BIOLOGICALLY ACTIVE PYRROLIZIDINE ALKALOIDS FROM THE TRUE FORGET-ME-NOT, *MYOSOTIS SCORPIOIDES*[1]

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ABSTRACT.—Four pyrrolizidine alkaloids have been isolated from the aerial parts of *Myosotis scorpioides*. They are 7-acetylscorpioidine (**1**), scorpioidine (**2**), symphytine (**4**), and myoscorpine (**5**). Their free bases cause ataxia, lethargy, and death in mice, accounting for the previously described "curare-like" action of the crude alkaloid fraction.

The pyrrolizidine alkaloids are an important class of plant toxins whose hepato- and pneumotoxicity have been extensively studied (1). Other pharmacological properties of these compounds, including inhibition of intestinal smooth muscle and atropine-like anticholinergic action (2), as well as the occurrence of neuro-muscular block (3), have also been noted but are less well understood. The pharmacology of alkaloid preparations from several species of the Boraginaceae, a plant family rich in pyrrolizidine alkaloids, was reported in 1953 by Gessner (4). One of these, the true forget-me-not, *Myosotis scorpioides* L. (syn. *palustris* L.), was said to contain a "curare-like" alkaloid which had occasionally been employed in homeopathy. However, neither experimental studies of toxicity nor cases of accidental poisoning in livestock or man appear to have been reported, despite general toxicological interest in plants containing pyrrolizidine alkaloids. We therefore undertook an investigation of the identity and toxicity of the alkaloids of this plant.

RESULTS AND DISCUSSION

Isolation of alkaloids from *M. scorpioides* by standard procedures afforded a mixture of pyrrolizidine alkaloids in their free base and amine oxide forms. After separation of the free bases from the amine oxides, solutions of each were administered to mice by intraperitoneal injection. Mice receiving the free bases showed no signs of poisoning at low doses (63 and 125 mg alkaloid/kg body weight). The group receiving 250 mg/kg was lethargic and ataxic for one to three hours following dosing. A 500 mg/dg dose killed all mice tested within five minutes. In contrast, mice receiving corresponding dose levels of the amine oxides were perfectly normal and survived until killed two weeks later.

We focused further work, therefore, on the free base forms. Chemical reduction of the amine oxides gave a mixture of alkaloids indistinguishable from the free base fraction already isolated. Thin layer chromatographic analysis showed three components, designated A, B, and C in order of increasing polarity. After successive chromatography on Sephadex LH-20 and silica gel, these three components were isolated in an 85:3:12 ratio, respectively.

Alkaloid A was characterized by means of its ^{1}H nmr spectrum as a pyrrolizidine alkaloid of the retronecine-7,9-diester type (5). Signals for both acetate and tiglate residues were present, as well as methyl signals corresponding to expectations for a viridiflorate ester (6). The secondary alcohol of the viridiflorate moiety was also esterified, as evidenced by a strongly deshielded one proton quartet at δ5.19, coupled only to a methyl doublet at δ1.30.

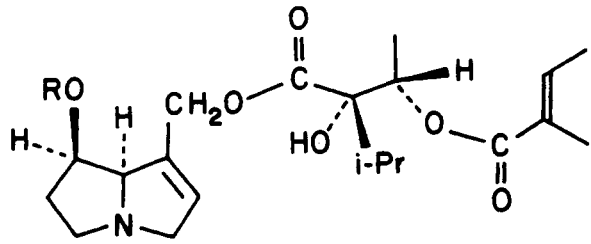
The mass spectrum of alkaloid A permitted a preliminary assignment of structure. The exact mass of the molecular ion at m/e 423 gave a molecular formula

[1]Parts of this work have appeared previously in communication form; see ref. 12.

of $C_{22}H_{33}O_7N$. An intense fragment ion at *m/e* 180, typical of 7-acetyl retronecine derivatives (7), suggested that the acetate occupied position 7, while the remaining allylic alcohol at the 9-position of the retronecine nucleus was esterified with β-tiglylviridifloric acid.

