Table II. Precision and Accuracy Data for the Determination of Olaquindox in Feeds by 2nd Derivative Spectrophotometry

Olaquindox added, ppm	Olaquindox found,[a] ppm	recovery, %	% RSD
20.0	20.7 ± 1.4	103	7
30.0	31.0 ± 1.1	103	3
60.0	59.5 ± 1.7	99	3
90.0	87.4 ± 0.7	97	1
120.0	116.2 ± 1.2	97	1
150.0	143.8 ± 1.7	96	1

[a] Mean of six replicates ±SD.

Table III. Recovery from Feed Samples Containing 60 ppm of Olaquindox and Various Levels of Other Additives

additive	level in feed, ppm	% recovery of Olaquindox[a]
Amprolium (+ Ethopabate)	125 (+8)	96
Carbadox	50	102
Clopidol	125	98
Erythromycin thiocyanate	200	95
Furaltadone	500	102
Furazolidone	150	102
Monensin	125	96
Nitrofurazone	125	108
Oxytetracycline	500	98
Ronidazole	100	96
Sulfamethazine	200	102
Sulfaquinoxaline	125	96
Tetracycline	500	98
Tylosin	200	102

[a] Mean of two replicates.

significantly influenced by the copresence in the feed of other additives examined.

To evaluate the precision and accuracy of the method, 10-g samples of a poultry feed, fortified with 1 mL of Olaquindox solutions at levels ranging from 20–150 ppm, were analyzed according to the procedure. The results based on six independent determinations at each fortification level are summarized in Table II.

The estimated recovery and relative standard deviation values (RSD) were found to be comparable to those reported for the determination of Olaquindox with the prementioned methods. It appears, however, that a fairly large RSD (7%) should be expected when analyzing samples with an Olaquindox content of 20 ppm, due to matrix effects. In such a case and because of the procedure's simplicity, the method of standard addition may be conveniently applied to compensate for those effects. This alternative was successfully applied on a sample fortified with as low as 10 ppm.

Possible interferences with Olaquindox analysis from the presence of each of the additives shown in Table III were also examined; the determination was significantly affected by Nitrofurazone only, due to overlapping of the 2nd derivative spectra. The presence of this additive at a 125 ppm level resulted in an Olaquindox recovery of 108%, whereas much poorer recovery values (120%) were obtained when Nitrofurazone was added at a 300 ppm level.

In conclusion, the current study shows that the use of 2nd derivative spectrophotometry provides a rapid, accurate, and precise method for the determination of Olaquindox in feeds. Its advantages, i.e., minimal sample preparation, no need for costly reagents and instrumentation, render this method valuable for routine analysis.

Registry No. Olaquindox, 23696-28-8; amprolium, 121-25-5; carbadox, 6804-07-5; clopidol, 2971-90-6; erythromycin thiocyanate, 7704-67-8; furaltadone, 139-91-3; furazolidone, 67-45-8; monensin, 17090-79-8; nitrofurazone, 59-87-0; oxytetracycline, 79-57-2; ronidazole, 7681-76-7; sulfamethazine, 57-68-1; sulfaquinoxaline, 59-40-5; tetracycline, 60-54-8; tylosin, 1401-69-0; ethopabate, 59-06-3.

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Received for review April 2, 1985. Accepted July 5, 1985.

Identification of Toxic Alkaloids from the *calcaratus* Subspecies of *Lupinus arbustus*

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A recent chemical study of the previously uninvestigated *Lupinus arbustus* subspecies *calcaratus* resulted in the isolation and characterization of the novel bipiperidyl–indole alkaloid gramodendrine. The present study describes the identification of three additional structurally diverse alkaloids from the title plant. These include the quinolizidine base lusitanine, the piperidine derivative ammodendrine, and the indole alkylamine gramine. The total alkaloid fraction as well as the first three of the above individual alkaloids were tested for gross locomotor activity and for rotorod performance in mice. Pharmacologic data indicated that gramodendrine and ammodendrine were moderately potent (300 mg/kg, ip) in reducing spontaneous motor activity (30% of control) and in causing central nervous system depression (50% of control).

