Isolation and Characterization of Two Guanidines from *Galega orientalis* Lam. Cv. Gale (Fodder Galega)

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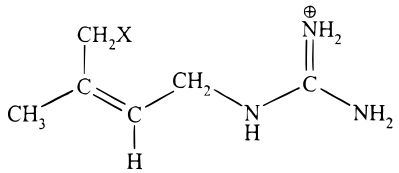
The vegetative parts of *Galega orientalis* Lam. cv. Gale, being tested in Canada as a new cold-hardy fodder crop, were found to contain two hemiterpenoid guanidines: the know alkaloid smirnovine, and its previously unknown (*Z*)-4-hydroxy derivative. The synthesis of smirnovine picrate is also described for the first time. A sample of *G. orientalis* from Estonia yielded smirnovine in similar concentrations (0.2%). Although these levels are low, more research is required to confirm that the alkaloids in fodder galega do not pose a threat to livestock in Canada.

Keywords: *Galega orientalis; fodder galega; guanidines; smirnovine; (Z)-4-hydroxysmirnovine*

INTRODUCTION

The genus *Galega* [Fabaceae (Leguminosae)] is currently considered to comprise six species (Mabberley, 1987). Of these, *G. officinalis* L., known as goat's rue, has long been used as an ornamental and medicinal plant. *G. orientalis* Lam., also commonly known as goat's rue, has recently been developed in Estonia as a fodder crop (Raig, 1994). This latter species is a winter-hardy tap-rooted perennial herb that can spread and propagate vegetatively by underground stolons. The epigeal parts have a high concentration of protein and are recommended for conservation as hay, silage, haylage, or dehydrated meal (Raig, 1994). The agronomic potential of the plant has been assessed for cool-temperate regions (Varis, 1986), and a proposal has been made by the Estonian Research Institute of Agriculture and Land Improvement to the International Seed Testing Association to have the species included in their list of field and forage crops under the name "fodder galega" (Raig, 1994). That common name was chosen to minimize confusion of *G. orientalis* with the other goat's rue, *G. officinalis*, which contains the guanidino-alkaloids galegine (**1**; Tanret, 1914; Späth and Prokopp, 1924) and 4-hydroxygalegine (**2**; Pufahl and Schreiber, 1961; Schreiber *et al*., 1964), and the pyrroloquinazoline vasicine (peganine, Schreiber *et al*., 1962). Of these components, galegine has been established as toxic to sheep (Gresham and Booth, 1991; Keeler *et al*., 1992, 1986; Huxtable *et al*., 1993). In contrast to the findings for *G. officinalis*, galegine was not detected in the cultivars of *G. orientalis* developed by the Estonians, although 4-hydroxygalegine was found in the inflorescences. In addition, the vasicine content (<0.001%) of *G. orientalis* was below the level that would cause poisoning of animals using the plant as fodder (Laakso *et al*., 1993; Nômmsalu, 1993). *G. orientalis* cv. Gale is being tested as a new crop in Northern Alberta, so we decided to check its alkaloid content.



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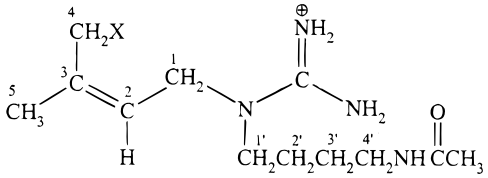

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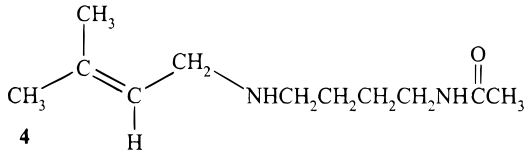

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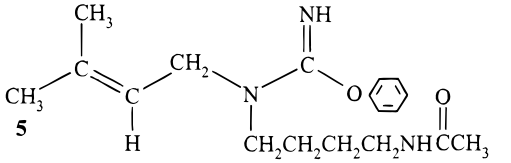

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EXPERIMENTAL PROCEDURES

Plant Material. *G. orientalis* cv. Gale was grown at Beaverlodge, Alberta, Canada, from seed provided by Dr.

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Helmut Raig, Estonian Research Institute of Agriculture. The plant was harvested at the early to mid flowering stages of growth, at a cutting height of ~10 cm, in the first and second years of growth. The plant material was freeze-dried and then pulverized in a Wiley mill.