Degradation by hydrogenolysis and hydrolysis afforded proof of this structure and permitted the assignment of absolute stereochemistry at all four chiral centers. Hydrogenolysis of the allylic ester gave 7-acetylretronecanol (8), without a trace of 7-(2-methylbutyryl)retronecanol, the expected hydrogenolysis product of a 7-tiglylretronecine diester. Alkaline hydrolysis gave (+)-(7*R*,8*R*)-retronecine, identical to an authentic sample, as the sole basic hydrolysis product. The acid fraction consisted of acetic, tiglic, and viridifloric acids. The latter was shown to be the (−)-(2*S*,3*S*) enantiomer by its optical rotation and by mixture melting point with an authentic sample. The structure and absolute stereochemistry of alkaloid A is therefore represented by structure **1**.

1, R = Ac
2, R = H



Alkaloid C, unlike alkaloid A, showed no acetate peak in the ^1H nmr. Additionally, the H–7 proton displayed an upfield shift of 1.0 ppm relative to that of alkaloid A. Its molecular ion at *m/e* 381 gave an exact mass corresponding to $C_{20}H_{31}O_6N$, which suggested that it lacked only the acetyl moiety of structure **1**. Mild acetylation of C with acetic anhydride in pyridine gave a monoacetate with spectral properties identical to those of alkaloid A. Alkaloid C, for which the name scorpioidine is proposed, is therefore represented by structure **2**. Scorpioidine is thus epimeric with the known alkaloid anadoline (**3**), which has been isolated from *Symphytum orientale* (9) and *S. tuberosum* (10), also members of the Boraginaceae.

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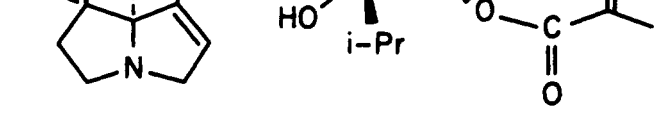
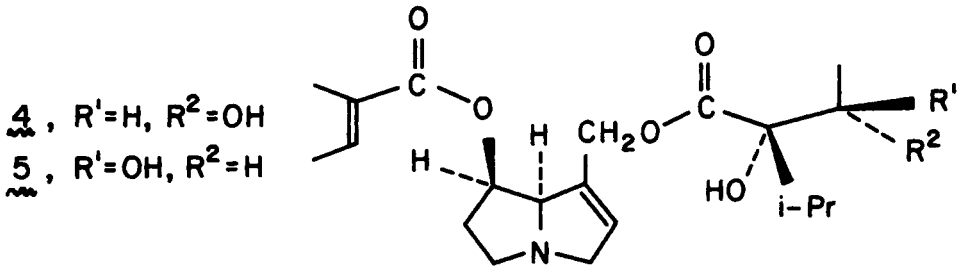


The nature of component B, available only in small quantities, remained to be determined. Although B appeared as a single spot upon thin layer chromatography, gas chromatography-mass spectrometry of the methyl boronate derivative showed two peaks in a 1:1 ratio. Both gave parent ions corresponding to a molecular weight before derivatization of 381 and showed fragment ions at *m/e* 220, as would be expected for 7-tiglylretronecine diesters. The high resolution ^1H nmr spectrum of the mixture showed one of the components to be symphytine (**4**), which was first isolated from *Symphytum officinale* (11). Despite repeated efforts, separation of the two compounds could not be accomplished. Alkaline hydrolysis of the mixture gave retronecine as the sole basic product, and tiglic, viridifloric, and trachelanthic acids as the acidic products. By means of chiral-chelated osmate esters (12), the absolute stereochemistry of the viridifloric and

trachelanthic acids was shown to be (2*S*,3*S*) and (2*S*,3*R*) respectively. Fraction B is therefore a 1:1 mixture of the epimeric alkaloids symphytine (**4**) and a new alkaloid **5**, for which the name myoscorpine is proposed.



Of the four alkaloids isolated from *M. scorpioides*, only 7-acetylscorpioidine (**1**) and scorpioidine (**2**) were available in sufficient quantity for biological testing. Intraperitoneal administration of the two alkaloids produced indistinguishable effects. Whether mice received 7-acetylscorpioidine or scorpioidine, doses of 63 and 125 mg/kg produced no effects; 250 mg/kg produced lethargy and ataxia followed by recovery. A 500 mg/kg dose again rapidly induced lethargy and ataxia followed by brief clonic convulsions, gasping, and death within four minutes of dosing. Body weights of all mice tested were monitored for two weeks after testing; neither dose level nor compound administered significantly affected body weight gain as a percentage of original weight when examined by two way analysis of variance. Histological examination of the liver from a mouse that received 250 mg/kg of 7-acetylscorpioidine and another that received the same amount of scorpioidine showed no lesions that could be attributed to the pyrrolizidine alkaloids.

