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Isolation and NMR Study on Swainsonine from Locoweed, *Astragalus strictus*

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Abstract

Locoweed is a poisonous plant wildly distributed in most area of the world and can cause livestock poisoning or death with significant economic loss. The principal responsible for its toxicity is indolizidine alkaloid swainsonine, a new potential anticancer and antiviral drug. *Astragalus strictus* is mainly distributed in Tibet of China and is a serious hazard to the local livestock industry. To analyze its main toxic ingredients and supply more structural information and more accurate data, swainsonine has been isolated from this plant by D101 macroporous resin and the ^{1}H and ^{13}C chemical shifts of the compound has been assigned by 1D-NMR and 2D-NMR techniques. At the same time, complete assignments of swainsonine's ^{13}C spectral signals are reported.

Key words: chemical shift assignment, locoweed, NMR, swainsonine

INTRODUCTION

Locoweed, known as poisonous *Astragalus* and *Oxytropis* genera, is mainly distributed in Inner Mongolia, Ningxia, Gansu, Shaanxi, Qinghai, Xinjiang, Tibet and Sichuan of China. It causes chronic neurological disease and a mass death of livestock in the area of the west grassland, which has seriously inhibited the sustainable development of livestock industry in China (Zhao *et al.* 2006). The main toxic ingredient of locoweed is the indolizidine alkaloid swainsonine (SW), an inhibitor of α-mannosidase, which was first isolated and identified from *Swainsona canescens* in 1979 (Colegate *et al.* 1979), and then from locoweed *Astragalus lentiginosus* in 1982 (Molyneux and James 1982). Follwing that, swainsonine was isolated from many other locoweed populations. The study on its bioactivity indicated that swainsonine and its analogs and derivates inhibited the activity of glycosidase, especially Golgi α-mannosidase II (GM II), a key enzyme in the biosynthesis of N-linked glycoproteins. Swainsonine was found to reduce tumor growth and metastasis (Fujita *et al.* 2004). Cancer research on swainsonine is performed in laboratories in the United States, Canada, and Japan. A phase I study on swainsonine administered i.v. at 50-550 μg kg^{-1} d^{-1}, as a new clinical anticancer drug in patients with advanced malignancies, showed that it was well tolerated in cancer patients (Goss *et al.* 1994). It is now in phase III trials for cancer. Furthermore, swainsonine has antiviral effects such as respiratory syncytial virus (RSV) as found in a recent research (McDonald *et al.* 2006). In China, swainsonine was first isolated from *Oxytropis ochrocephala* (Cao *et al.* 1989), and then from *Oxytropis kansuensis*, *Oxytropis glacialis*, *Oxytropis serioopetala* and *Astragalus variabilis*, successively. There was no related report on *Astragalus strictus*. The structure of swainsonine has been identified for more than 20 years, revealing an indolizidine ring with four contiguous stereogenic

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carbons, with C-1 and C-2 representing a 1,2-*syn*-diol functionality and C-8 and C-8a representing a 1,2-anti-amino-alcohol moiety (Molyneux and James 1982; Bennett *et al.* 1989; Pearson and Hembre 1996; Hunt and Roush 1997; Mukai *et al.* 1998; Zhao *et al.* 2001; Lindsay and Pyne 2002; Buschmann *et al.* 2002; Miller and Chamberlin 1990), but there is no 2D-NMR research on it, nor is there a report on the complete assignment of the ^{13}C signals.

Astragalus strictus, the most popular poisonous plant in Tibet, is a locoweed. Its coverage rate may be up to 70-80% in some areas. It causes such a huge economic loss in Tibet every year that 116 752 animals have been poisoned and 46 630 have died from 1976 to 1979, according to a survey of the Tibetan Agriculture and Animal Husbandry Bureau in 1980 (Zhao 1994). In this experiment, swainsonine is isolated from locoweed and the signals of ^{1}H and ^{13}C-NMR are assigned completely to ^{1}H- and ^{13}C-NMR including DMPT, ^{1}H-^{1}H COSY, HMQC, HMBC and EI-MS.

