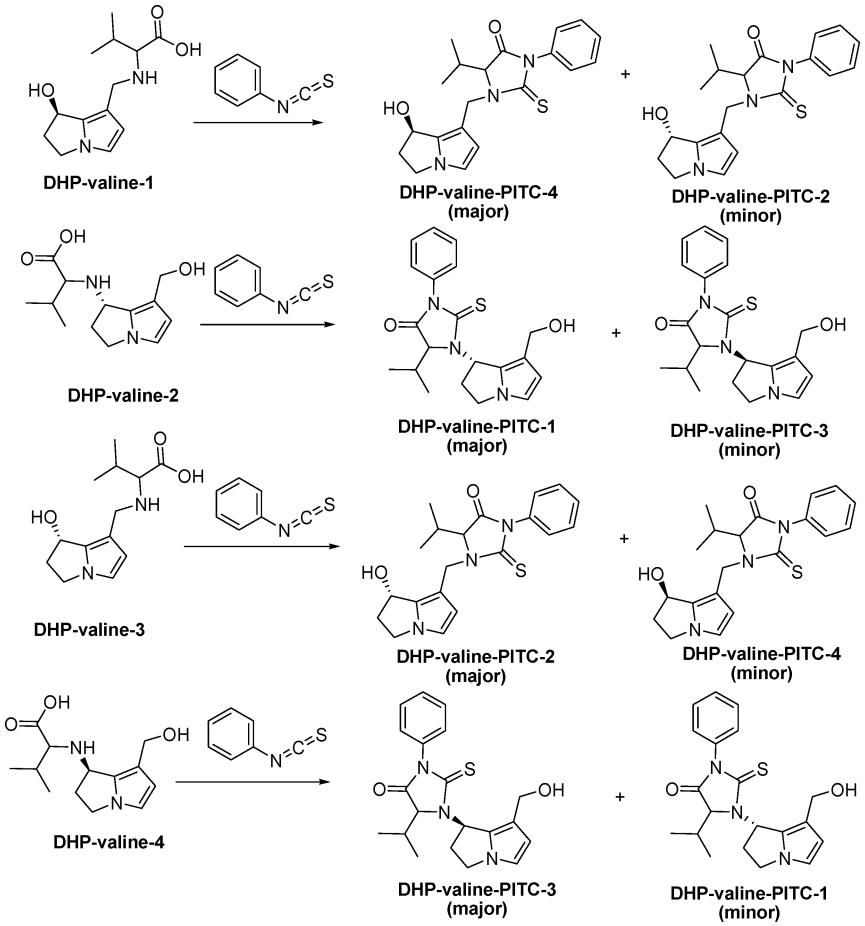

Figure 7. Structures of four DHP−valine adducts and the relationship with the four DHP−valine−PITC adducts.

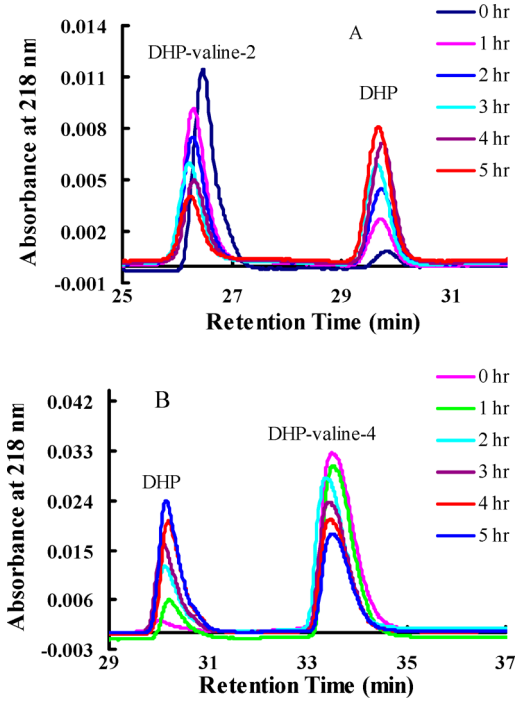

Figure 8. Kinetic analysis of decomposition of DHP−valine adducts in acetontrile/water (v/v, 1:20). Decomposition of (A) DHP−valine-2 adduct to DHP and (B) DHP−valine-4 adduct to DHP.

room temperature DHP−valine−PITC-1 and DHP−valine−PITC-3 dissolved in 10% acetonitrile in water (v/v, 1:1) were interconvertible. HPLC analysis indicated that DHP−valine−PITC-1 was converted into DHP−valine−PITC-3 gradually and reached an approximately equal ratio in 3 days (Figure 5A). Similar results occurred when the experiment was started with pure DHP−valine−PITC-3, but the rate of interconversion was slower, with an equal ratio of both epimers being reached at 6 days of incubation (Figure 5C). These results indicate that conversion of DHP−valine−PITC-1 is faster than that of DHP−valine−PITC-3.