In the Rocky Mountain region of North America, acute toxicoses and death of livestock grazing on certain species of the legume genus *Lupinus* has been attributed to the quinolizidine alkaloid content of these plants (Couch, 1937; Kingsbury, 1964; Keeler, 1975). The age and species of lupine together with the species of animal contribute to a variation in the symptoms and severity of poisoning (Couch, 1937; Beath et al., 1953). Recently, it was shown that the ingestion of certain lupine species by pregnant

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0021-8561/85/1433-0909$01.50/0 © 1985 American Chemical Society

cows causes crooked calf disease in the offspring (Shupe et al., 1967). It has been suggested that the quinolizidine alkaloid anagyrine is the teratogen responsible for the congenital deformities (Keeler, 1973).

In our search for new alkaloids from potentially toxic lupines, the unusual yellow flowered *Lupinus arbustus* Dougl. ex Lindl. subsp. *calcaratus* (Kell.) Dunn has been collected and examined chemically. The initial part of this study resulted in the isolation and characterization of a novel bipiperidyl–indole alkaloid which we named gramodendrine (Keller and Hatfield, 1982).

The present report describes the identification of additional, structurally diverse alkaloids in the *calcaratus* subspecies of *L. arbustus*. A preliminary pharmacologic evaluation of the total alkaloid fraction as well as individual components of this mixture is also reported.

EXPERIMENTAL SECTION

Apparatus. Melting points were determined on a Fisher 355 digital melting point analyzer and are uncorrected. The IR spectra were recorded neat on a Perkin-Elmer 257 grating infrared spectrophotometer. Both ^1H NMR (360 MHz) and ^{13}C NMR (90.56 MHz) spectra were measured in $CDCl_3$ on a Bruker spectrometer. With use of a Hewlett-Packard 5880 A, GC was carried out over OV-17 (10 m × 0.25 mm fused silica capillary column) with a temperature program of 4 °C per min from 130–230 °C. The same GC system was combined with a DuPont 321 Dimaspec low-resolution mass spectrometer (GC–MS) interfaced with a 320 data reduction system.

Spontaneous motor activity of male Swiss-Webster mice was measured by using a Stoelting Model 31410 Electronic Activity Monitor System.

Plant Material. The *Lupinus arbustus* Dougl. ex Lindl. subsp. *calcaratus* (Kell.) Dunn (Leguminosae) used in this investigation was collected 10 miles east of the Hailey city limits in the Cove Creek area of Blaine County, ID, on July 9, 1979. The plant was identified by Dr. David B. Dunn and a voucher (preserved) specimen (UMO-130847) representing the material collected is available for inspection at the University of Missouri Herbarium, Columbia, MO 65201.

Extracts of *L. chamissonis* Eschsch., *L. hirsutissimus* Benth., and *L. sparsiflorus* Benth. were obtained from Dr. Alan D. Kinghorn, University of Illinois at the Medical Center, Chicago, IL 60680. Extraction procedures and identification of these lupines have been reported elsewhere (Kinghorn et al., 1980).

Extraction and Isolation of Alkaloids. The dried, powdered (40 mesh) aboveground plant parts (200 g) were homogenized with MeOH and processed as usual (Keller and Zelenski, 1978) to give 1.30 g (0.65% of dry weight) of a crude alkaloid extract. TLC of this mixture over 0.25-mm layers of silica gel 60 GF 254 (Merck) with $CHCl_3$–MeOH–18 M NH_4OH (100:10:1) indicated the presence of three alkaloids located at R_f 0.73, 0.44, and 0.31 after spraying with Dragendorff reagent. Further TLC with the same system revealed the presence of trace quantities of an additional, Ehrlich-positive compound at R_f 0.18. A GC analysis demonstrated the presence of three peaks with t_r of 5.75, 10.65, and 11.43 min.

The major alkaloids were isolated by first chromatographing the 1.30 g of alkaloid extract over an 80-g (50 cm × 2 cm) column of 70–230 mesh ASTM silica gel 60 (Merck). Fractions (20 mL) were collected during the gradient elution with 450-mL volumes of each of the following solvent combinations: $CHCl_3$–MeOH 12:1, 8:1, 4:1, and 2:1. This process yielded 47 mg of R_f 0.73 (fractions 15–26), 542 mg of R_f 0.44 (fractions 31–57), and 522 mg of a binary mixture containing R_f 0.44 and R_f 0.31 (fractions 58–102). The Ehrlich-positive compound (R_f 0.18) did not elute from the column.

Preparative TLC of pooled fractions 58–102 over 1-mm layers of silica gel 60 PF_{254} (Merck) with cyclohexane–diethylamine (7:3) afforded 170 mg of R_f 0.44 and 236 mg of R_f 0.31.