These results suggest an explanation for the lack of observed toxicity of the whole plant. An animal might have to eat at least its body weight of this forget-me-not within a short time to ingest a lethal amount of the toxic alkaloids. We have not investigated the toxicity of repeated small doses of these alkaloids over long periods of time. Such exposure may be an unlikely event in nature, since we have observed that dairy cattle in our region avoid lush foliage of *M. scorpioides* while closely cropping nearby plants. Gessner (4) has reported similar avoidance of the related *Symphytum officinale*. It is therefore not surprising that poisoning of livestock by *M. scorpioides* is not described in the literature.

EXPERIMENTAL[2]

PLANT MATERIAL.—The plant material for this study was collected in the flowering stage on the Cornell University campus, Ithaca, New York, in July 1980. The plants were identified by Mr. Ed Cope of the Bailey Hortorium of this university, and voucher specimens are deposited at the Cornell University Herbarium.

EXTRACTION OF ALKALOIDS.—Fresh above-ground portions, 617 g, were immersed in 95% ethanol upon collection and then stored at −20° for three weeks. The ethanol was then decanted, the plants macerated in methanol and extracted with methanol in a Soxhlet apparatus for 10 hr. The dry weight after extraction was 58.6 g. The methanolic and ethanolic extracts were combined and evaporated at reduced pressure. The residue was partitioned between

[2]Melting points were taken on a Kofler hot stage melting point unit and are uncorrected. Optical rotations were measured on a Perkin-Elmer model 141 polarimeter, uv spectra were obtained on a Varian Cary 118 recording spectrophotometer, and ir spectra were determined on a Perkin-Elmer 299-B infrared spectrophotometer. ^{1}H nmr spectra were recorded at 300 MHz on a Bruker WM-300 spectrometer, and ^{13}C nmr spectra at 20 MHz on a Varian CFT-20 spectrometer, with tetramethylsilane as internal standard and chemical shifts recorded in δ (ppm) units. High and low resolution ei-ms were obtained at 70 eV with an AEI MS-902 mass spectrometer, and gc-ms were recorded with a Finnigan model 3300 instrument with a 2.1 m by 2.0 mm OV-1 column with temperature programming 160° to 290° at 6°/min, and carrier gas (N_2) at 27 ml/min. Silica gel plates for preparative and analytical thin-layer chromatography were from Machery-Nagel and were used with a solvent system of chloroform-methanol-ammonium hydroxide (85:14:1).

ether (350 ml) and 0.5 *N* H_2SO_4 (350 ml), and the ether layer was extracted further with 0.5 *N* H_2SO_4 (2 x 350 ml). The aqueous layers were combined, filtered through Celite, and washed with ether (4 x 500 ml). The aqueous extract was basified with conc. NH_4OH to pH 10 and then exhaustively extracted with chloroform (8 x 500 ml). The chloroform layers were combined, dried ($MgSO_4$), and evaporated to afford 473 mg of mixed free base and amine oxide alkaloids as a brown foam. Reduction of the residual aqueous layer (Zn dust in 2 *N* H_2SO_4) afforded no further yield of alkaloids, indicating extraction of amine oxides with chloroform had been complete. The mixed alkaloids were redissolved in 0.5 *N* H_2SO_4 (25 ml) and extracted with chloroform (3 x 25 ml) to remove the last traces of neutral impurities. The aqueous layer was then made basic to pH 10 with conc. NH_4OH, and the free base alkaloids extracted with ether (3 x 25 ml). The ether layers were combined, (dried $MgSO_4$), and evaporated to give 125 mg of mixed free base alkaloids, R_f .67 (major), .48 (trace), and .42 (minor). The aqueous layer was then extracted with chloroform (5 x 25 ml), and the combined chloroform layers were dried ($MgSO_4$) and evaporated to afford 330 mg of mixed amine oxides, R_f .33 (major), .19 (trace), and .16 (minor). Both free bases and amine oxides gave a positive color reaction with Dragendorff's reagent; amine oxides also gave a brown color with acetic anhydride on heating.

