Original Article

Absolute configuration, stability, and interconversion of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine valine adducts and their phenylthiohydantoin derivatives

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ABSTRACT

Pyrrolizidine alkaloid-containing plants are widespread in the world and probably the most common poisonous plants affecting livestock, wildlife, and humans. Pyrrolizidine alkaloids require metabolic activation to form dehydropyrrolizidine alkaloids that bind to cellular proteins and DNA leading to hepatotoxicity, genotoxicity, and tumorigenicity. At present, it is not clear how dehydropyrrolizidine alkaloids bind to cellular amino acids and proteins to induced toxicity. We previously reported that reaction of dehydrodromonocrotaline with valine generated four highly unstable 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)-derived valine (DHP-valine) adducts that upon reaction with phenyl isothiocyanate (PITC) formed four DHP-valine-PITC adduct isomers. In this study, we report the absolute configuration and stability of DHP-valine and DHP-valine-PITC adducts, and the mechanism of interconversion between DHP-valine-PITC adducts.

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1. Introduction

Pyrrolizidine alkaloids are common phytochemical constituents of hundreds of plant species worldwide [1-6] and about half of them exhibit hepatotoxic activity [2,5]. Pyrrolizidine alkaloids are among the first naturally occurring carcinogens identified in plants, and pyrrolizidine alkaloid-containing plants are probably the most common type of poisonous plants affecting livestock, wildlife, and humans [2,5]. Humans are exposed to toxic pyrrolizidine alkaloids through staple foods, food contaminants, herbal teas, herbal medicines, and dietary supplements [2,5,7-12].

Pyrrolizidine alkaloids are biologically inert, requiring metabolic activation to generate dehydropyrrolizidine alkaloids that bind to cellular proteins and DNA, leading to toxicity, including cytotoxicity, genotoxicity, and tumorigenicity [2,5]. Dehydropyrrolizidine alkaloids are highly chemically and biologically reactive, easily hydrolyze to (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP), react with cellular proteins to form DHP-protein adducts, and bind to DNA to produce DHP-DNA adducts (Fig. 1) [2,5,12]. We previously demonstrated that metabolism of different types of tumorigenic pyrrolizidine alkaloids generated the same set of DHP-derived DNA adducts in vivo, but that these DNA adducts were not formed from the nontumorigenic pyrrolizidine alkaloids [6,13,14]. These results suggest that this set of DNA adducts can serve as a common biological biomarker of pyrrolizidine alkaloid-induced tumorigenicity and exposure [14]. Taking monocrotaline as an example, Fig. 1 shows the metabolic activation to form a dehydropyrrolizidine alkaloid that binds to cellular proteins and DNA leading to the formation of DHP-protein and DHP-DNA adducts and initiate cytotoxicity, genotoxicity, and tumorigenicity.

It has been proposed that DHP-protein adducts formed in vivo in the liver are responsible for pyrrolizidine alkaloid-induced cytotoxicity, specifically hepatotoxicity [2,5]. However, due to their highly unstable nature, the structures of DHP-protein adducts have never been characterized at the molecular level. With regard to DHP-amino acid adducts, 7-cysteine-DHP [15] and DHP-valine [16] are the only two DHP-amino acid adducts that have had their structures fully elucidated. 7-Cysteine-DHP was prepared from the reaction of dehydroretronecine with cysteine [15]. DHP-valine adducts, consisting of four structural isomers, were prepared by the reaction of valine with dehydropyrrolizidine alkaloids, including dehydromonocrotaline, dehydroriddelliine, and dehydroheliotrine (Fig. 2) [16]. For structural identification, DHP-valine adducts were converted to the corresponding isothiohydantoin (DHP-valine-PITC) derivatives by reacting with phenyl isothiocyanate (PITC) [16]. DHP-valine-PITC adducts were also found from the reaction of hemoglobin-DHP adducts with PITC through an Edman rearrangement, which is the most commonly employed method for quantitation of metabolite-bound protein adducts in vivo [17]. Since DHP-valine-PITC adducts can potentially be used for quantitation of pyrrolizidine alkaloid bound protein adducts in vivo, it is important to study their chemical properties associated with pyrrolizidine alkaloid-induced toxicology.

We previously determined that DHP-valine adducts formed from the above-described reactions consist of two pairs of epimeric isomers [16]. The first pair, designated as DHP-valine-1 and DHP-valine-3, has the amino group of valine linked to the C9 position of the necine base, and the other pair, DHP-valine-2 and DHP-valine-4, has the amino group of valine linked to the C7 position of the necine base (Fig. 2) [16]. We have also determined that DHP-valine-PITC-1 was interconvertible with DHP-valine-PITC-3, and that...