Alkaloid Identification. The R_f 0.73 alkaloid has recently been identified as a novel bipiperidyl–indole derivative, gramodendrine, based on spectral data, degradation to methoxymethylindole, and synthesis from ammodendrine (Keller and Hatfield, 1982).

The isolated R_f 0.44 alkaloid was identified as ammodendrine based on previously recorded chromatographic and spectral parameters (Keller and Hatfield, 1982). Ammodendrine was further characterized by preparing the perchlorate derivative, mp 210–211 °C (lit. mp 210–211 °C (Fitch et al., 1974)).

The R_f 0.31 alkaloid was chromatographically identical with lusitanine. This preliminary identification was substantiated with the following: mass spectrum, m/z (relative intensity) 208 (M^+, 100), 179 (35), 166 (79), 149 (14), 136 (31), and 110 (9); ^1H NMR ($CDCl_3$) δ 8.36 (d, 1), 6.66 (d, 1), and 2.09 (s, 3). In addition to these signals, a broad multiplet integrating for 15 protons was observed at δ 3.20–1.20. The most significant IR adsorptions were the following: γ_{max} (neat) 2940 and 2860 (*trans*-quinolizidine) and 1645 cm^{-1} (C=O). Free base lusitanine was crystallized from acetone, mp 183–185 °C (lit. mp 184–186 °C (Steinegger and Wicky, 1965)).

Gramine (R_f 0.18) was tenatively identified after chromatographic comparisons with reference material. This identification was confirmed with the following: mass spectrum, m/z (relative intensity) 174 (M^+, 23), 131 (19), 130 (100), and 77 (13).

Rotorod Testing. Male Swiss-Webster mice, weighing 25–35 g, were placed on a 2.8 cm diameter × 16 cm long wooden rod rotating at 5 rpm (Bourn et al., 1978; Bourn et al., 1979). After a 30-min training period, six animals per treatment group were injected intraperitoneally with either a solution of normal saline (control), alkaloid extract hydrochloride in saline (300 mg/kg), or isolated alkaloids in saline (300 mg/kg of gramodendrine, 200 mg/kg of ammodendrine hydrochloride, or 100 mg/kg of lusitanine). Tween 80 (10%) aided solubilization of gramodendrine and lusitanine. Single doses were evaluated due to minimal amounts available after lengthy alkaloid isolation. Dosage levels were chosen based on preliminary screening of extract effects.

At fifteen minute intervals after injection, mice were placed on the rotating rod and the length of time each animal was able to remain on the rod was determined. These measurements continued for 2 h after treatment. A cutoff time of 90 s was used for animals which did not fall from the rod.

Locomotor Activity. Disturbance of an electromagnetic field by individual male Swiss-Webster mice (25–35 g) in cages within the monitor system resulted in voltage pulses proportional in magnitude to animal motor activity (Bourn et al., 1978; Bourn et al., 1979). Disturbances were counted with electronic counters and activity was measured as counts per min. Total observation time was 2 h after treatment. Test periods commenced after intraperitoneal administration to six animals per treatment group of either normal saline (control), alkaloid extract hydrochloride in saline (300 mg/kg) or isolated alkaloids in saline (300 mg/kg of gramodendrine, 300 mg/kg of ammodendrine hydrochloride, or 100 mg/kg of lusitanine). Tween 80

Table I. Pharmacological Measurements following Alkaloid Treatment[a]

compound	dose, mg/kg	rotorod, s	locomotor activity, counts/min
control (saline)	0	88 ± 1	24 ± 1
extract	300	32 ± 10[b]	8 ± 1[b]
gramodendrine	300	36 ± 10[b]	7 ± 1[b]
ammodendrine	300	47 ± 11[b]	7 ± 2[b]
lusitanine	100	67 ± 6[b]	7 ± 1[b]

[a] $n = 6$ animals per treatment group; ±SEM. [b] Significantly different from control ($p < 0.05$) as determined by Dunnett's test (Dunnett, 1964).

(10%) aided solubilization of gramodendrine and lusitanine.

RESULTS AND DISCUSSION

Recently we reported the isolation and characterization of a novel bipiperidyl–indole alkaloid from the *calcaratus* subspecies of *Lupinus arbustus* (Keller and Hatfield, 1982). The name gramodendrine was selected because of the close structural relationship to gramine and ammodendrine. An important part of this initial study was a demonstration of the thermal decomposition of gramodendrine to the bipiperidyl alkaloid ammodendrine during the course of GC.