Interconversion between DHP−Valine−PITC-2 and DHP−Valine−PITC-4 Adducts. A study of interconversion between DHP−valine−PITC-2 and DHP−valine−PITC-4 was similarly conducted. It was found that the rate of interconversion between DHP−valine−PITC-2 and DHP−valine−PITC-4 was much faster than that between DHP−valine−PITC-1 and DHP−valine−PITC-3. Interconversion of DHP−valine−PITC-2 to DHP−valine−PITC-4 to an equal ratio required only 3 h (Figure 5B). Interconversion of DHP−valine−PITC-4 to a near equal ratio of DHP−valine−PITC-2 took 7 h (Figure 5D), a little longer time than that of DHP−valine−PITC-2 but much shorter than those between DHP−valine−PITC-1 and DHP−valine−PITC-3 (Figure 5A,C).

Stability of DHP−Valine−PITC Adducts. It was determined that DHP−valine−PITC adducts dissolved in acetonitrile with and without a trace of water were stable for up to a week. However, in fully aqueous medium or in a solvent consisting of 20% acetonitrile in water during HPLC

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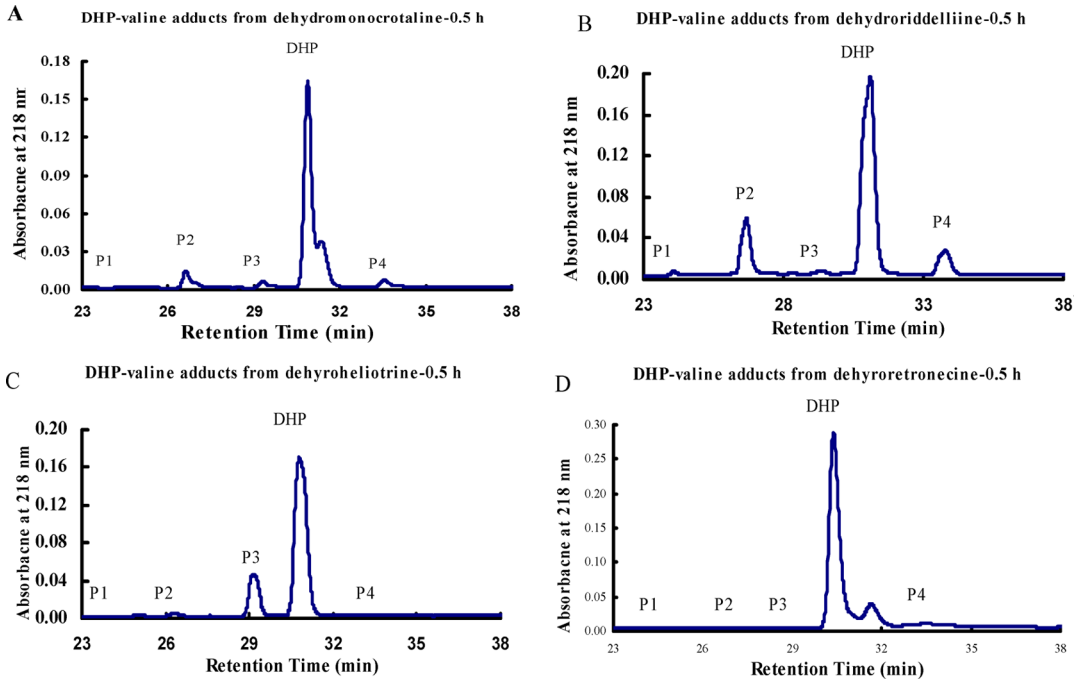

Figure 9. HPLC chromatogram of four DHP−valine adducts formed from the reaction valine with (A) dehydroriddelliine, (B) dehydromonocrotaline, (C) dehyroheliotrine, and (D) dehyroretronecine (DHR). In the figure, P1, P2, P3, and P4 designate DHP−valine-1, DHP−valine-2, DHP−valine-3, and DHP−valine-4, respectively. HPLC conditions: column, Phenomenex C18 Luna (250 × 4.6 nm); flow rate: 1 mL/min; mobile phase (linear gradient): 0−10 min, 100% water; 10−13 min, 0−2% acetonitrile in water; 13−15 min, 2−3% acetonitrile in water; 15−18 min, 3−4% acetonitrile in water; 18−50 min, 4−8% acetonitrile in water.

separation, the DHP−valine−PITC adducts gave rise to many decomposition products.