MATERIALS AND METHODS

Plant materials

The aerial part of *Astragalus strictus* was collected in Tibet in July 2005, and taxonomically identified by Professor Zhang Zhenwan, Northwest A & F University, China.

Extraction and isolation

The plant sample (10 kg) was placed in EtOH trice, seven days per test, and the EtOH was recovered under reduced pressure. Crude extraction was obtained, water-resolved with a volume adjusted to 2 L, and then extracted by petroleum ether, chloroform, ethyl acetate, and n-butanol successively. The extraction of n-butanol was chromatographed through D101 macroporous resin (eluted by increasing EtOH in H_2O). The first eluted fractions were obtained and detected by TLC. The results were compared with those of the standard swainsonine sample. The fractions containing SW (H_2O eluting fraction) were collected, concentrated and then chromatographed by G gel chromatography (adjust the pH to ca. 6-7, eluted by EtOAc:EtOH=4:1, v/v). The second eluted fractions were obtained and detected by TLC. The results were compared with the standard swainsonine sample. These swainsonine containing fractions were put together, and at last purified by sublimation after a treatment of alkaline chloroform. The experimental route is available in Fig.1.

Identification

TLC detection was performed on silica gel G precoated plates (Qingdao Marine Chemical Factory, China) with the solvent of chloroform-methanol-ammonia-water (70:26:2:2, v/v) as the developing reagent and H_2O_2/10% acetic anhydride in EtOH/Ehrlich's reagent as the color developer. The melting point was measured by X-6 micro melting apparatus (uncorrected). Furthermore, the IR spectrum was recorded by NICOLET AVATAR 360 FT-IR spectrometer, and UV was detected by the UV1102 spectrometer.

1D-NMR and 2D-NMR assessments

^{1}H and ^{13}C DMPT NMR (ppm, *J* in Hz) spectra and 2D-

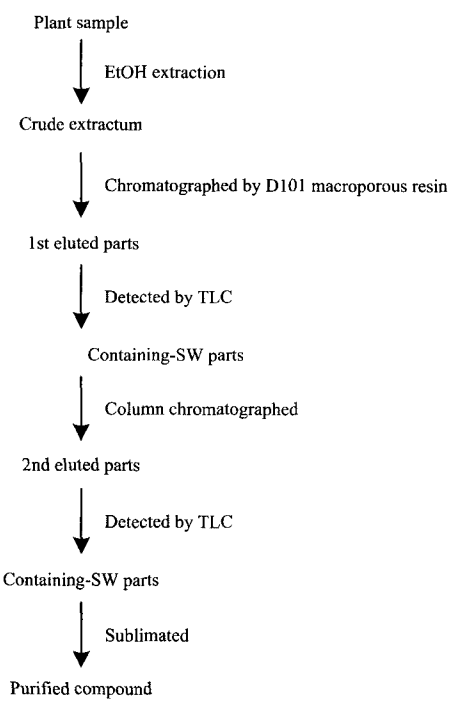

Fig. 1 Experimental route.

NMR spectra were detected by Bruker AM-400 and DRX-500 instruments with C_5D_5N (^{13}C: 150.0, 135.6, and 123.6; and ^1H: 8.71, 7.57, and 7.20) as an internal standard. EI-MS was measured by VG Autospec-3000 mass spectrometer.

RESULTS

Crude extraction of 889 g was obtained from 10 kg plant samples with an extraction rate of 8.89%. Of this, 440 g was extracted by different polar solvents successfully, and petroleum extraction (90 g), chloroform extraction (33 g), ethyl acetate extraction (30 g), and n-butanol extraction (163 g) were obtained. 50 g of the n-butanol extraction was eluted. The results of the first eluted fractions detected by TLC are listed in Table 1. It indicated that the H_2O eluting fraction contained swainsonine. Therefore the H_2O part was eluted and five fractions were obtained after putting together parts of the same composition, through column chromatography. The results of the second eluted fractions detected by TLC are also listed in Table 1. The containing swainsonine fractions were put together and 30 mg of the purified compound was isolated by sublimation. The isolation yield of swainsonine was 0.006%.