Isolation of Alkaloids. Typically, 50 g of the powdered plant was extracted several times with methanol at room temperature. The combined extracts were concentrated under reduced pressure and at 40 °C (bath), and the residue was suspended in 250 mL of hot water containing 20 g of Celite filter-aid. The suspension was centrifuged at 27000*g* for 2 min, and the supernatant was removed and concentrated, as before, to a small volume. This supernatant was diluted 10 times with methanol to give a suspension of yellow particles that was again sedimented by centrifugation. The supernatant was concentrated under reduced pressure to yield ~6 g of a syrupy residue. Half of this residue was dissolved in the minimum volume of methanol and loaded onto a column of neutral alumina (20 × 3 cm) packed in chloroform. The column was eluted with 100 mL of chloroform, and then 10 × 50 mL of chloroform:methanol (9:1 → 1:9). The individual fractions, each 25 mL, were assayed by TLC (silica gel 60, developed with chloroform−methanol−acetic acid−water, 62:25:5:5), and alkaloidal components were detected with a modified Dragendorff reagent (0.2 g of bismuth subnitrate, 5 g of potassium iodide, 2 g of iodine, and 10 mL of acetic acid diluted to 250 mL with water). Fractions 8−11 contained a single Dragendorff-positive substance (**A**; R_f, 0.60), and fractions 12−18 contained mixtures of **A** with another Dragendorff-positive substance (**B**; R_f, 0.46). The latter mixture was separated by centrifugally accelerated TLC (Chromatotron) on a 2-mm rotor, prepared from neutral aluminum oxide 60 GF containing 10% calcium sulfate hemihydrate, using a stepwise gradient (1:4 → 3:2) of chloroform−methanol and a flow rate of 3 mL min^{-1}. As before, individual 25-mL fractions were collected and assayed by TLC. By these means, **A** and **B** were isolated from the dry plant in yields of ~0.2 and ~0.05%, respectively.

Alkaloid A was a colorless glass and was insoluble in chloroform, but readily soluble in water or methanol; ^{1}H NMR (400 MHz, in D_2O, TSP-d_4 as ref = 0 ppm) δ_H 5.20 (1H, t, J = 6.5 Hz), 3.97 (2H, d, J = 6.5 Hz), 3.32 (2H, t, J = 7.6 Hz), 3.19 (2H, t, J = 6.6 Hz), 1.99 (3H, s), 1.77 (3H, s), 1.71 (3H, s), 1.64 (2H, pent, J = ~7.0 Hz), and 1.53 (2H, pent, J = ~7.0 Hz); ^{13}C (100 MHz, in D_2O, TSP-d_4 as ref 0 ppm) δ_c 176.8(s), 158.8-(s), 141.9(s), 119.7(d), 50.9(t), 49.6(t), 41.7(t), 28.4(t), 27.6(q), 26.7(t), 24.7(q), and 20.0(q). Addition of a saturated aqueous solution of picric acid to a solution of alkaloid **A** in water gave a bright yellow precipitate that was recrystallized from water or dilute aqueous ethanol to afford lemon-yellow needles: mp, 152−154 °C; lit. mp (Ryabinin 1947, 1948) for smirnovine picrate, 154 °C. The ^{1}H and ^{13}C NMR spectra of this salt were identical with those of synthetic smirnovine picrate.

Alkloid B was also obtained as a colorless glass with solubility properties similar to those of alkaloid **A**: ^{1}H NMR (400 MHz, D_2O, TSP-d_4 as ref = 0 ppm) δ_H 5.38 (1H, t, J = 6.7 Hz), 4.15 (2H, s) 4.06 (2H, d, J = 6.7 Hz), 3.34 (2H, t, J = 7.6 Hz), 3.20 (2H, t, J = 6.6 Hz), 1.99 (3H, s), 1.82 (3H, s), 1.65 (2H, pent, J = ~7 Hz), and 1.54 (2H, pent, J = ~7 Hz); ^{13}C NMR (D_2O, TSP-d_4 as ref = 0 ppm) δ_c 176.9(s), 158.9(s), 142.4-(s), 123.8(d), 62.9(t), 51.0(t), 49.0(t), 41.7(t), 28.4(t), 26.7(t), 24.7(q), 23.5(q); EI-MS (70 eV) m/z 256 (M^+, 1) and 239.1892 (18) (M-OH; calcd for $C_{12}H_{23}N_4O$, 239.1872), 196(8), 114(20), 84(32), and 43(100). These spectra identified alkaloid **B** as 4-hydroxysmirnovine.