Structural Identification of DHP−Valine Adducts. To determinate the structures of DHP−valine adducts (DHP−valine-1, DHP−valine-2, DHP−valine-3, and DHP−valine-4) formed from reaction of dehydromonocrotaline and valine, we isolated the materials contained in each of the chromatographic peaks shown in Figure 3A to react with PITC, and we then compared their HPLC retention times and UV spectra with those of the four DHP−valine−PITC adducts, whose structures had been elucidated by mass spectrometry and ^{1}H NMR. Upon derivatization with PITC, each of the four peaks labeled as DHP−valine-1 (eluted at 24.1 min), DHP−valine-2 (26.7 min), DHP−valine-3 (29.2 min), and DHP−valine-4 (33.5 min) in Figure 3A formed one major and one minor DHP−valine−PITC adducts (Figure 6). As described above, interconversion occurred between DHP−valine−PITC-1 and DHP−valine−PITC-3 as well as between DHP−valine−PITC-2 and DHP−valine−PITC-4. Therefore, it is reasonable to assume that the major DHP−valine−PITC was generated from its parent DHP−valine adduct and that the minor DHP−valine−PITC was formed by interconversion from the major one. As a result, DHP−valine−PITC-4 was derived from DHP−valine-1 (Figure 6A), DHP−valine−PITC-1 was derived from DHP−valine-2 (Figure 6B), DHP−valine−PITC-2 was derived from DHP−valine-3 (Figure 6C), and DHP−valine−PITC-3 was derived from DHP−valine-4 (Figure 6D).

Because the structures of DHP−valine−PITC adducts have been characterized, the structures of four DHP−valine adducts are subsequently deduced as shown in Figure 7.

Stability of DHP−Valine Adducts. A sample of DHP−valine-2 adduct was incubated in acetonitrile/H_2O (v/v, 1:20) at room temperature and was monitored by HPLC at 0, 1, 2, 3, 4, and 5 h. As shown in Figure 8A, the amount of DHP−valine-2 adduct decreased gradually, and DHP formed increasingly in a time-dependent manner. The sum of the integrated peak areas of the two peaks, DHP−valine-2 and DHP, in each analysis is almost equal. No other peak was found in the HPLC analysis, which suggested that the DHP−valine-2 adduct decomposed to DHP and valine. The same phenomenon was found for DHP−valine-4 adduct under similar experimental conditions. As shown in Figure 8B, the amount of DHP−valine-4 adduct decreased gradually and DHP formed increasingly in a time-dependent manner.

Reaction of Dehydromonocrotaline, Dehydroriddelliine, Dehydroheliotrine, and DHR with Valine. As already described, DHP−valine adducts are unstable, undergoing interconversion between epimers and, as other dehydropyrrolizidine alkaloids, are quickly hydrolyzed into DHP (the racemic form of DHR) in aqueous medium. To compare the relative yield, reactions of dehydromonocrotaline, dehydroriddelliine, dehydroheliotrine, and DHR with valine were conducted for 30 min. It was found that reaction of dehydromonocrotaline, dehydroriddelliine, and dehydroheliotrine all produced DHP−valine adducts, but with different yields and patterns. In contrast, reaction of DHR and valine did not generate DHP−valine at a detected level (Figure 9).

Reaction of Dehydromonocrotaline and DHR with Hemoglobin Followed by Reaction with Phenyl Isothiocyanate. Similar to the reaction of valine with dehydromonocrotaline, a reaction of hemoglobin (Hb) with dehydromonocrotaline in K_2CO_3 and DMF was conducted, followed by reaction with phenyl isothiocynante. The reaction products were analyzed by HPLC, and the eluate was analyzed by ultraviolet detection and mass spectrometry. There were four chromatographic peaks, each of which had retention time,