A recent report described the alkaloid distribution in 21 New World *Lupinus* species (Kinghorn et al., 1980). Three of these plants, *L. chamissonis*, *L. hirsutissimus*, and *L. sparsiflorus*, were shown to contain ammodendrine, several known quinolizidine bases, and an unknown alkaloid. Based on our observation that gramodendrine readily decomposes during GC to give ammodendrine as the detectable product, it was postulated that gramodendrine could be the unknown alkaloid in the above lupine species. However, TLC analysis of extracts of these lupines confirmed the presence of ammodendrine but revealed the absence of gramodendrine. Therefore, *L. arbustus* subsp. *calcaratus* is presently the only plant known to produce and accumulate gramodendrine.

In addition to gramodendrine, the isolation of ammodendrine, lusitanine, and gramine demonstrated a great alkaloid biosynthetic diversity in *L. arbustus* subsp. *calcaratus*. The major alkaloid was ammodendrine, a piperidine derivative that has been isolated from species of several legume genera including *Lupinus* (Mears and Mabry, 1971; Kinghorn, et al., 1980). Lusitanine, a bicyclic quinolizidine base, has a much more restricted distribution having been isolated from only two other plants, the leguminous *Genista lusitanica* L. (Steinegger and Wicky, 1965) and *Chamaecytisus austriacus* (L.) Link, subsp. *stefanoffii* (Stoj.) Kuzm (Daily and Dutschewska, 1979). The detection of trace quantities of the indole alkaloid gramine in *L. arbustus* subsp. *calcaratus* was not surprising in view of the concurrent presence of gramodendrine in this plant. Gramine was originally obtained from the leaves of germinating barley (Von Euler and Erdtmann, 1935) and has since been isolated from a number of different plants including several species of lupine (Mears and Mabry, 1971; Anderson and Martin, 1976). These phytochemical data demonstrate *L. arbustus* subsp. *calcaratus* to be the most versatile of the lupines with respect to production of diverse structural types of alkaloids.

An accurate GC quantitation of the individual components of the alkaloid mixture was precluded by the thermal decomposition of gramodendrine. A careful measurement of spot size after analytical TLC yielded semiquantitative data. This process indicated the following relative concentrations of the *L. arbustus* subsp. *calcaratus* alkaloids: ammodendrine 86%, lusitanine 10%, gramodendrine 3%, and gramine 1%.

An initial pharmacologic screen of the total alkaloid hydrochlorides produced unusual behavioral responses in rats at an intraperitoneal dose of 300 mg/kg (Keller, 1984). Within 5 min after administration of these compounds, the test animals became lethargic, lost their righting reflex, and exhibited ptosis while remaining motionless. These animals were easily aroused but quickly relapsed into their quasi-hypnotic state. It was also unusual to note that these effects were still evident two days after dosing with the total alkaloid hydrochlorides.

These gross observations prompted further pharmacologic evaluations of the entire mixture as well as the individual alkaloids. Gramine, however, was not tested because of its very low concentration in the alkaloid fraction. Table I shows the effects of the extract and the individual alkaloids on motor coordination (rotorod performance) and spontaneous motor activity (locomotor activity).

At a 300 mg/kg intraperitoneal dose, ammodendrine and gramodendrine both produced a significant ($p < 0.05$) reduction in spontaneous motor activity to about 30% of control values. At the same dose, ammodendrine and gramodendrine significantly ($p < 0.05$) reduced time spent on the rotorod to approximately 50% of control values. Lusitanine was too toxic to test at doses above 100 mg/kg. Mice died at doses of 200 and 300 mg/kg. However, at 100 mg/kg, spontaneous motor activity was significantly ($p < 0.05$) reduced to 30% of control values and rotorod performance was reduced to 76% of control values. Therefore, ammodendrine and gramodendrine were essentially equipotent to the alkaloidal extract (Table I).

Data from these pharmacologic tests indicate the *L. arbustus* subsp. *calcaratus* alkaloids impair motor function in mice. These compounds would undoubtedly contribute to the toxicity that may be observed in livestock that have grazed on *L. arbustus* subsp. *calcaratus*. The pharmacologic action of ammodendrine that was demonstrated in this study would contribute to the toxicity of other North American lupines known to contain this alkaloid (Kinghorn et al., 1980). A more precise description of the pharmacologic action of these moderately toxic alkaloids can be ascertained only from extensive further testing which is currently in progress.