![Fig. 1 – Metabolic activation of monocrotaline to form dehydromonocrotaline, 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)-protein adduct and DHP-DNA adducts leading to cytotoxicity, genotoxicity, and tumorigenicity.](image-url)
DHP-valine-PITC-2 was interconvertible with DHP-valine-PITC-4.

As a continuation of our investigation, in this paper we determine absolute configurations of each of the DHP-valine and DHP-valine-PITC adducts, study the stability of DHP-valine and DHP-valine-PITC adducts, and elucidate the mechanism of the interconversion between DHP-valine-PITC epimers.

2. Materials and methods

2.1. Chemicals

Monocrotaline was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylformamide (DMF) and PITC were obtained from Fisher Scientific (Pittsburg, PA, USA). Acetonitrile, potassium carbonate, chloroform, and diethyl ether were purchased from Bio-Rad (Hercules, CA, USA). Dehydromonocrotaline was synthesized by dehydrogenation of monocrotaline in chloroform with o-chloranil as previously described [18,19]. H\textsuperscript{18}O (isotopic purity 97%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Alkaline phosphatase was acquired from Roche Diagnostics Corporation (Indianapolis, IN, USA).

2.2. Synthesis of DHP-valine and DHP-valine-PITC adducts

DHP-valine-PITC adducts were prepared following previously published procedures [16]. Briefly, a solution of valine (10 mg) and K\textsubscript{2}CO\textsubscript{3} (10 mg) in 400 \(\mu\)L distilled water and 800 \(\mu\)L DMF was sonicated until the solution turned clear, and then added drop-wise into a solution of dehydromonocrotaline (10 mg) in 1200 \(\mu\)L of DMF. After 2 hours, the reaction mixture was filtered through a 0.4 \(\mu\)m regenerated cellulose membrane, and products were purified by high-performance liquid chromatography (HPLC) with a 250 mm \(\times\) 10 mm Prodigy column (Phenomenex, Torrance, CA, USA) with eluting conditions: 0–5 minutes: 2% acetonitrile in water; 5–45 minutes: 2–8% acetonitrile in water at a flow rate of 1 mL/min, and monitored by UV absorbance at 218 nm.

The resulting DHP-valine adducts mixture dissolved in 5 mL of acetonitrile was reacted with PITC (10 \(\mu\)L) under shaking in a water bath at 45 °C for 48 hours. The resulting isothiohydantoin adducts (DHP-valine-PITC) were purified by HPLC with a Phenomenex C18 Luna (2) column (250 mm \(\times\) 4.6 mm) eluted with acetonitrile in water (v/v, 43/57) at a flow rate of 1 mL/min, and monitored at 268 nm. Each DHP-valine-PITC adduct was characterized by comparison of its UV absorption spectrum, HPLC retention time, mass spectrometric data with those of the previously prepared authentic sample [16].

For determination of the absolute configuration of the four DHP-valine adducts, the adducts were synthesized by a similar reaction of dehydromonocrotaline with valine.

2.3. Mechanistic studies of interconversion between DHP-valine-PITC-1 and DHP-valine-PITC-3 adducts and between DHP-valine-PITC-2 and DHP-valine-PITC-4

To determine the mechanism of the interconversion between DHP-valine-PITC-1 and DHP-valine-PITC-3, DHP-valine-PITC-3 (~200 \(\mu\)g), dissolved in 100 \(\mu\)L acetonitrile/H\textsuperscript{2}O\textsuperscript{18} (v/v, 43/57), was incubated at room temperature for 2 days. The resulting DHP-valine-PITC-1 and DHP-valine-PITC-3 adducts were separated by HPLC. HPLC conditions were: ACE C18 AR column (4.6 mm \(\times\) 250 mm, 5 \(\mu\)m; Mac-Mod Analytical, Inc., Chadds Ford, PA, USA); gradient program: 0–5 minutes: 10% acetonitrile in H\textsubscript{2}O; 5–50 minutes: 10–18% acetonitrile in H\textsubscript{2}O; with a flow rate of 1 mL/min, and monitored at 268 nm. The resulting DHP-valine-PITC-1 and DHP-valine-PITC-3 adducts were collected by HPLC for liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis.

