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CO-OCCURRENCE OF 2-PYRROLIDINEACETIC ACID WITH THE PYRROLIZIDINES TUSSILAGINIC ACID AND ISOTUSSILAGINIC ACID AND THEIR 1-EPIMERS IN *ARNICA* SPECIES AND *TUSSILAGO FARFARA*

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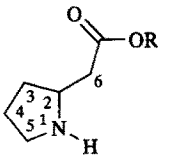
Key Word Index—*Arnica* species; *Tussilago farfara*; Compositae; flowerheads; tussilaginic acid; isotussilaginic acid; pyrrolizidine alkaloids; 2-pyrrolidineacetic acid; biosynthesis.

Abstract—Flowerheads of *Arnica montana*, *A. chamissonis* ssp. *foliosa*, *A. amplexicaulis* and *A. sachalinensis* contained, in addition to the pyrrolizidine alkaloids, tussilaginic acid and isotussilaginic acid, 2-pyrrolidineacetic acid and the C-1 epimers of the two pyrrolizidine acids. Biosynthetic aspects are discussed on the basis of this co-occurrence. The pyrrolizidines tussilagine and isotussilagine, formerly reported for Asteraceae species, are artefacts caused by Soxhlet extraction with methanol.

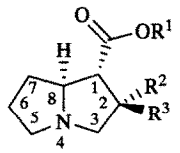
INTRODUCTION

Recently we reported the identification of the pyrrolizidine alkaloids tussilagine **2b** and its C-2 epimer isotussilagine **3b** in flowerheads of four *Arnica* species [1]. Previously these two atypical and non-toxic pyrrolizidines bearing a methyl group at C-2 were only found in *Tussilago farfara*, *Echinacea purpurea*, and *E. angustifolia* [2, 3]. Up to now no other pyrrolizidine alkaloids bearing a methyl group at C-2 have been found [4, 5]. The present paper deals with the isolation of 2-pyrrolidineacetic acid **1a** and its methyl ester **1b** from the alkaloid fraction of the flowers of *Arnica montana* and *A. amplexicaulis*, and their identification in *A. chamissonis* ssp. *foliosa*, *A. sachalinensis* and *T. farfara* by GC-mass spectral analysis. Beside this, it could be shown that **1b** and the pyrrolizidines **2b** and **3b**, formerly reported for *Arnica* species [1], *T. farfara* [2] and *Echinacea* species [3], are artefacts produced by Soxhlet extraction with methanol.

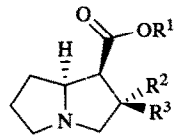
Moreover the occurrence of the 1-epimers **4b** and **5b** of the pyrrolizidines **2b** and **3b** could be demonstrated in the alkaloid fractions from the four *Arnica* species and *T. farfara* by GC-mass spectral analysis. Based on the co-occurrence of 2-pyrrolidineacetic acid along with tussilaginic acid and isotussilaginic acid, it is speculated that this β-amino acid may be an intermediate in the biosynthesis of these pyrrolizidine alkaloids bearing a methyl group at C-2.



	R
1a	H
1b	Me



	R^1	R^2	R^3
2a	H	OH	Me
2b	Me	OH	Me
3a	H	Me	OH
3b	Me	Me	OH



	R^1	R^2	R^3
4a	H	OH	Me
4b	Me	OH	Me
5a	H	Me	OH
5b	Me	Me	OH

RESULTS AND DISCUSSION

The GC of the alkaloid fractions from the flowers of the four *Arnica* species, obtained by extraction with methanol and further preparation in the usual way used for pyrrolizidine alkaloids [2], showed in each case, besides tussilagine (**2b**) and isotussilagine (**3b**), another nitrogen-containing compound ($[M]^+$ at *m/z* 143; $[M-CH_2COOMe]^+$ at *m/z* 70, base peak) as the main component. This compound was isolated from *A. montana* and *A. amplexicaulis* by droplet counter current chromatography (DCCC). The ^{13}C NMR spectrum clearly indicated the presence of seven carbon atoms, which were identified as one Me-, four CH_2-, one CH-/ and one quaternary carbon signal using the ^{13}C DEPT technique (Table 1). ^1H NMR spectroscopy, including 2D-COSY and 2D-HETCOR experiments (Table 2) established its structure as the methyl ester of the 2-pyrrolidineacetic acid (**1b**), already synthesized by Cassal *et al.* [6].