REDUCTION OF AMINE OXIDES.—Mixed amine oxides (250 mg) were dissolved with stirring in 2 *N* H_2SO_4 (10 ml), and zinc dust (1 g) was added. After vigorous stirring for four hours, the solution was filtered and the filtrate adjusted to pH 10 by cautious addition of NH_4OH. Extraction of the aqueous layer with chloroform (5 x 30 ml) gave 207 mg of mixed free base alkaloids, R_f .67 (major), .48 (trace), and .42 (minor), all unreactive to acetic anhydride on heating.

7-ACETYLSCORPIOIDINE (**1**).—Chromatography of mixed free base alkaloids (200 mg) on a column of Sephadex LH-20 in methylene chloride-methanol (1:1) gave pure 7-acetylscorpioidine (147 mg) followed by a mixture of symphytine, myoscorpine, and scorpioidine (35 mg). For 7-acetylscorpioidine, a colorless oil, R_f .67, $[\alpha]^{24.5}D$ −8.17° (c 1.78, MeOH); uv, λmax (MeOH) 215 nm (log ε 4.03); ir, ν max (CCl_4) 3530 cm^{-1} (br), 1740, 1733 (sh), 1713, 1651, 1375, 1240, 1160, 1075, 1025; ^1H nmr ($CDCl_3$) δ0.85 (d, 3H, *J*=7.0 Hz), 0.95 (d, 3H, *J*=7.0 Hz), 1.30 (d, 3H, *J*=6.6 Hz), 1.76 (d, 3H, *J*=7 Hz with additional fine coupling), 1.77 (br s, 3H), 1.99 (s, 3H), 2.08 (m, 2H), 2.14 (heptet, 1H, *J*=7.0 Hz), 2.67 (br q, 1H, *J*=8.1 Hz), 3.37 (m, 2H), 3.40 (br s, 1H) (O*H*), 3.96 (br d, 1H, *J*=15.4 Hz), 4.34 (m, 1H), 4.63 and 4.85 (AB q, 2H, *J*=13.6 Hz), 5.19 (q, 1H, *J*=6.6 Hz), 5.33 (m, 1H), 5.81 (m, 1H), 6.77 (q, 1H, *J*=7 Hz with additional fine coupling); ^{13}C nmr ($CDCl_3$) δ11.8 (q), 14.2 (q), 15.1 (q), 15.8 (q), 17.2 (q), 20.9 (q), 32.1 (d), 34.1 (t), 53.5 (t), 62.4 (t), 62.6 (t), 73.8 (d), 75.5 (d), 78.7 (d), 81.7 (s), 127.8 (d), 128.3 (s), 132.8 (s), 137.4 (d), 166.8 (s), 170.0 (s), 173.6 (s); ei-ms *m/e* (rel. int.) 423 (0.8), 181 (23), 180 (92), 179 (17), 136 (23), 121 (10), 120 (57), 119 (45), 95 (6.0), 94 (35), 93 (57), 83 (100), 80 (15), 55 (39), 43 (30); exact mass for M^+ at *m/e* 423, 423.2271 ($C_{22}H_{33}O_7N$ requires 423.2257), for *m/e* 180, 180.1029 ($C_{10}H_{14}O_2N$ requires 180.1024).

HYDROGENOLYSIS OF 7-ACETYLSCORPIOIDINE.—7-Acetylscorpioidine (15 mg) was dissolved in absolute ethanol (12 ml), PtO_2 (5 mg) was added, and the solution was stirred vigorously under 1 atm of hydrogen gas for 4.5 hr. The solution was then filtered and the solvent evaporated. The residue was taken up in water (5 ml) adjusted to pH 10 with NH_4OH. The aqueous layer was extracted with chloroform (4 x 5 ml). The combined organic layers were dried ($MgSO_4$) and evaporated to afford 7-acetylretronecanol as a colorless oil (5.5 mg), identical to an authentic sample (8) by ^1H nmr, ir, and ms.

