Characterization of Two Structural Forms of Otonecine-Type Pyrrolizidine Alkaloids from *Ligularia hodgsonii* by NMR Spectroscopy

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Clivorine (**1**) and ligularine (**2**), two hepatotoxic otonecine-type pyrrolizidine alkaloids isolated from *Ligularia hodgsonii*, an antitussive traditional Chinese medicine, were investigated in $CDCl_3$ and D_2O by various NMR techniques to delineate why this type of alkaloid displays uncharacteristic solubility properties by dissolving in both nonpolar organic and aqueous solutions. The results demonstrated that both alkaloids exist in a non-ionized form in $CDCl_3$, but in an ionized form in D_2O, suggesting that this unique dual solubility may play a role in the intoxication resultant from consumption of water extracts of herbs, including herbal teas, containing otonecine-type pyrrolizidine alkaloids.

Pyrrolizidine alkaloids (PAs) are generally esters composed of amino alcohol and acid components termed the necine base and necic acid, respectively (Figure 1), and are found in a wide variety of plant species worldwide. They are a major cause of human and animal poisonings through consumption of PA-containing plants, vegetables, herbal teas, and herbal remedies. PAs having a structure with an 1,2-unsaturated necine base are hepatotoxic, causing veno-occlusive disease of the liver, and many of them are also carcinogenic. In addition, certain unsaturated PAs are known to induce pulmonary arterial hypertension in experimental and farm animals.[1−6]

Toxic 1,2-unsaturated PAs are generally classified into two types according to the structure of the necine base: retronecine-type [or heliotridine-type, a 7(*S*)-isomer of 7(*R*)-retronecine] and otonecine-type with necine bases with dehydropyrrolidino[1,2-*a*]pyrrolidine and hexahydroazocine heterocyclic ring systems, respectively (Figure 1). In previous studies on PA metabolism-induced hepatotoxicity[7] and its prevention,[8] we observed that, whereas retronecine-type PAs dissolved only in organic solvents, the available otonecine-type PAs dissolved in both nonpolar organic solvents and aqueous solutions. Consequently, the good aqueous solubility of the latter type PA may give an explanation for health problems that arise from the general practice of ingestion of otonecine-type PA-containing herbal remedies by means of oral consumption of the aqueous decoction.

Previous IR and X-ray crystallographic analyses of the otonecine-type PAs have suggested that there is a transannular interaction between N-4 and C-8 in the necine base such that they may exist in a charged form.[9−12] Therefore, their dual solubility in both organic and aqueous solutions may be mainly due to structural variation of the necine base. However, there is no definitive study to prove that otonecine-type PAs exist in two different non-ionized lipophilic and ionized hydrophilic forms in different media.

Two otonecine-type PAs, clivorine (**1**) and ligularine (**2**), isolated from *Ligularia hodgsonii* Hook. (family, Compositae), an antitussive traditional Chinese medicine, were utilized for the present study. Both these PAs have been reported to affect toxicity; for example, **1** has shown mutagenic,[13] genotoxic,[14] and carcinogenic[15] activities. The two PAs were examined in a nonpolar organic solvent ($CDCl_3$) and aqueous solution (D_2O) by various one- and two-dimensional ^1H and ^{13}C NMR techniques. The data obtained clearly demonstrated that the non-ionized, monocyclic ring form of the necine base existed when these two alkaloids were dissolved in $CDCl_3$, whereas the ionized, bicyclic ring form of the necine base existed when they were dissolved in D_2O.

The one- and two-dimensional ^1H and ^{13}C NMR spectra of **1** were obtained in both $CDCl_3$ and D_2O. The pertinent data for distinguishing the two structural forms of **1** in the different solvents are summarized in Table 1, and chemical shift data assignments for all hydrogen and carbon atoms are available in the Supporting Information. The correlation between hydrogen and carbon atoms was determined on the basis of direct correlation HMQC spectra in both $CDCl_3$ and D_2O. The assignments of the ^1H and ^{13}C NMR signals in $CDCl_3$ were consistent with previously reported data obtained in either $CDCl_3$ for ^1H resonances[16] or deuterated dioxane for ^{13}C resonances.[11,16] Using $CDCl_3$ as solvent, the ^{13}C NMR spectrum showed a C-8 signal at δ 192.17, which corresponded to a carbonyl group of a typical α,β-unsaturated ketone. These data indicate that in $CDCl_3$ **1** exists in the non-ionized form (Figure 1). In contrast, in D_2O solution, the resonance of C-8 significantly shifted upfield to δ 145.14, thereby indicating that in this solvent C-8 exists as an atypical carbonyl carbon atom with a negatively charged oxygen that produces a shielding effect on C-8. In addition, the chemical shift of C-22 of the methyl group attached to the cyclic nitrogen atom markedly shifted downfield from δ 40.09 (in $CDCl_3$) to δ 45.74 (in D_2O). This suggested a significant deshielding effect of the C-22 atom mainly due to a positive charge located at the nitrogen atom. Furthermore, the protons adjacent to the nitrogen atom appeared further upfield in $CDCl_3$ (δ 3.35 and 3.17 for 2 × H-3; δ 2.84−2.90 and 2.70 for 2 × H-5; δ 2.04 for H-22) than in D_2O (δ 3.82 and 3.69 for 2 × H-3; δ 3.39 and 3.21 for 2 × H-5; δ 2.40 for H-22). Similarly, the signals