Table 1. ^{13}C NMR spectral data of compound **1b** (75 MHz, $CDCl_3$, TMS as int. standard)

Carbon	δ (ppm)	DEPT
2	55.8	CH
3	30.5	CH_2
4	23.6	CH_2
5	44.8	CH_2
6	36.1	CH_2
COOMe	170.8	C
COOMe	52.2	Me

The non-esterified 2-pyrrolidineacetic acid (**1a**) has already been found in *Nicotiana tabacum* [7] and its *N*-methyl derivative was described as a possible intermediate in the biosynthesis of cocaine [8, 9]. In addition to **1b**, the free 2-pyrrolidineacetic acid **1a** could be isolated from the alkaloid fraction of *A. amplexicaulis* and it was identified by FAB-mass spectrometry $\{[M+H]^+$ at *m/z* 130; $[(M-MeCOOH)+H]^+$ at *m/z* 70, base peak$\}$ and ^1H NMR (Table 2).

From the co-occurrence of free 2-pyrrolidineacetic acid (**1a**) and its methyl ester **1b**, it can be supposed that **1b** is an artefact from **1a**, which is formed during Soxhlet extraction of the flowers with methanol. To prove this, flowers of *A. montana* were extracted with acetone–water. In the alkaloid fraction prepared from this extract, in the same manner, **1b** could not be detected by TLC and GC. Surprisingly, tussilagine (**2b**) and isotussilagine (**3b**) were also not detectable. Due to the use of methanol as solvent while first isolating tussilagine and isotussilagine from *T. farfara* by Röder *et al.* [2], the leaves of this plant were extracted as well without using methanol. Both compounds were not detectable by TLC and GC in the alkaloid fraction, and appeared only after treatment with methanol for a longer time under reflux conditions. Therefore, tussilagine and isotussilagine are also artefacts.

After treatment with methanol, 2-pyrrolidineacetic acid (**1a**) was also detectable as its methyl ester **1b** by GC and GC-mass spectrometry in the alkaloid extract of *T. farfara*. The chromatograms of alkaloid extracts from all four *Arnica* species and *T. farfara* included two further compounds (**4b** and **5b**) which were also formed during Soxhlet extraction with methanol. The R_ts of both compounds were close to those of tussilagine (**2b**) and isotussilagine (**3b**). Their mass spectra showed the same fragmentation patterns as **2b** and **3b**, indicating that these compounds could be the hitherto unknown C-1 epimers of **2b** and **3b**, namely, neo-tussilagine (**4b**) and neo-isotussilagine (**5b**). Conspicuous differences appeared only in the relative intensity of some fragment ions, especially those at *m/z* 156 and 124. By analogy to **2b** and **3b** both compounds occur naturally as the corresponding free acids **4a** and **5a**.

The simultaneous occurrence of 2-pyrrolidineacetic acid and tussilaginic and isotussilaginic acid leads to the assumption that the β-amino acid **1a** or a more activated precursor could be an intermediate in the biosynthesis of these two pyrrolizidine alkaloids bearing a methyl group at C-2. Thus, reaction of a keto compound, like pyruvate or acetoacetyl-CoA, with the activated methylene group of **1a**, followed by ring closure of the N atom of the pyrrolidine ring could explain the simultaneous formation of the C-2 epimeric tussilaginic and isotussilaginic acid and their C-1 epimers. Further investigations to confirm this hypothesis are planned.

EXPERIMENTAL

Plant material. Arnica montana L. and *Tussilago farfara* L.: Fa. Caesar & Loretz, identity and purity proved. *Arnica chamissonis* Less. ssp. *foliosa* (Nutt.) Maguire, *A. amplexicaulis* Nutt. and *A. sachalinensis* (Regl.) A. Gray were cultivated on the proving fields of the Botanical Garden of the University of Düsseldorf. Vouchers are deposited at the herbarium of the Institute of Pharmaceutical Biology.

Extraction and isolation. Arnica montana: dried flowers (1 kg) were extracted with MeOH (24 hr). A portion (200 g) of the crude extract obtained (280 g) was treated with 0.25 M H_2SO_4/Zn to reduce *N*-oxides of alkaloids and then extracted after filtration with Et_2O and CH_2Cl_2. Alkalinization of the aq. phase up to pH 9 with NH_3 and further extraction with CH_2Cl_2 gave 220 mg of an alkaloid-containing extract. The upper phase after DCCC (descending) of this extract with $CHCl_3$–MeOH–H_2O (5:6:4) gave 28 mg of **1b**. *Arnica amplexicaulis*: Dried and powdered flowerheads (2.2 kg) were defatted by extraction with $CHCl_3$ and then extracted with MeOH. From 490 g of this extract (642 g) alkaloids were extracted as described above. The resulting crude alkaloid extract (890 mg) was further purified by DCCC and gave 11.8 mg of **1a** and 23 mg **1b**. *Arnica chamissonis* ssp. *foliosa*: dried and powdered flowerheads (500 g) were defatted by extraction with CH_2Cl_2 and then extracted with MeOH. From 45 g of the MeOH extract (170 g) alkaloids were extracted as described in ref. [10]. Pyrrolizidine alkaloid-containing fr.: 17 mg (0.013%). *Arnica sachalinensis*: dried and powdered flo-