The study of interconversion between DHP-valine-PITC-2 and DHP-valine-PITC-4 was studied similarly by mixing DHP-valine-PITC-4 (~200 \(\mu\)g) with 100 \(\mu\)L acetonitrile/H\textsuperscript{2}O\textsuperscript{18} (v/v, 43/57) at room temperature for 2 days, and the resulting DHP-valine-PITC-2 and DHP-valine-PITC-4 adducts were first separated by HPLC and followed by LC/MS/MS analysis.
2.4. LC/MS/MS analysis of DHP-valine-PITC adducts

DHP-valine-PITC adducts were analyzed by LC/MS/MS. The LC 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-visible detector (Shimadzu Scientific Instrument, Columbia, MD, USA), and an automated switching valve (TPMV; Rheodyne, Cotati, CA, USA) used to divert the column effluent to either waste or to MS instrument. The HPLC was coupled with an AB Sciex 4000 QTrap LC/MS/MS system (AB Sciex, Foster City, CA, USA), equipped with a Turbo V ion source. Desolvation temperature was set at 500°C. Nitrogen was used as curtain gas, nebulizer gas, heater gas, and collision gas. The samples were acquired in positive IonSpray mode using MS full scan and enhanced product ion scan. The IonSpray Voltage was 4500 V; the ion source gas 1 and gas 2 were set to 60 and 50, respectively. The declustering potential was 50 V; and the collision energy was 25 eV [16].

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, USA), with a water/acetonitrile gradient at 0.2 mL/min. The column chamber temperature was set to 45°C. The mobile phase program: water, 0.5 minutes; a linear gradient up to 15% acetonitrile in water over 50 minutes; up to 95% in water, 1.5 minutes; and 95% acetonitrile in water, 5 minutes [16].

3. Results

3.1. Absolute configuration of DHP-valine products

We previously studied the reaction of dehydromonocrotaline with valine using two different reaction times, 30 minutes and 48 hours; and under both conditions, DHP-valine-1, DHP-valine-2, DHP-valine-3, and DHP-valine-4 adducts were formed [16]. These four DHP-valine adducts are structural isomers and their UV-visible absorption spectra are mainly attributed from the necine moiety. Thus, it is reasonable to assume that they have an identical or nearly identical UV-visible absorption molar extinction coefficient. Consequently, based on HPLC peak areas of these adduct products, the relative yields of DHP-valine adducts obtained from the above-described reaction can be compared.

However, from the reaction of 30 minutes, all the HPLC peak areas for each of DHP-valine adducts [16] are too small to compare, and thus, the relative yield of these adducts could not be determined. Further, the results from the reaction of 48 hours could not be used for determining which stereoisomer is formed predominantly because interconversion between isomers would occur during reaction. Therefore, in order to determine the absolute configuration of these adducts, we conducted the reaction of dehydromonocrotaline with valine for 2 hours.

Previously we determined that both DHP-valine-1 and DHP-valine-3 have the valine moiety linked to the C9 position of the DHP base and the hydroxyl group at the C7 position (Fig. 2), whereas DHP-valine-2 and DHP-valine-4 have the valine moiety linked at the C7 position of the DHP base, and the hydroxyl group sited at the C9 position (Fig. 2) [16].

As the HPLC chromatogram in Fig. 3 shows, the HPLC peak area of the DHP-valine-3 is greater than that of DHP-valine-1, indicating that the yield of DHP-valine-3 is greater than DHP-valine-1. Since both DHP-valine-1 and DHP-valine-3 were produced, the reaction of dehydromonocrotaline with valine should not be a pure S<sub>n</sub>2 mechanism because an S<sub>n</sub>2 mechanism produces only one enantiomer product. It is not a pure S<sub>n</sub>1 mechanism either, because such a mechanism would produce both enantiomers in an equal or nearly equal quantity. Since it is apparent that the formation of DHP-valine-1 and DHP-valine-3 adducts occurred in an unequal ratio, this suggests that adduct formation involves a combination of S<sub>n</sub>1 and S<sub>n</sub>2 mechanisms. An S<sub>n</sub>2 reaction produces the product with its absolute configuration opposite to the substrate at the reaction site, e.g., in this case the C7 position. Thus, since dehydromonocrotaline, the starting material of the reaction possesses a 7R absolute configuration [2,5], the major product DHP-valine-3, should possess a 7S absolute configuration; and the minor product DHP-valine-1 should have a 7R absolute configuration.

Similarly, for the enantiomer pair DHP-valine-2 and DHP-valine-4, the predominant product DHP-valine-2 should have a 7S absolute configuration; and DHP-valine-4, the minor product, holds a 7R absolute configuration. The assigned absolute configurations of these DHP-valine adducts are shown in Fig. 2.