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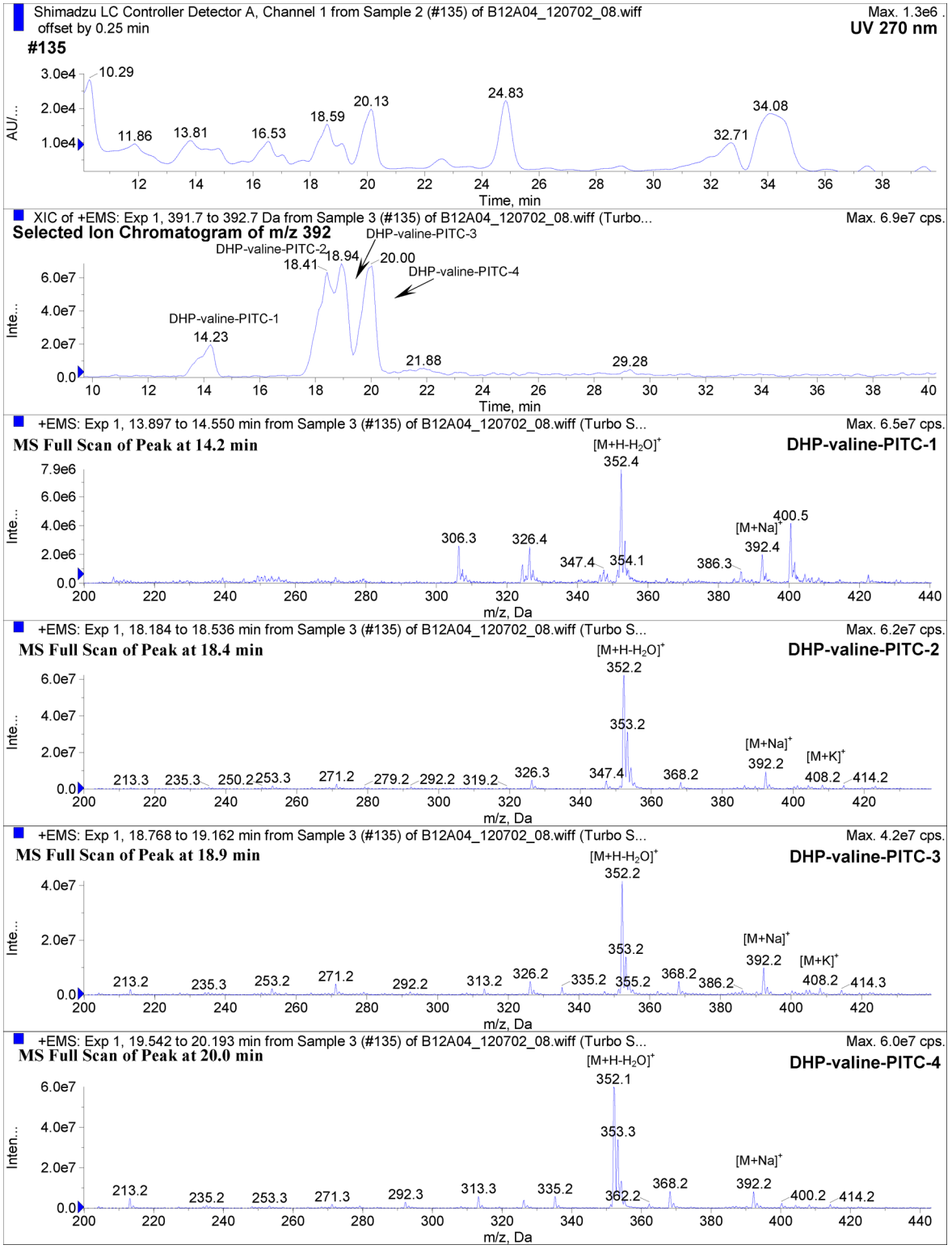

Figure 10. HPLC chromatogram and mass spectrometric profiles of DHP−valine−PITC adducts formed from reaction of dehydromonocrotaline with hemoglobin followed by reaction with PITC.

UV−visible absorption spectra, and mass spectra evidencing ions at m/z 352 that corresponded to the loss of water by the protonated molecules and $(M + Na)^+$ at 392 m/z (Figure 10) identical to those of the four DHP−valine−PITC adducts formed from the reaction of dehydromonocrotaline with valine. In contrast, on the basis of similar spectral analyses, the reaction of DHR with Hb did not generate DHP−valine−PITC adducts.

Analysis of DHP−Hemoglobin Adducts Formed *in Vivo*. The hemoglobin samples of rats treated with riddelliine and monocrotaline by gavage were similarly treated with phenyl isothiocyanate. LC/MS/MS analysis of the reaction mixtures indicated that no DHP−valine−PITC adducts were detected in the samples using LC/MS full scan and MS/MS methods as well as using MRM.