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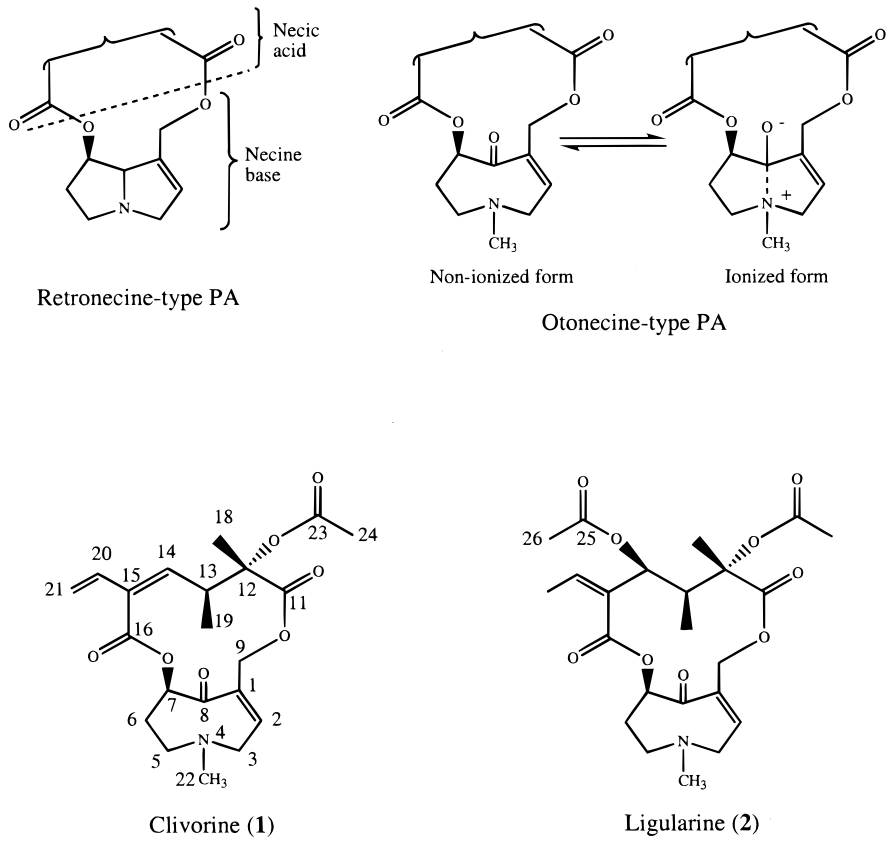

Figure 1. Structures of retronecine-type PA, otonecine-type PA, clivorine (**1**), and ligularine (**2**).

Table 1. Selective Chemical Shifts of Clivorine (**1**) and Ligularine (**2**)

	δ_H		δ_C[a]		published data[b]	
position	($CDCl_3$)	(D_2O)	($CDCl_3$)	(D_2O)	δ_H ($CDCl_3$)	δ_C (*d*-dioxane)
		Clivorine (**1**)				
3	3.35 (1H, d, J = 18.3 Hz)	3.82 (1H, d, J = 17.1 Hz)	58.80 (2)	66.71 (2)	N/A	58.7
	3.17 (1H, d, J = 18.3 Hz)	3.69 (1H, dd, J = 17.2, 2.2 Hz)				
5	2.84−2.90 (1H, m)	3.39 (1H, m)	53.26 (2)	60.82 (2)	N/A	53.4
	2.70 (1H, m)	3.21 (1H, m)				
8			192.17 (0)	145.14 (0)		194.8
22	2.04 (3H, s)	2.40 (3H, s)	40.09 (3)	45.74 (3)	2.07	41.4
		Ligularine (**2**)				
3	3.55 (1H, m)	3.91 (1H, m)	58.75 (2)	64.45 (2)	3.60	N/A
	3.29 (1H, m)	3.78 (1H, m)			3.30	N/A
5	2.91−2.80 (2H, m)	3.36−3.25 (2H, m)	53.06 (2)	57.76 (2)	2.9	N/A
8			191.72 (0)	141.29 (0)		N/A
22	2.16 (3H, s)	2.42 (3H, s)	40.36 (3)	43.48 (3)	2.145	N/A