3.2. Absolute configuration of phenylthiohydantoin (DHP-valine-PITC) adducts

Following the experimental procedure previously described [16], the DHP-valine adduct reaction mixture was reacted with PITC. The resulting phenylthiohydantoin adducts, DHP-valine-PITC-1, DHP-valine-PITC-2, DHP-valine-PITC-3, and DHP-valine-PITC-4, were separated by reversed-phase HPLC (Fig. 4). These adducts eluted at 17.1 minutes, 25.3 minutes, 27.2 minutes, and 29.8 minutes; and each had its UV absorption spectrum and mass spectral pattern, with preponderant ions at m/z 392 and ions at m/z 352 corresponding to the loss of water by the protonated adduct ([M-H2O+H]+), identical to those previously reported [16].

From each reaction using a pure DHP-valine adduct to react with PITC, two phenylthiohydantoin (DHP-valine-PITC) adducts were generated. The structures of the predominant products from each reaction are shown in Fig. 2. These results are identical to those previously published [16]. As shown in Fig. 2, the corresponding predominant products from DHP-valine-1, DHP-valine-2, DHP-valine-3, and DHP-valine-4 are DHP-valine-PITC-4, DHP-valine-PITC-1, DHP-valine-PITC-2, and DHP-valine-PITC-3, respectively. Since, in each DHP-valine reaction, no chemical bonds of the necine base were involved (broken) in the reaction, each predominant DHP-valine-PITC adduct should retain the same absolute configuration as that of the DHP-valine starting material. The absolute configurations of each DHP-valine-PITC are shown in Fig. 2.

3.3. Interconversion of DHP-valine-PITC-3 to DHP-valine-PITC-1 adduct in the presence of H218O

To determine the mechanism of interconversion between DHP-valine-PITC-1 and DHP-valine-PITC-3, DHP-valine-PITC-1...
3 was dissolved in acetonitrile/H$_2$O (v/v, 43/57) and kept at room temperature for 2 days. The resulting DHP-valine-PITC-1 adduct was collected and analyzed by LC/MS. The mass spectrum of the unlabeled DHP-valine-PITC-1 had characteristic ions at m/z 352 ([M - H$_2$O + H]), m/z 392 ([M + Na]), and m/z 136 ([DHP-OH]) (Fig. 5). The mass spectrum of the [18O] DHP-valine-PITC-1 had characteristic ions at m/z 354 ([M - H$_2$O + H]), m/z 394 ([M + Na]), and m/z 138 ([DHP-18OH]) (Fig. 5). Comparison of the mass of these fragments ions and the assigned structures in Fig. 5 indicates that the 18O atom is specifically located at the 9-hydroxyl group of the necine base.

3.4. Interconversion of DHP-valine-PITC-4 to DHP-valine-PITC-2 adduct in the presence of H$_2$O$_{18}$

Similarly, DHP-valine-PITC-4 was dissolved in acetonitrile/H$_2$O$_{18}$ (v/v, 43/57), kept at room temperature for 2 days, and the resulting adducts were analyzed by LC-MS. The mass spectrum of the unlabeled DHP-valine-PITC-2 had characteristic ions at m/z 352 ([M - H$_2$O + H]), m/z 392 ([M + Na]), and m/z 136 ([DHP-OH]) (Fig. 5). The mass spectrum of the [18O] DHP-valine-PITC-2 had characteristic ions at m/z 354 ([M - H$_2$O + H]), m/z 394 ([M + Na]), and m/z 138 ([DHP-18OH]) (Fig. 5). Comparison of the mass of these fragments ions and the assigned structures in Fig. 5 indicates that the 18O atom is specifically located at the 9-hydroxyl group of the necine base.

4. Discussion

To date, none of the pyrrolizidine alkaloid-derived protein adducts have had their structures characterized at the molecular level. With regard to DHP-derived amino acid adducts, 7-cysteine-DHP and DHP-valine are the only DHP-amino acid adducts that their structures are fully elucidated [15,16]. There are four DHP-valine adduct isomers, and their structures were characterized by spectral analysis of their corresponding phenylthiohydantoin derivatives, DHP-valine-PITC.
adducts, which are formed from reaction of DHP-valine with PITC. The most commonly employed method for identification and quantitation of metabolite-bound protein adducts in vivo is the reaction of hemoglobin adducts with PITC. In this reaction, the PITC interacts at the valine terminal amino group of the hemoglobin adducts, resulting in the replacement of the hemoglobin moiety by the phenylthiohydantoin group [17]. Thus, for identification of pyrrolizidine alkaloid-derived protein adducts, e.g., DHP-hemoglobin adducts, DHP-valine-PITC will be formed from the reaction of DHP-hemoglobin adducts with PITC. Consequently, our current study on the properties of DHP-valine-PITC adducts provides a better understanding of the feasibility of using this approach for identification and quantitation of DHP-hemoglobin adducts in vivo.