Synthesis of Smirnovine Picrate. To a stirred mixture of 10 mL of saturated aqueous potassium carbonate and 30 mL of dichloromethane was added 0.352 g of the hydrochloride salt of **4** (Heesing and Eckard, 1970) at room temperature. The stirring was continued for 1 h, and the organic layer was then separated and dried (K_2CO_3), and the solvent was removed under reduced pressure to provide 256 mg (87%) of the free base **4**.

A solution of 198 mg of this base and 119 mg of phenyl cyanate (freshly prepared according to Martin and Bauer, 1990) in anhydrous diethyl ether was kept for 10 h at room temperature. Evaporation under reduced pressure left 317 mg of crude **5** as a residual oil [^{1}H NMR (200 MHz, $CDCl_3$ δ_H 7.27 ref) δ_H 7.45−6.81 (6H, m, phenyl and NH), 5.28 (1H, m, J = 6.9 and 1.4 Hz), 3.97 (2H, d, J = 6.9 Hz), 3.41−3.27 (4H, m), 1.95 (3H, s), 1.76 (3H, s), 1.68 (3H, s), and 1.71−1.53 (4H, m)]. This oil was dissolved in 20 mL of methanol, which had been saturated with ammonia at room temperature, and 100 mg of ammonium nitrate was added. This solution was kept at room temperature for 7 days, and the solvent then removed under reduced pressure. The residue was taken up in 10 mL of water, and the solution was passed through a column of 10 g of Amberlite IRA-400 resin (OH form), followed by 100 mL of water. The combined eluates were evaporated under reduced pressure, and the residue was taken up in the minimum volume of ethanol. A solution of 112 mg of picric acid in 5 mL of ethanol was added, and the mixture was stored in ice for 30 min, and then filtered. The filter cake was washed with a little ethanol and then recrystallized from 16 mL of hot water to afford 194 mg (40%) of smirnovine picrate as yellow needles: mp, 152−153 °C; lit. mp, 154 °C (Ryabinin, 1947, 1948); ^{1}H NMR (400 MHz, CD_3OD, residual methanol δ_H 3.31 as ref) δ_H 8.74 (2H, s, H-2″ and 5″), 5.16 (1H, mt, J = 6.7 and 1.3 Hz, H-2), 3.96 (2H, d, J = 6.7 Hz, H-1), 3.31 (2H, t, partly obscured by the solvent, H-4′), 3.18 (2H, t, J = 6.8 Hz, H-1′), 1.93 (3H, s, Ac), 1.74 (3H, d, J = 0.8 Hz, H-4), 1.73 (3H, s, H-5), 1.60 (2H, m, H-2′), and 1.50 (2H, pent, J = ~6.8 Hz, H-3′); ^{13}C NMR (CD_3OD, δ_c 49.0 as ref) δ_c 174.0 (CO), 163.6 (picrate-1″), 157.8 (guanidino-C), 143.6 (picrate-2″,6″), 139.8 (C-3), 128.5 (picrate-4″), 126.9 (picrate-3″,5″), 118.7 (C-2), 47.7 (C-1), 39.8 (C-4′), 27.7 (C-4), 26.0 (C-2′), 25.5 (acetate-CH_3), 22.8 (C-3′), and 18.2 (C-5), the C-1′ methylene resonance is buried under the solvent signal.

RESULTS AND DISCUSSION

We isolated two alkaloids (**A** and **B**) from *G. orientalis* foliage. Both alkaloids were highly polar substances, readily soluble in water or methanol, but virtually insoluble in chloroform or diethyl ether.

The ^{1}H NMR spectrum of the major alkaloid (**A**, ~0.2% plant dry weight) contained a set of resonances that corresponded to the hemiterpenoid, prenyl unit of galegine (**1**), and signals for an acetyl and four methylene groups. Selective decoupling and homonuclear COSY spectra revealed that the four methylene groups formed a chain, and the chemical shifts of the terminal ones suggested that they were attached to electronegative heteroatoms, most probably nitrogen. The ^{13}C NMR spectra of **A** (broad band and DEPT) are in accord with these conclusions, and in addition revealed two low-field resonances (δ_c 176.8 and 158.8) that could be ascribed to an acetate-carbonyl and a guanidino-C, respectively.