[a] Numbers of hydrogen atoms attached, indicated in parentheses, were determined by ^{13}C DEPT NMR spectra. [b] ^1H NMR[16] and ^{13}C NMR[11,16] data were cited from references. N/A: not available.

for the cyclic carbons vicinal to the nitrogen atom also showed markedly upfield shifts in $CDCl_3$ (δ 58.80 for C-3; δ 53.26 for C-5) as compared to these same resonances in D_2O (δ 66.71 for C-3; δ 60.82 for C-5). These observed deshielding effects provide further evidence for a positively charged nitrogen atom in the necine base and hence that **1** exists in the ionized form in an aqueous solution (Figure 1).

HMBC long-range correlations between ^{13}C and ^1H of **1** were also investigated using both $CDCl_3$ and D_2O solvents. Whereas many of the long-range correlations were common to both solvents, some were not. Particularly noteworthy is that the three-bonded long-range correlation between C-8 and the proton signal at δ 2.40 ($N-CH_3$) was exclusive to the D_2O solvent data. This latter observation gives further evidence that in D_2O solution **1** exists with a $^-O-C_8-N^+-CH_3$ linkage across the eight-membered ring.

Similar NMR studies were also conducted for **2**. The proton signals obtained in $CDCl_3$ were consistent with published data;[16] however, no ^{13}C NMR data for **2** were reported previously. The relevant data for the elucidation of two different structural forms of **2** are also shown in Table 1. Results similar to those for **1** were obtained with respect to the necine base in the two different solvents. In particular, the resonance for C-8 markedly shifted upfield from δ 191.72 ($CDCl_3$) to δ 141.29 (D_2O), indicating the existence of the non-ionized and ionized form in organic and aqueous solutions, respectively. Also, the deshielding effects produced by the positively charged nitrogen atom in the ionized form of the necine base of **2** were observed in both proton and carbon spectra. Thus, the vicinal carbon atoms had greater downfield chemical shifts in D_2O (δ 64.45 for C-3; δ 57.76 for C-5; δ 43.48 for C-22) than in $CDCl_3$ (δ 58.75 for C-3; δ 53.06 for C-5; δ 40.36 for C-22).

Furthermore, the proton signals for the hydrogen atoms immediately adjacent to the nitrogen atom were further downfield in D_2O (δ 3.91 and 3.78 for 2 × H-3; δ 3.36−3.25 for 2 × H-5; δ 2.42 for H-22) than in $CDCl_3$ (δ 3.55 and 3.29 for 2 × H-3; δ 2.91−2.80 for 2 × H-5; δ 2.16 for H-22). In addition, a long-range correlation between C-8 and the proton signal of $N-CH_3$ (δ 2.42) indicative of the $^{-}O-C_8-N^{+}-CH_3$ linkage was observed only in the HMBC spectrum obtained in D_2O. Therefore, on the basis of the NMR spectroscopic evidence **2** was also shown to exist in two different forms, namely, the non-ionized form in the nonpolar solvent and the ionized form in water.

PA **1** originally isolated from *Ligularia clivorum* Maxim. was structurally identified based on various techniques including MS, IR, NMR, and X-ray crystallography.[11,17] The IR spectrum of **1** showed a stretching band at 1603 cm^{-1} in chloroform, which was absent in the spectrum of its perchloric acid salt.[17] In addition, some of the bond distances determined by X-ray crystallographic analysis were unusual; for example, the C_8═O distance (1.258 Å) was significantly longer than a normal C═O bond length (1.215 Å).[11] Therefore, it was suggested that there is a transannular interaction between the nitrogen atom and C-8 in the structure of **1**. Similar suggestions have also been made previously in view of various X-ray crystallographic studies, regarding the structure of otonecine base[1−3,5,12] and other otonecine-type PAs,[18−21] including **2**, which was originally isolated from *Ligularia elegans* Cass.[10] However, it should be noted that in all these X-ray crystallographic studies the compounds were examined as the dried crystalline form, and consequently, there has been no previous report regarding the dependency of the type of media in determining whether otonecine-type PAs exist in non-ionized or ionized forms.