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DISCUSSION

Probably because of their highly unstable nature, limited information on the structures of DHP−protein adducts and DHP−amino acid adducts have been reported. 7-Cysteine-DHP, prepared from reaction of DHR with cysteine by Robertson et al.,[44] is the only DHP−amino acid adduct with the structure fully elucidated by MS and NMR analysis. Estep et al.[45] isolated *N*-acetylcysteine−DHP in the urine of rats administered monocrotaline and senecionine. Its identity was based on an Ehrlich-reagent-positive result, which indicates the presence of a pyrrolic moiety, and its molecular weight was determined by MS. In this present study, the reaction of dehydromonocrotaline, dehyroriddelliine, and dehydroheliotrine with valine and the reaction of dehydromonocrotaline with hemoglobin were studied. The structures of the resulting DHP−valine-1, DHP−valine-2, DHP−valine-3, and DHP−valine-4 were elucidated through the full structural characterization of the four corresponding DHP−valine−PITC derivatives. This represents the first full structural elucidation of an amino acid−DHP adduct that was prepared from the reaction between an amino acid and dehydropyrrolizidine alkaloids, the primary reactive metabolites of pyrrolizidine alkaloids.

In aqueous medium, DHP−valine adducts proved to be highly unstable, facilely decomposed, and interconverted between DHP−valine epimers. Moreover, both DHP−valine-2 and DHP−valine-4 can facilely lose the valine moiety at the C7 position of the necine base to form DHP in a time-dependent manner (Figures 8). These findings are highly significant because they suggest that DHP−protein adducts may also dissociate into protein and DHP *in vivo* and *in vitro*. This observation may shed light on the role of DHP−protein adducts on hepatotoxicity and carcinogenesis induced by pyrrolizidine alkaloids.

In the field of chemical carcinogenesis, hemoglobin adducts can provide noninvasive biomarkers of reactive species involved in liver tumorigenicity, if there is a correlation between the liver tumor potency and the level of hemoglobin adducts in the blood. The most commonly employed method for quantitation of metabolite-bound protein adducts *in vivo* is the use of Edman rearrangement reaction.[46] Edman reaction involves the use phenyl isothiocyanate and derivatives, such as pentafluorophenyl isothiocyanate, to react with the valine terminal amino group of the hemoglobin adducts, resulting in the replacement of the hemoglobin moiety by a phenylthiohydantoin group. Quantitation can then be accomplished by LC/MS/MS.[47] There are limitations as to whether a specific hemoglobin adduct can be validated as a biomarker. At minimum, these include the yield and the stability of the hemoglobin adduct that is formed by linking the valine terminal amino group of the hemoglobin to the metabolite, the yield of the Edman rearrangement reaction, the stability of the final products, the ease of the separation of final products by HPLC, the detection efficiency of the final products by LC/MS/MS, and the availability of the deuterated final products used as internal standards for detection and quantification by LC/MS/MS.

In the present study, we demonstrate that DHP−valine adducts are highly unstable in aqueous media, which suggests that DHP−protein adducts that are formed through linking the valine terminal amino group to the DHP moiety should be highly unstable as well. Thus, our data indicate that quantification of DHP−hemoglobin adducts *in vivo* via the Edman rearrangement reaction pathway may not be feasible or may severely underestimate the total reactive metabolite produced or that the reactive metabolite does not get into circulation. Consistently, as reported in the present study, no DHP−valine−PITC adducts were detected from the hemoglobin of rats administered riddelliine or monocrotaline by gavage.

In a previous study, we determined[35] that calf thymus DNA, dG, and dA bind to dehydroriddelliine preferentially at the C9 position of the necine base. Furthermore, in an *in vivo* study, we determined that female rats gavaged with riddelliine, monocrotaline, retrorsine, heliotrine, lasiocarpine, clivorine, senkirkine, and riddelliine N-oxide afforded the same pattern of DNA adducts, forming two sets of epimeric dG and dA adducts with the nucleic bases linked to the C9 position of the necine base. We previously hypothesized that the regioselectivity of these reaction is mainly due to steric hindrance at the C7 position.[36] In contrast, it was determined that dehydropyrrolizidine alkaloids and DHR bind with nucleophiles preferentially at the C7 position of the necine base.[1,7,34] The binding of DHR with cysteine and glutathione (GSH) also preferentially occurred at the 7-position to form 7-cysteine−DHP and 7-GSH−DHP; neither 9-cysteine−DHP nor 9-GSH−DHP were formed.[44,48] On the other hand, our findings in the present study clearly indicate that reactions of valine with dehydromonocrotaline and dehydroriddelliine are not regioselective, although reaction at the C7 position to form DHP−valine-2 and DHP−valine-4 proceeded at higher yields than that at the C9 position, which yields DHP−valine-1 and DHP−valine-3 adducts. Overall, in order to better understand the mechanism of pyrrolizidine alkaloid-induced hepatotoxicity and liver tumor formation, determination of the reaction of dehydropyrrolizidine alkaloids with cellular macromolecules warrants further investigation.