In this study, we determined that DHP-valine-PITC-2 to DHP-valine-PITC-4 to give an equal ratio required only 3 hours; while the interconversion of DHP-
valine-PITC-4 to DHP-valine-PITC-2 to give an equal ratio required 7 hours [16]. By contrast, the interconversion of DHP-valine-PITC-1 to give a nearly equal ratio of DHP-valine-PITC-3 took 3 days; and the interconversion of DHP-valine-PITC-3 to give a nearly equal ratio of DHP-valine-PITC-1 took 7 days [16]. Thus, the interconversion between DHP-valine-PITC-2 and DHP-valine-PITC-4 is much faster than the interconversion between DHP-valine-PITC-1 and DHP-valine-PITC-3 adducts. These results suggest that the stability of these adducts is in the order: DHP-valine-PITC-3 > DHP-valine-PITC-1 > DHP-valine-PITC-4 > and DHP-valine-PITC-2.

The interconversion between DHP-valine-PITC-2 and DHP-valine-PITC-4 requires the dissociation/association of the hydroxyl group at the C7 position, while the interconversion of DHP-valine-PITC-1 and DHP-valine-PITC-3 adducts involves the dissociation/association of the phenylthiohydantoin.
group at the C7 position. These results suggest that DHP-valine-PITC adducts with the bulky phenylthiohydantoin group sited at the C7 position (vs. the C9 position) are more stable than the isomers possessing a hydroxyl group sited at the C7 position.

We also analyzed the polarity of these adducts by comparison of their HPLC retention times shown in Fig. 4 and nuclear magnetic resonance chemical shifts reported previously [16]. These data are listed in Table 1. Based on the comparison of their HPLC retention times and nuclear magnetic resonance chemical shifts, we propose that DHP-valine-PITC-1 is more polar than DHP-valine-PITC-3; and that DHP-valine-PITC-2 is more polar than DHP-valine-PITC-4 (Table 1).

In the present study, we determine the mechanism of interconversion by dissolving DHP-valine-PITC-4 in acetoni-

triene/H$_2^{18}$O. LC/MS spectral analysis revealed that the resulting DHP-valine-PITC-2 contained $^{18}$O at its C7 hydroxyl group (Fig. 6). These results indicate that during interconversion, the hydroxyl group at the C7 position dissociates from the molecule and is replaced by an $^{18}$O-containing hydroxyl group from the H$_2^{18}$O. Based on these results, we propose that this interconversion requires only one transaction reaction (Fig. 7).

From the reaction of DHP-valine-PITC-3 dissolved in the presence of H$_2^{18}$O, the resulting DHP-valine-PITC-1 adduct also had the C9 hydroxyl group contain $^{18}$O. Thus, this interconversion involves at least two separate transitions: (1) formation of a carbonium ion at the C9 position and then regenerate the C9 hydroxyl group by reaction with H$_2^{18}$O, resulting in the C9 $^{18}$O-containing hydroxyl group; and (2) dissociation and association of the proton at the C7 position (Fig. 7). The proposed mechanism may also interpret that interconversion between DHP-valine-PITC-1 and DHP-valine-3 takes a longer time than that of DHP-valine-PITC-2 and DHP-valine-4.

**Table 1** Comparison of chemical properties of enantiomers between 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)-valine-phenyl isothiocyanate (PITC)-1 and DHP-valine-PITC-3 and between DHP-valine-PITC-2 and DHP-valine-PITC-4.

| Absolute configuration | DHP-valine-PITC-1 | DHP-valine-PITC-3 | DHP-valine-PITC-2 | DHP-valine-PITC-4 |
|------------------------|-------------------|-------------------|-------------------|-------------------|
| Polarity               | More polar        | Less polar        | More polar        | Less polar        |
| HPLC retention time (Difference) | 17.1 min (10.1 min) | 27.2 min | 25.3 min (4.5 min) | 29.8 min |
| NMR chemical shift (Difference) | 6.41–6.44 ppm (~0.33 ppm) | 6.08 ppm | 5.19–5.20 ppm (~0.08 ppm) | 5.11–5.12 ppm |
| Stability interconversion time (reach to equal ratio) | 3 d | More stable | 6 d | More stable |

HPLC = high-performance liquid chromatography; NMR = nuclear magnetic resonance.

![Fig. 7 – Proposed mechanism of interconversion between 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)-valine-phenyl isothiocyanate (PITC)-1 and DHP-valine-PITC-3 and between DHP-valine-PITC-2 and DHP-valine-PITC-4.](image-url)
Conflicts of interest

The authors declare no competing financial interest.

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