The isolated compound was white acicular crystal, with mp 144-145°C; R_f 0.47 [purple-red, chloroform: methanol:ammonia:water (70:26:2:2, v/v) as the developing reagent and H_2O_2/10% acetic anhydride in EtOH/ Ehrlich's reagent as the color developer]; IR_{max} (KBr, cm^{-1}) 3423 (-OH), 2943, 2891 (C-H), 2828 (Bohlman zone), 1072 (C-O). δ_H and δ_C are listed in Table 2.

DISCUSSION

The compound was obtained as a white acicular crystal. It was assigned a molecular formula of $C_8H_{15}NO_3$ on the basis of the IR, UV, EI-MS [173 (M+)], ^1H-NMR (500 MHz), and ^{13}C-NMR (including DMPT) (125 MHz) (Table 2). With the data of ^1H-^1H COSY (500 MHz), HMQC (500 MHz) and HMBC (500 MHz) (Table 2), the structure of this compound was established as swainsonine.

Swainsonine can cause an accumulation of oligosaccharides and result in significant cellular vacuolation in parenchymatous organs (Elbein *et al.* 1981; Tulsiani *et al.* 1990; Stegelmeier *et al.* 2005). A previous study on the toxicity of *Astragalus strictus* in goats (Zhao *et al.* 1992) has shown a disordered activity of the

Table 1 Results of the 1st and 2nd eluted fractions detected by TLC

Samples		Developing agent	R_f value	Color developer
1st eluted fractions	H_2O elution	$CHCl_3$:MeOH:NH_3:H_2O:H_2O (70:26:2:2)	0.13 A, 0.46 A, 0.50 A, 0.60 A	Ehrlich's
	20% EtOH elution	EtOAc:EtOH:NH_3:H_2O:H_2O (11:2:1:1)	0.16 B, 0.23 C, 0.27 A, 0.39 A, 0.52 A	5% sulphuric acid ethanol solution
	40% EtOH elution	$CHCl_3$:MeOH:H_2O (16:6:1)	0.09 D, 0.14 D, 0.22 D, 0.34 B, 0.52 B, 0.67 B, 0.72 B	5% sulphuric acid ethanol solution
	60% EtOH elution	$CHCl_3$:MeOH:H_2O (32:13:1)	0.07 D, 0.16 B, 0.28 D, 0.47 B, 0.59 B, 0.64 D, 0.75 D	5% sulphuric acid ethanol solution
	100% EtOH elution	$CHCl_3$:MeOH:H_2O (32:13:1)	No colored spot	5% sulphuric acid ethanol solution
2nd eluted fractions	Fr. 1 (1-100)	$CHCl_3$:MeOH:NH_3:H_2O (90:10:1)	No colored spot	Ehrlich's
	Fr. 2 (101-126)	$CHCl_3$:MeOH:NH_3:H_2O (85:15:1)	0.43 A	Ehrlich's
	Fr. 3 (127-147)	$CHCl_3$:MeOH:NH_3:H_2O (85:15:1)	0.43 A, 0.36 A	Ehrlich's
	Fr. 4 (148-165)	$CHCl_3$:MeOH:NH_3:H_2O (85:15:1)	0.36 A	Ehrlich's
	Fr. 5 (166)	$CHCl_3$:MeOH:NH_3:H_2O:H_2O (70:26:2:2)	0.12 A, 0.47 A	Ehrlich's
Standard SW		$CHCl_3$:MeOH:NH_3:H_2O:H_2O (70:26:2:2)	0.46 A	Ehrlich's
		$CHCl_3$:MeOH:NH_3:H_2O (85:15:1)	0.43 A	Ehrlich's

Different letters stand for different color. A, purple-red; B, yellow; C, purple; D, brown.