ASSOCIATED CONTENT

Supporting Information

Ultraviolet, LC/MS, and $^{1}H-^{1}H$ COSY NMR spectra of DHP−valine−PITC products. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

This article is not an official U.S. Food and Drug Administration (FDA) guidance or policy statement. No official support or endorsement by the U.S. FDA is intended or should be inferred.

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DHR, dehydroretronecine or (−)-*R*-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine; DHP, (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine; PITC, phenyl isothiochanate; DHP−valine-1 and DHP−valine-3, an epimeric pair of 7-hydroxy-9-valine-dehydrosupinidine; DHP−valine-2 and DHP−valine-4, an epimeric pair of 7-valine-dehydrosupinidine; DHP−valine−PITC-2 and DHP−valine−PITC-4, an epimeric pairs of 7-hydroxy-9-valine-dehydrosupinidine; DHP−valine−PITC-1 and DHP−valine−PITC-3, an epimeric pairs of 7-valine-dehydrosupinidine; Hb, hemoglobin; LC/MS/MS, high-performance liquid chromatography electrospray ionization tandem mass spectrometry; NCTR, National Center for Toxicological Research

■ REFERENCES

(1) Fu, P. P., Chou, M. W., Churchwell, M., Wang, Y., Zhao, Y., Xia, Q., Gamboa da Costa, G., Marques, M. M., Beland, F. A., and Doerge, D. R. (2010) High-performance liquid chromatography electrospray ionization tandem mass spectrometry for the detection and quantitation of pyrrolizidine alkaloid-derived DNA adducts *in vitro* and *in vivo*. *Chem. Res. Toxicol. 23*, 637−652.

(2) Fu, P. P., Chou, M. W., Xia, Q., Yang, Y. C., Yan, J., Doerge, D. R., and Chan, P. C. (2001) Genotoxic pyrrolizidine alkaloids and pyrrolizidine alkaloid N-oxides—mechanisms leading to DNA adduct formation and tumorigenicity. *J. Environ. Sci. Health, Part C: Environ. Carcinog. Ecotoxicol. Rev. 19*, 353−386.

(3) Fu, P. P., Xia, Q., Chou, M. W., and Lin, G. (2007) Detection, hepatotoxicity, and tumorigenicity of pyrrolizidine alkaloids in Chinese herbal plants and herbal dietary supplements. *J. Food Drug Anal. 15*, 400−415.

(4) Fu, P. P., Xia, Q., Lin, G., and Chou, M. W. (2004) Pyrrolizidine alkaloids—genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. *Drug Metab. Rev. 36*, 1−55.

(5) Fu, P. P., Yang, Y.-C., Xia, Q., Chou, M. W., Cui, Y., and Lin, G. (2002) Pyrrolizidine alkaloids—tumorigenic components in Chinese herbal medicines and dietary supplements. *J. Food Drug Anal. 10*, 198−211.

(6) (1989) *Pyrrolizidine alkaloids: health and safety guide 26*, International Programme on Chemical Safety, World Health Organization, Geneva, Switzerland.

(7) Mattocks, A. R. (1986) *Chemistry and Toxicology of Pyrrolizidine Alkaloids*, Academic Press, London.

(8) Roeder, E. (2000) Medicinal plants in China containing pyrrolizidine alkaloids. *Pharmazie 55*, 711−726.

(9) Roeder, E. (1995) Medicinal plants in Europe containing pyrrolizidine alkaloids. *Pharmazie 50*, 83−98.

(10) Roeder, E., and Wiedenfeld, H. (2009) Pyrrolizidine alkaloids in medicinal plants of Mongolia, Nepal and Tibet. *Pharmazie 64*, 699−716.

(11) Smith, L. W., and Culvenor, C. C. (1981) Plant sources of hepatotoxic pyrrolizidine alkaloids. *J. Nat. Prod. 44*, 129−152.

(12) Betz, J. M., Eppley, R. M., Taylor, W. C., and Andrzejewski, D. (1994) Determination of pyrrolizidine alkaloids in commercial comfrey products (Symphytum sp.). *J. Pharm. Sci. 83*, 649−653.

(13) Edgar, J. A., Roeder, E., and Molyneux, R. J. (2002) Honey from plants containing pyrrolizidine alkaloids: a potential threat to health. *J. Agric. Food Chem. 50*, 2719−2730.

(14) (1989) *Human Health Implications of Pyrrolizidine Alkaloids and Herbs Containing Them* (Huxtable, R. J., Ed.) Vol. *1*, CRC Press Inc., Boca Raton, FL.