ACKNOWLEDGMENT

We acknowledge the technical assistance of Cheryl B. Keller in locating and collecting the plant material and of Professor David B. Dunn who identified the voucher specimens. We also wish to thank Professors John N. Davisson and William M. Bourn for their technical advice. We greatly appreciate the donation of reference ammodendrine from Professor Carl Djerassi, of gramine from Professor J. L. McLaughlin, and of lusitanine from Professor E. Steinegger.

Registry No. Gramodendrine, 83905-67-3; lusitanine, 5121-36-8; ammodendrine, 494-15-5; gramine, 87-52-5.

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Received for review February 4, 1985. Accepted May 20, 1985. This research was supported, in part, by a grant from the Northeast Louisiana University (NLU) Faculty Research Fund.

A Study on Gelation of Soybean Globulin Solutions. 5. The Effect of Protein Concentration on the Extent of Conversion in Gelation Process According to Data of Sol-Analysis

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A study has been undertaken on the effect of protein concentration, C, on gel yield, G, in the process of thermotropic gelation of three soybean globulin preparations (5% of β-conglycinin + 95% of glycinin; 85% of β-conglycinin + 15% of glycinin; 50% of β-conglycinin + 50% of glycinin) at pH 7. G equals zero until a certain concentration C^* and then increases with concentration to some constant level. The master concentration dependences of G vs. $\tilde{C} = C/C^*$ for the preparations are the same at low $\tilde{C}$ and are different at high $\tilde{C}$. The initial slope of these dependences equals 2. Sol fractions of all preparations consisted of acidic subunits of glycinin. It has been suggested that gelling ability of proteins should be estimated by the value of the average hydrophobicity parameter $H\phi_{av}$ according to Tanford-Bigelow. The gelling ability of the monomeric forms of main soybean globulins diminishes in the series 2.8S globulin > B > β > α > A, where A and B are acidic and basic subunits of glycinin and α and β are heavy and light subunits of β-conglycinin.

INTRODUCTION

At the present time a thermotropic gelation of proteins is being studied widely in connection with the great significance of this phenomenon for food technology. A number of studies (Babajamopoulus et al., 1983; Mori et al., 1982a,b; Nakamura et al. 1983, 1984a,b; Utsumi et al., 1983) was devoted to the elucidation of the molecular mechanism of thermotropic gelation. One can also mention the works of van Kleef et al. (1978) and Richardson and Ross-Murphy (1981a,b), where the methodology of modern physical chemistry of polymers was applied to study some interesting aspects of protein gelation. In all of these works only some properties of gel networks were considered. However, it is well-known that the gelation of polymers leads to the formation not only of networks but also some soluble products (the finite clusters) (Flory, 1953). He calls these products the sol-fraction of the gel. The investigation of qualitative and quantitative aspects of the sol-fraction formation permits us to understand some important details of the gelation mechanism (Irzshak et al., 1979).

In this report the qualitative and quantitative aspects of sol-formation for thermotropic gelation of soybeans globulins are considered. Since this approach to studying thermotropic gelation of proteins is being stated for the first time, some general information from the physical chemistry of polymers is cited in the paper to facilitate understanding the data obtained.

The most important characteristics of the gelation process appear to be the content of a soluble sol-fraction (S) in the gel and its equilibrium elasticity modulus (E_e) (Flory, 1953). Sol-fraction content and the modulus have similar information but not identical. They outline the extent of conversion in this process. The lower the content of sol-fraction and the greater the modulus the higher is the extent of conversion. The value (1 - S), which is termed as gel yield (G), may be identified to a larger degree with the extent of conversion, while the modulus depends not only upon the extent of conversion, but also upon the gel structure.

The statistical theory considers gelation as a result of the formation of multiple random bonds between the particles that can be either polyfunctional monomers or macromolecules. It provides a connection between gel yield of the elasticity modulus and the extent of conversion for random bonds (p). Analysis of experimental dependences $G(p)$ and $E_e(p)$ makes it possible to study the gelation mechanism in the given system.

In the case of thermotropic gelation of polymers, the extent of conversion in the reaction of association or aggregation of macromolecules is usually unknown. It directly relates to the concentration of a polymer in com-

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