The present study of the two otonecine-type PAs by ^1H and ^{13}C NMR techniques definitively demonstrated their existence as two structural forms in different media. The NMR spectra of both PAs in chloroform exhibited the chemical shifts for C-8 as a typical α,β-unsaturated carbonyl carbon atom, thereby indicating that the necine base component of the structure exists as a monocyclic eight-membered ring. Therefore, otonecine-type PAs exist in a non-ionized form in lipophilic organic solutions. However, only acid-free $CDCl_3$ produced appropriate ^{13}C NMR spectra, while the signal for C-8 was too broad to be detected in the carbon spectra for both alkaloids using $CDCl_3$ without pretreatment with anhydrous sodium bicarbonate. These observations are particularly indicative of an interaction between the carbonyl carbon in the necine base and trace acid content in $CDCl_3$ and thus give further indication for the dependency of the structure and hence NMR spectra on the nature of the medium. On the other hand, the signals for C-8 in the spectra of the two otonecine-type PAs in D_2O clearly showed a shielding effect due to the interaction between C-8 and the tertiary nitrogen, indicating a negative charge located on C-8, namely, $C-O^{-}$. Furthermore, the significant downfield shifts of the signals corresponding to the carbon and hydrogen atoms most directly adjacent to the nitrogen confirmed the presence of a positively charged nitrogen atom in the necine base. Moreover, a long-range correlation between C-8 and H-22 observed in the HMBC experiment in D_2O indicated the presence of the $^{-}O-C_8-N^{+}-CH_3$ linkage and provided further evidence for the existence of the ionized form of this type of PA. Consequently, in aqueous solution this type of PA exists in an ionized hydrophilic form with the necine base component as a bicyclic structure.

It is worthy of note that the unique dual solubility of otonecine-type PAs may play an important role regarding their well-established intoxication. For example, since herbal remedies, including herbal teas, are commonly given orally via an aqueous decoction, the hydrophilic ionized form of otonecine-type PAs can facilitate dissolution in the decoction. In contrast, this type of PA will exist in the lipophilic non-ionized form in the lipid membranes and lipid sites of the body and hence will play a role in its absorption, distribution, and elimination.

Experimental Section

General Experimental Procedures. NMR spectra, including ^1H, ^{13}C, and ^{13}C DEPT NMR, HMQC (^1H-detected, one-bond $^1H-^{13}C$ heteronuclear correlation), and HMBC (^1H-detected, long-range $^1H-^{13}C$ heteronuclear correlation), were all performed on a Bruker AMX 500 with a Bruker Dual $^1H/^{13}C$ 5 mm probe. The DEPT experiment used the "dept135" pulse program showing CH and CH_3 signals positive and CH_2 negative. HMQC and HMBC spectra were operated by using the "inv4tp" and "inv4lplrnd" pulse programs, respectively. ^1H NMR spectra were recorded at 500.13 MHz, while ^{13}C NMR spectra were recorded at 125.76 MHz. $CDCl_3$ utilized in the present study was prepared as an acid-free $CDCl_3$ solution by freshly saturating $CDCl_3$ with anhydrous sodium bicarbonate, followed by filtration. Aliquots (0.7 mL) of the samples containing 5 mg of **1** for all experiments or 5 mg of **2** for one-dimensional and 2 mg of **2** for two-dimensional experiments were utilized in the present study.

Plant Material. *L. hodgsonii* was collected by Professor Liang-ko Song (Sichuan School of Chinese Materia Medica, Emei, People's Republic of China) and Dr. X. G. Zhao on September 19, 1993, at Emei County, Sichuan Province, People's Republic of China. The plant identity was authenticated by two of the authors (Professor Z. T. Wang and Dr. X. G. Zhao). Voucher specimens are deposited in the Department of Pharmacognosy, China Pharmaceutical University.

Extraction and Isolation. 1 and **2** were isolated from *L. hodgsonii* by a standard extraction method for PAs.[10,22] The purity of the isolated PAs was determined by HPLC and shown to be higher than 99%. Their identities were confirmed by UV, IR, NMR, and MS comparison to previously published data.[10−12,16,17,22]

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Supporting Information Available: NMR chemical shift data for clivorine (**1**) and ligularine (**2**) and $^1H-^{13}C$ HMQC spectra of clivorine (**1**) in (a) $CDCl_3$ and (b) H_2O. These materials are available free of charge via the Internet at http://pubs.acs.org.

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