(15) Huxtable, R. J. (1980) Herbal teas and toxins: novel aspects of pyrrolizidine poisoning in the United States. *Perspect. Biol. Med. 24*, 1−14.

(16) Stegelmeier, B. L., Edgar, J. A., Colegate, S. M., Gardner, D. R., Schoch, T. K., Coulombe, R. A., and Molyneux, R. J. (1999) Pyrrolizidine alkaloid plants, metabolism and toxicity. *J. Nat. Toxins 8*, 95−116.

(17) Schoental, R., head, M. A., and Peacock, P. R. (1954) Senecio alkaloids: primary liver tumours in rats as a result of treatment with (1) a mixture of alkaloids from *S. jacobaea* lin.; (2) retrorsine; (3) isatidine. *Br. J. Cancer 8*, 458−465.

(18) IARC (1976) Monocrotaline. *IARC Monogr. Eval. Carcinog. Risk Chem. Man 10*, 291−302.

(19) Chan, P. C., Haseman, J. K., Prejean, J. D., and Nyska, A. (2003) Toxicity and carcinogenicity of riddelliine in rats and mice. *Toxicol. Lett. 144*, 295−311.

(20) Galloway, S. M., Armstrong, M. J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A. D., Nakamura, F., Ahmed, M., Duk, S., et al. (1987) Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. *Environ. Mol. Mutagen. 10*, 1−175.

(21) Li, N., Xia, Q., Ruan, J., Fu, P. P., and Lin, G. (2011) Hepatotoxicity and tumorigenicity induced by metabolic activation of pyrrolizidine alkaloids in herbs. *Curr. Drug Metab. 12*, 823−834.

(22) (2011) *NTP 12th Report on Carcinogens (RoC)*, U.S. Department of Health and Human Services.

(23) Cooper, R. A., and Huxtable, R. J. (1996) A simple procedure for determining the aqueous half-lives of pyrrolic metabolites of pyrrolizidine alkaloids. *Toxicon 34*, 604−607.

(24) Lin, G., Wang, J. Y., Li, N., Li, M., Gao, H., Ji, Y., Zhang, F., Wang, H., Zhou, Y., Ye, Y., Xu, H. X., and Zheng, J. (2011) Hepatic sinusoidal obstruction syndrome associated with consumption of Gynura segetum. *J. Hepatol. 54*, 666−673.

(25) Coulombe, R. A., Jr., Drew, G. L., and Stermitz, F. R. (1999) Pyrrolizidine alkaloids crosslink DNA with actin. *Toxicol. Appl. Pharmacol. 154*, 198−202.

(26) Hincks, J. R., Kim, H. Y., Segall, H. J., Molyneux, R. J., Stermitz, F. R., and Coulombe, R. A. (1991) DNA cross-linking in mammalian cells by pyrrolizidine alkaloids: structure-activity relationships. *Toxicol. Appl. Pharmacol. 111*, 90−98.

(27) Kim, H. Y., Stermitz, F. R., and Coulombe, R. A. (1995) Pyrrolizidine alkaloid-induced DNA-protein cross-links. *Carcinogenesis 16*, 2691−2697.

(28) Kim, H. Y., Stermitz, F. R., Li, J. K., and Coulombe, R. A. (1999) Comparative DNA cross-linking by activated pyrrolizidine alkaloids. *Food Chem. Toxicol. 37*, 619−625.

(29) Lin, G., Cui, Y. Y., and Hawes, E. M. (1998) Microsomal formation of a pyrrolic alcohol glutathione conjugate of clivorine. Firm evidence for the formation of a pyrrolic metabolite of an otonecine-type pyrrolizidine alkaloid. *Drug Metab. Dispos. 26*, 181−184.

(30) Chou, M. W., Yan, J., Nichols, J., Xia, Q., Beland, F. A., Chan, P. C., and Fu, P. P. (2003) Correlation of DNA adduct formation and riddelliine-induced liver tumorigenesis in F344 rats and B6C3F(1) mice. *Cancer Lett. 193*, 119−125.

(31) Chou, M. W., Yan, J., Williams, L., Xia, Q., Churchwell, M., Doerge, D. R., and Fu, P. P. (2003) Identification of DNA adducts derived from riddelliine, a carcinogenic pyrrolizidine alkaloid, *in vitro* and *in vivo*. *Chem. Res. Toxicol. 16*, 1130−1137.

(32) Xia, Q., Chou, M. W., Kadlubar, F. F., Chan, P. C., and Fu, P. P. (2003) Human liver microsomal metabolism and DNA adduct formation of the tumorigenic pyrrolizidine alkaloid, riddelliine. *Chem. Res. Toxicol. 16*, 66−73.