HYDROLYSIS OF 7-ACETYLSCORPIOIDINE.—7-Acetylscorpioidine (69.9 mg) was refluxed for 3 hr in water (5 ml) containing $Ba(OH)_2 \cdot 8H_2O$ (300 mg). After cooling, the solution was saturated with CO_2 and filtered. The filtrate was acidified to Congo red by the addition of 2 *N* H_2SO_4 and continuously extracted with ether for two days. Gentle evaporation of the ether gave the acidic fraction containing acetic acid (verified by ^1H nmr). Extraction of the acidic fraction with pentane gave crude tiglic acid (8 mg), which was recrystallized from water and was identical to an authentic sample by ^1H nmr, ir, mp and mmp. The remaining residue (29 mg) was recrystallized from ether-petroleum ether to give (−)-(2*S*,3*S*)-viridifloric acid, identical to an authentic sample by ^1H nmr, ir, $[\alpha]D$, mp and mmp. The aqueous layer remaining from the continuous extraction was made basic to pH 12 with Na_2CO_3 and again continuously extracted with ether for two days. Evaporation of the ether left a crystalline residue (20 mg), which was recrystallized from acetone to give (+)-(7*R*, 8*R*)-retronecine, identical to an authentic sample available in our laboratory by ^1H nmr, ir, $[\alpha]D$, mp and mmp.

Scorpioidine **2**.—A mixture of scorpioidine, myoscorpine, and symphytine from LH-20 chromatography (35 mg) was separated by preparative thin-layer chromatography to afford mixed symphytine-myoscorpine (5.0 mg), scorpioidine (22.3 mg), and a small quantity of 7-acetylscorpioidine (4.6 mg). For scorpioidine, a colorless oil, R_f .42; $[\alpha]^{24.0}D$ −5.06° (c 1.72, MeOH); uv, λmax 216 nm (log ε 4.10); ir, νmax (CCl_4) 3530 cm^{-1} (br), 1734 (sh), 1719, 1651, 1381, 1268, 1232, 1159, 1140, 1130, 1072, 1023; ^1H nmr ($CDCl_3$) δ0.87 (d, 3H, *J*=6.6 Hz), 0.97 (d, 3H, *J*=7.0 Hz), 1.34 (d, 3H, *J*=6.6 Hz), 1.76 (d, 3H, *J*=7 Hz with additional fine coupling), 1.77 (br s, 3H), 1.99 (m, 2H), 2.16 (heptet, 1H, *J*=6.6 Hz), 2.20 (br s, 2H) (O*H*), 3.30 (m, 1H), 3.39 (ddd, 1H, *J*=15.8, 5.5, and 1.8 Hz), 3.95 (br d, 1H, *J*=15.8 Hz), 4.21 (m, 1H), 4.32 (m, 1H), 4.77 and 4.84 (AB q, 2H, *J*=13.1 Hz), 5.20 (q, 1H, *J*=6.6 Hz), 5.86 (m, 1H), 6.75 (q, 1H, *J*=7 Hz with additional fine coupling); ^{13}C nmr ($CDCl_3$) δ 12.0, 14.4, 15.2, 15.9, 17.4, 32.6, 36.2, 53.9, 62.7, 62.7, 71.0, 74.2, 78.2, 82.2, 128.4, 129.1, 132.8, 137.9, 167.2, 173.7; ei-ms *m/e* (rel. int.) 381 (7.1), 199 (6.8), 139 (11), 138 (69), 137 (28), 136 (13), 120 (9.3), 101 (8.0), 95 (3.7), 94 (21), 93 (41), 83 (100), 80 (8.7), 67 (5.9), 57 (5.8), 55 (34), 53 (6.3), 43 (10); exact mass of M^+ at *m/e* 381, 381.2143 ($C_{20}H_{31}O_6N$ requires 381.2151). Acetylation with excess acetic anhydride in

pyridine at room temperature for four hours gave a monoacetate identical to 7-acetylscorpioidine by R_f, ^1H nmr, and ir.