Table 2. ^1H NMR spectral data (300 MHz) of compounds **1** ($CDCl_3$, TMS) and **2** (D_2O, TSP)

H	**1a**	**1b**	*J* (Hz) **1a**	**1b**
2	4.15 *m*	3.91 *m*	—	—
3a	1.73 *m*	1.75 *dq*	—	8.7 and 12.8
3b	2.23 *m*	2.28 *dq*	—	8.7 and 12.8
4a,b	2.03 *m*	2.04 *m*	—	—
5a,b	3.34 *t*-like *m*	3.4 *t*-like *m*	6.3 and 7.8	6.7 and 7.4
6a	2.6 *dd*	2.82 *dd*	7.3 and 16.3	7 and 17
6b	2.65 *dd*	3.18 *dd*	6.4 and 16.2	7 and 17
COOMe	—	3.72 *s*		

werheads (1.2 kg) were defatted with CH_2Cl_2 and then extracted with MeOH. From 45 g of this extract (475 g) alkaloids were extracted and further purified as described for *A. montana*. Alkaloid-containing extract: 15 mg (0.013%). *Tussilago farfara*: powdered leaves (1 kg) were stirred at room temp. with frequent changes of Me_2CO–H_2O (1:1). A portion (10 g) of the freeze-dried extract (160 g) was extracted as described for *A. chamissonis*. Alkaloid-containing extract: 21 mg.

GC. Column OV-01, 25 m × 0.25 mm. Temp. prog. 100° (10 min isothermal) to 280° at 10° min^{-1}. Inj./det. temp. 300°. Carrier N_2 at 1.3 ml min^{-1}. R_t (min): **1b**: 4.5; **2b**: 14.78; **3b**: 14.15; **4b** and **5b**: 14.64 and 15.03.

TLC. Silica gel 60 F_{254}, CH_2Cl_2–MeOH–NH_3 (25%) (85:14:1) [11]. Detection: Dragendorff's reagent and reagents of refs [10, 11]. R_f: **1a**: 0.02; **1b**: 0.42; **2b** and isomers: 0.13, 0.18, 0.23. Silica gel 60 F_{254}, *n*-BuOH–HOAc–H_2O (3:1:1). Detection as described above. R_f: **1a**: 0.34; **1b**: 0.4.

2-Pyrrolidineacetic acid (**1a**). FAB-MS (*m/z*, rel. int.): 130 $[M+H]^+$, 70 $[(M-CH_2COOH)+H]^+$ (100).

2-Pyrrolidineacetic acid methyl ester (**1b**). GC-MS 70 eV, (*m/z*, rel. int.): 143 $[M]^+$ (12), 128 $[M-Me]^+$ (10), 115 $[M-CO]^+$ (20), 110 (28), 82 (28), 70 $[M-CH_2COOMe]^+$ (100), 56 (48), 43 (50). FAB-MS (*m/z*, rel. int.): 144 $[M+H]^+$, 70 (100).

Tussilagine (**2b**). GC-MS 70 eV, (*m/z*, rel. int.): 199 $[M]^+$ (10); 168 $[M-OMe]^+$ (12); 156 (17); 124 (22); 114 (5); 83 (100); 70 (45); 43 (45).

Isotussilagine (**3b**). GC-MS 70 eV (*m/z*, rel. int.): 199 $[M]^+$ (10); 168 (10); 156 (13); 126 (7); 124 (20); 114 (5); 83 (100); 70 (25); 43 (42).

neo-Tussilagine (**4b**). GC-MS 70 eV (*m/z*, rel. int.): 199 $[M]^+$ (5); 168 (5); 156 (80); 126 (25); 124 (18); 114 (70); 83 (100); 70 (38); 43 (55).

neo-Isotussilagine (**5b**). GC-MS 70 eV (*m/z*, rel. int.): 199 $[M]^+$ (15); 168 (22); 156 (38); 124 (40); 114 (5); 83 (100); 70 (50); 43 (58).

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