(33) Yan, J., Nichols, J., Yang, Y. C., Fu, P. P., and Chou, M. W. (2002) Detection of riddelliine-derived DNA adducts in blood of rats fed riddelliine. *Int. J. Mol. Sci. 3*, 1019−1026.

(34) Yang, Y. C., Yan, J., Doerge, D. R., Chan, P. C., Fu, P. P., and Chou, M. W. (2001) Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation in vivo. *Chem. Res. Toxicol. 14*, 101−109.

(35) Zhao, Y., Xia, Q., Gamboa da Costa, G., Yu, H., Cai, L., and Fu, P. P. (2012) Full structure assignments of pyrrolizidine alkaloid DNA adducts and mechanism of tumor initiation. *Chem. Res. Toxicol. 25*, 1985−1996.
(36) Xia, Q., Zhao, Y., Von Tungeln, L. S., Doerge, D. R., Lin, G., Cai, L., and Fu, P. P. (2013) Pyrrolizidine alkaloid-derived DNA adducts as a common biological biomarker of pyrrolizidine alkaloid-induced tumorigenicity. *Chem. Res. Toxicol. 26*, 1384−1396.
(37) Hsu, I. C., Robertson, K. A., and Allen, J. R. (1976) Tissue distribution, binding properties and lesions produced by dehydroretronecine in the nonhuman primate. *Chem.−Biol. Interact. 12*, 19−28.
(38) Hsu, I. C., Robertson, K. A., Shumaker, R. C., and Allen, J. R. (1975) Binding of tritiated dehydroretronecine to macromolecules. *Res. Commun. Chem. Pathol. Pharmacol. 11*, 99−106.
(39) Mattocks, A. R., Jukes, R., and Brown, J. (1989) Simple procedures for preparing putative toxic metabolites of pyrrolizidine alkaloids. *Toxicon 27*, 561−567.
(40) Zhao, Y., Xia, Q., Yin, J. J., Lin, G., and Fu, P. P. (2011) Photoirradiation of dehydropyrrolizidine alkaloids—formation of reactive oxygen species and induction of lipid peroxidation. *Toxicol. Lett. 205*, 302−309.
(41) von Stedingk, H., Vikstrom, A. C., Rydberg, P., Pedersen, M., Nielsen, J. K., Segerback, D., Knudsen, L. E., and Tornqvist, M. (2011) Analysis of hemoglobin adducts from acrylamide, glycidamide, and ethylene oxide in paired mother/cord blood samples from Denmark. *Chem. Res. Toxicol. 24*, 1957−1965.
(42) Antunes, A. M., Godinho, A. L., Martins, I. L., Justino, G. C., Beland, F. A., and Marques, M. M. (2010) Amino acid adduct formation by the nevirapine metabolite, 12-hydroxynevirapine—a possible factor in nevirapine toxicity. *Chem. Res. Toxicol. 23*, 888−899.
(43) Fennell, T. R., Snyder, R. W., Krol, W. L., and Sumner, S. C. (2003) Comparison of the hemoglobin adducts formed by administration of *N*-methylolacrylamide and acrylamide to rats. *Toxicol. Sci. 71*, 164−175.
(44) Robertson, K. A., Seymour, J. L., Hsia, M. T., and Allen, J. R. (1977) Covalent interaction of dehydroretronecine, a carcinogenic metabolite of the pyrrolizidine alkaloid monocrotaline, with cysteine and glutathione. *Cancer Res. 37*, 3141−3144.
(45) Estep, J. E., Lame, M. W., Jones, A. D., and Segall, H. J. (1990) *N*-Acetylcysteine-conjugated pyrrole identified in rat urine following administration of two pyrrolizidine alkaloids, monocrotaline and senecionine. *Toxicol. Lett. 54*, 61−69.
(46) Törnqvist, M., Fred, C., Haglund, J., Helleberg, H., Paulsson, B., and Rydberg, P. (2002) Protein adducts: quantitative and qualitative aspects of their formation, analysis and applications. *J. Chromatogr. B 778*, 279−308.
(47) Farmer, P. B. (1994) Carcinogen adducts: use in diagnosis and risk assessment. *Clin. Chem. 40*, 1438−1443.
(48) Mattocks, A. R., and Jukes, R. (1990) Recovery of the pyrrolic nucleus of pyrrolizidine alkaloid metabolites from sulphur conjugates in tissues and body fluids. *Chem.−Biol. Interact 75*, 225−239.