On the basis of these spectra, we tentatively identified **A** as an *ω-N*-acetyl putrescinyl derivative of galegine [i.e., an *N*-(4-acetamidobutyl)-*N*-(3-methyl-2-butenyl) guanidinium salt (**3**)]. This derivative corresponds to smirnovine, which is an alkaloid that was originally isolated from *Smirnovia turkestana* Bge., a leguminous shrub from the desert regions of Central Asia (Ryabinin, 1947, 1948). The structure of smirnovine was finally established by Heesing and Eckard (1970) via the synthesis of a dihydro derivative of deacetylsmirnovine, that is, dihydrosphaerophysine.

Support for this conclusion was provided by the formation from **A** of a nicely crystalline picrate salt whose melting point was in excellent agreement with that reported for smirnovine picrate (Ryabinin, 1947, 1948). However, because we were unable to obtain an authentic specimen for comparison purposes, we confirmed our identification by synthesizing smirnovine picrate as follows.

A sample of *N*-(4-acetamidobutyl)-*N*-(3-methyl-2-butenyl)amine (**4**) was prepared as described by Heesing

and Eckard (1970). All our attempts to convert this secondary amine to a guanidine by its reaction with *S*-methylisothiourea failed (which may account for the absence of a previous synthesis of smirnovine). However, we were able to overcome this problem by an approach that is, to the best of our knowledge, a novel procedure; that is, addition of the amine to the electrophilic activated nitrile of phenyl cyanate gave a product (**5**; Martin and Bacalaglu, 1980), which upon ammonolysis followed by treatment with picric acid yielded smirnovine picrate. This synthetic material and the picrate of **A** were identical.

We were unable to obtain a crystalline salt of the minor base (**B**, ~0.05% of the dry plant), but the following spectrometric evidence revealed that it was the apparently previously unknown (*Z*)-*N*-(4-acetamidobutyl)-*N*-(4-hydroxy-3-methyl-2-butenyl)guanidinium analogue of **A**; that is, (*Z*)-4-hydroxysmirnovine (**6**). The ^{1}H NMR spectrum of **B** differed from that of **A** only by the replacement of a methyl resonance by a methylene singlet at δ_H 4.09 ppm, corresponding to the hydroxylation of either C-4 or -5 in smirnovine. Irradiation of the methyl resonance (δ_H 1.8 ppm) resulted in a 10% NOE in the methine resonance at δ_H 5.3, whereas in the reverse experiment, irradiation of the methine gave a 4% NOE in the methyl resonance. This result established the *cis* relationship of these two functions and the (*Z*)-geometry of the alkenyl systems. The ^{13}C NMR data (see Experimental Procedures) for **B** were also consistent with the proposed structure. Further support was provided by the EI-MS experiments in which a prominent high-mass fragmention was shown by high-resolution measurement to have the composition $C_{12}H_{23}N_4O$, corresponding to the expected facile loss of OH from the guanidinium cation.

It is interesting that the alkaloids of *G. orientalis* correspond to conjugated forms of the two guanidines (galegine and hydroxygalegine) previously found in *G. offinalis* (Leonard and Playtis, 1972). However, unlike *G. offinalis*, which may be toxic and is considered a weed (Keeler *et al*., 1992) there have been no reports on the toxicity of fodder galega, which has been established as a perennial legume in Estonia since 1972 and in Finland since 1978 (Varis, 1986; Nômmsalu, 1983). We also analyzed a sample of *G. orientalis* from Estonia and isolated smirnovine in 0.2% yield. Thus, smirnovine, the conjugated form of galegine, appears to be much less toxic than galegine itself. This difference has been confirmed in toxicity tests with mice using subcutaneous injections of alcoholic extracts of *G. officinalis* and *G. orientalis* (Köhler, 1969).

From a biosynthetic viewpoint, the *G. orientalis* compounds seem likely to have been derived via the prenylation of arginine or agmatine (Steiniger and Reuter, 1974).

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