Table 2 ^{1}H- NMR, ^{13}C-NMR (including DMPT), ^{1}H-^{1}H COSY and HMBC assignments (ppm) of swainsonine

No.	^{1}H-NMR (500 MHz)	^{13}C-DMPT (125 MHz)	^{1}H-^{1}H COSY (500 MHz)	HMBC (500 MHz)
1	4.68 (1H, dd, J=6.2, 4.2)	70.9 (CH)	H-2, 8α	C-2, 3, 8a
2	4.44 (1H, t, J=6.5)	69.5 (CH)	H-1, 3β	C-1, 3, 8α
3	α 3.24 (1H, d, J=9.8)	63.3 (CH_2)		C-1, 2, 5, 8α
	β 2.41 (1H, dd, J=9.8, 7.0)		H-2	
5	α 2.90 (1H, d, J=10.4)	52.5 (CH_2)		C-3, 6, 7, 8α
	β 1.83 (1H, td, J=10.9, 2.9)		H-6α	
6	α 1.63 (1H, qt, J=13.0, 3.9)	24.6 (CH_2)	H-5β	C-5, 7, 8
	β 1.57 (1H, dt, J=13.0, 3.8)		H-7α	
7	α 1.49 (1H, qd, J=12.1, 3.6)	34.5 (CH_2)	H-8, 6β	C-5, 6, 8, 8α
	β 2.22 (1H, dd, J=12.1, 3.5)			
8	4.40 (1H, dd, J=9.9, 4.6)	66.5 (CH)	H-7α, 8α	C-1, 6, 7, 8α
8a	2.00 (1H, dd, J=8.8, 4.1)	75.5 (CH)	H-1, 8	C-1, 2, 3, 5, 7, 8

In C_5D_5N, J is coupling constants, Hz.

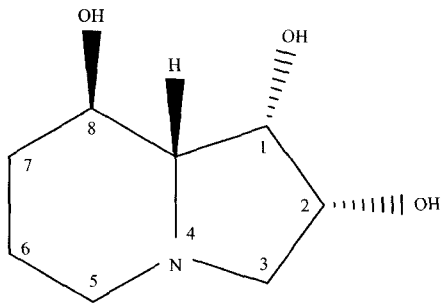

Fig. 2 The structure of swainsonine.

central nervous system (CNS) and cellular vacuolation in most organs, especially the neurons, after ingestion of this poisonous plant. It is similar with swainsonine toxicity. In this study, swainsonine is isolated from *Astragalus strictus*, thus it can be inferred that the principal toxin of this plant responsible for the toxicity of this plant is swainsonine. Further study should be carried out to confirm it.

The preferred conformation of swainsonine H-8a is β in indolizidines. The couple constant of H-7 and H-8 is 3.6 or 4.6 Hz, which infers that the preferred conformation of H-8 is α. The couple constant of H-8a and H-1 is 4.2 or 4.1 Hz, the couple constant of H-1 and H-2 is 6.2 or 6.5 Hz, and with the analysis of 3D chemistry, it is concluded that the preferred conformations of H-1 and H-2 are both β. In summary, swainsonine is confirmed as (1α, 2α, 8β, 8aβ)-trihydroxyindolizidine or (1*S*, 2*R*, 8*R*, 8a*R*)-1,2,8-trihydroxyindolizidine (Fig.2).

Table 3 The chemical shift of ^{13}C in previous studies

Author	Year	Solvent	δ of C (ppm)
Colegate	1979	D_2O	72.55, 69.42, 8.74, 66.07, 60.38, 51.33, 32.16, 22.89
Bennett	1989	D_2O	72.87, 69.72, 69.08, 66.37, 60.65, 51.72, 32.51, 23.21
Pearson	1996	D_2O	72.6, 69.4, 68.9, 66.0, 60.3, 51.6, 32.2, 22.9
Hunt	1997	D_2O	73.1, 70.0, 69.4, 66.7, 61.0, 52.0, 32.8, 23.6
Mukai	1998	D_2O	73.40, 70.24, 69.60, 66.95, 61.22, 2.24, 33.08, 23.78
Zhao	2001	D_2O	73.3, 70.0, 69.4, 66.8, 61.3, 52.0, 32.9, 23.6
Lindsay	2002	D_2O	71.8 (d), 68.5 (d), 67.9 (d), 65.2 (d), 59.7 (t), 50.6 (t), 31.5 (t), 22.2 (t)
Buschmann	2002	D_2O	72.5 (CH), 69.4 (CH), 68.8 (CH), 66.1 (CH), 60.3 (CH_2), 51.4 (CH_2), 32.2 (CH_2), 22.9 (CH_2)

To date, there are more than eight researches (Table 3) have reported the chemical shift of ^{13}C of swainsonine. None of them, however, has assigned the signals of ^{13}C.