Myoscorpine 5.—The 1:1 mixture of symphytine and myoscorpine obtained from the above preparative thin-layer chromatography could not be separated by further thin-layer or high performance liquid chromatography. By comparison with the spectrum of authentic symphytine obtained from *Symphytum officinale* (11)[3], ^1H nmr resonances for myoscorpine could be assigned: ($CDCl_3$) δ 0.97 (d, 6H, J=6.7 Hz), 1.18 (d, 3H, J=6.3 Hz), 1.76 (d, 3H, J=7.4 Hz with additional fine coupling), 1.76 (br s, 3H), 2.12 (heptet, 1H, J=6.7 Hz), 2.17 (m, 2H), 2.25 (br s, 2H) (O*H*), 2.78 (m, 1H), 3.50 (m, 2H), 4.07 (q, 1H, J=6.3 Hz), 4.13 (m, 1H), 4.57 (m, 1H), 4.58 and 4.90 (AB q, 2H, J=12.5 Hz), 5.42 (m, 1H), 5.85 (m, 1H), 6.76 (q, 1H, J=7.4 Hz with additional fine coupling).

After reaction with methylboronic acid in pyridine, gc analysis showed two peaks, R_t 12.3 and 12.6 min, in a 1:1 ratio. The first peak coinjected with authentic symphytine methylboronate, while the second gave the following mass spectrum for myoscorpine methylboronate: *m/e* (rel. int.) 405 (0.01), 305 (0.3), 221 (6.2), 220 (29), 141 (46), 136 (52), 127 (18), 121 (25), 120 (69), 119 (21), 118 (12), 117 (27), 100 (15), 99 (18), 95 (16), 94 (56), 93 (79), 90 (19), 89 (17), 85 (12), 83 (63), 54 (94), 41 (100).

HYDROLYSIS OF MIXED MYOSCORPINE–SYMPHYTINE.—The 1:1 mixture of alkaloids (7.3 mg) was refluxed with $Ba(OH)_2 \cdot 8H_2O$ (60 mg) in water (1 ml) for 2.5 hr. After cooling, the reaction mixture was saturated with CO_2 and filtered. The filtrate was acidified to Congo red with dilute H_2SO_4 and continuously extracted with ether for two days. Gentle evaporation of the ether left a residue which was extracted with pentane to afford tiglic acid (1.0 mg) identical to an authentic sample. The remaining residue (4.2 mg) was shown by ^1H nmr to consist of a 1:1 mixture of trachelanthic and viridifloric acids. These were sequentially treated with ethereal diazomethane, $Os_2O_6py_4$, and (*S,S*)-*trans*-N,N,N',N'-tetramethyl-1,2-cyclohexanediamine as previously described (12) to afford chiral-chelated osmate esters identical to those produced from a 1:1 mixture of (2*S*,3*S*)-viridifloric and (2*S*,3*R*)-trachelanthic acids. ^1H nmr (acetone-d_6, characteristic peaks) δ 2.544 (s, 6H), 2.598 (s, 3H), 2.626 (s, 3H), 2.928 (s, 3H), 2.968 (s, 3H), 3.018 (s, 3H), 3.028 (s, 3H). The remaining aqueous layer from the continuous extraction was made basic to pH 12 with Na_2CO_3 and again extracted continuously with ether. Evaporation of the ether left (+)-(7*R*,8*R*)-retronecine, (3.3 mg) identical to an authentic sample.

BIOLOGICAL EVALUATION.—Male BS1 mice (Blue Spruce Farms, Altamont, N.Y.) were conventionally housed in the Laboratory Animal Facility at the New York State College of Veterinary Medicine. The mice weighed 18–24 g. They were given weight-adjusted doses of 0.1 ml per 20 g. The compounds were dissolved in propylene glycol and then diluted with one-third volume of 0.85% saline. (Earlier trials showed that a semi-neutralized acid solution of the alkaloids produced abdominal adhesions.) Each dose level of each compound was tested on three individuals, except that only two mice per dose level received scorpioidine. Four mice received the vehicle only. All surviving animals were observed for two weeks and then sacrificed. Necropsies were performed on representative mice from each dose level and on all animals that died immediately.

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[3]We were surprised to find that extraction of *S. officinale* root afforded a chromatographic fraction of R_f .48 which contained not only symphytine, but also myoscorpine and symlandine (13) as minor components. The latter two alkaloids have not previously been reported from this species.