Pearson and Hembre (1996), Hunt and Roush (1997), Mukai *et al.* (1998), Zhao *et al.* (2001), Lindsay and Pyne (2002) and Buschmann *et al.* (2002) all reported the chemical shift of ^{1}H signals, however, they did not assign these signals. In the literatures listed in Table 4, Miller and Chamberlin (1990) gave a complete assignment of 1H signals, and the other researches assigned a part of the signals in ^{1}H-NMR.

The ^{1}H and ^{13}C chemical shifts in this experiment are a little different from those report (Table 3). The main reason may be the different influences between C_5H_5N and D_2O. D_2O is not a good solvent in the 2D-NMR experiment. It could influence the signals. Hence, D_2O

Table 4 Comparison of assignments (ppm) of ^1H among this experiment and literatures

No.	Zhao *et al.* (2006) (500 MHz, C_5H_5N)	Colegate *et al.* (1979) (90 MHz, D_2O)	Bennett *et al.* (1989) (300 MHz, D_2O)	Miller and Chamberlin (1990) (500 MHz, D_2O)
1	4.68 (1H, dd, J=6.2, 4.2)	4.42 (m)	4.24 (dd, J=6.1, 3.7)	4.26 (dd, J=6.0, 3.7)
2	4.44 (1H, t, J=6.5)	4.17 (m)	4.34 (m)	4.35 (ddd, J=8.0, 5.9, 2.5)
3	α 3.24 (1H, d, J≈9.8)	2.85 (m)	2.86 (dd, J=2.6, 11.0)	2.88 (dd, J=11.0, 2.4)
	β 2.41 (1H, dd, J=9.8, 7.0)	2.50 (dd, J=11.0, 6.0)	2.53 (dd, J=7.8, 11.0)	2.55 (dd, J=11.0, 7.9)
5	α 2.90 (1H, d, J≈10.4)	2.85 (m)	2.89-1.22	2.91 (dbrt, J=10.2)
	β 1.83 (1H, td, J=10.9, 2.9)	0.94-2.13 (m)	2.89-1.22	1.96 (dt, J=11.4, 2.9)
6	α 1.63 (1H, qt, J=13.0, 3.9)	0.94-2.13 (m)	2.89-1.22	1.52 (qt, J=13.5, 4.2)
	β 1.57 (1H, dt, J=13.0, 3.8)	0.94-2.13 (m)	2.89-1.22	1.72 (dq, J=13.9, 2.4)
7	α 1.49 (1H, qd, J=12.1, 3.6)	0.94-2.13 (m)	2.89-1.22	2.06 (dq, J=12.3, 3.7)
	β 2.22 (1H, dd, J=12.1, 3.5)	0.94-2.13 (m)	2.89-1.22	1.24 (qd, J=12.3, 4.5)
8	4.40 (1H, dd, J=9.9, 4.6)	3.79 (ddd, J≈10, 10, 4.5)	3.78 (ddd, J=3.9, 9.3, 10.7)	3.81 (ddd, J=12.6, 8.0, 4.6)
8a	2.00 (1H, dd, J=8.8, 4.1)	0.94-2.13 (m)	1.89 (dd, J=9.3, 3.7)	1.92 (dd, J=9.5, 3.7)

has not been chosen as a solvent in this experiment.

This study gives a complete assignment of C for the first time through 2D-NMR (^1H-^1H COSY, HMQC and HMBC). It will be helpful for the identification of indolizidines.

CONCLUSION

The isolated compound from locoweed *Astragalus strictus* was established as swainsonine by mp, IR, EI-MS and NMR. The principal toxin of this poisonous plant was confirmed to be swainsonine. Complete ^1H and ^{13}C signals were given by 1D-NMR and 2D-NMR. They revealed that the preferred conformation of H-8a is β and H-8 is α. The structure has been confirmed as (1α, 2α, 8β, 8aβ)-trihydroxyindolizidine or (1*S*, 2*R*, 8*R*, 8a*R*)-1,2,8-trihydroxyindolizidine.

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