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Structure and Toxicity of Alkaloids and Amino Acids of *Sophora secundiflora*

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Abstract □ Seeds from *Sophora secundiflora* were extracted into three major fractions: lipids, alkaloids, and amino acids. Most of the lipid material was composed of steroid esters. These esters were hydrolyzed, and the fatty acid compositions were determined. The major alkaloid component was cytisine, with *N*-methylcytisine, anagyrine, and termopsine in lower concentration. The major free ninhydrin-positive compound was γ-glutamyltyrosine. In addition to several common amino acids, pipecolic acid and 4-hydroxypipecolic acid were identified. Both the amino acid fraction and the alkaloid fractions caused minor pharmacological disorder when injected into rats. However, when both fractions were simultaneously administered, they were lethal.

Keyphrases □ *Sophora secundiflora*—seeds, alkaloids and amino acids isolated and identified, toxicity investigated in rats □ Alkaloids—isolated and identified, *Sophora secundiflora* seeds, toxicity, rats □ Amino acids—isolated and identified, *Sophora secundiflora* seeds, toxicity, rats

The toxicity of *Sophora secundiflora* (Ort.) DC to humans and domestic animals has been recognized for some time (1–3). The ingestion of the leaves was reported to lead to the production of poisonous milk (1), and *S. secundiflora* seeds (mescal beans) are known for their hallucinogenic effects among Indian tribes in the Southwestern United States and Mexico (4). Although the hallucinogenic effects of these seeds have been attributed to the presence of a high concentration of the alkaloid cytisine (4, 5), several species of *Laburnum, Ulex, Cytisus,* and *Sophora* containing this alkaloid are not hallucinogenic (6).

The presence of toxic amino acids in various plants has been documented (7, 8), and the toxic and/or hallucinogenic effects of *S. secundiflora* possibly may be due to nonalkaloid materials. Furthermore, atypical fatty acids and steroids are known to cause some neuropathies (9). Therefore, it was of interest to isolate and characterize these constituents and to determine which component(s) are responsible for the toxicity of these seeds.

EXPERIMENTAL

Extraction of Seeds—Mature seeds of *S. secundiflora*[1] were obtained from Mt. Bonnell, Austin, Tex. The hulled seeds (200 g) were machine ground and extracted with 500 ml of petroleum ether (lipid fraction). The residue was then extracted (soxhlet) with 800 ml of chloroform containing 0.5 ml of ammonium hydroxide (alkaloid fraction), and this residue was then extracted with 1000 ml of 50% ethanol (amino acid fraction). In some cases, the ground seeds were directly extracted with acidic chloroform from a methanolic extraction (soxhlet) of the ground seeds.

Fractionation and Analysis of Amino Acids—The amino acid fraction was reduced to 100 ml and applied to a 3.8 × 45-cm column of ion-exchange resin[2] (ammonium form). After washing with water, the column was eluted with 0.5 *N* ammonium hydroxide, and the ninhydrin-positive fractions were combined and reduced to dryness *in vacuo* to yield 3.8 g of solids. This mixture was then applied to a 2.8 × 45-cm column of ion-exchange resin[3] (acetate form) and washed with water to elute the neutral and basic amino acids. Subsequent elution with 0.5 *N* acetic acid resulted in the isolation of aspartic and glutamic acids and 350 mg of the unknown dipeptide.

The mixture of neutral and basic amino acids was then applied to a 2.2 × 100-cm column of ion-exchange resin[4] (hydrogen form), and the neutral amino acids were eluted with water. This fraction was applied to a 5.2 × 75-cm column of ion-exchange resin[3] (hydrogen form) and washed with water. Subsequent elution with 0.2 *N* HCl led to the isolation of 4-hydroxypipecolic acid and pipecolic acid.

[1] The plant material was identified as *Sophora secundiflora* L. by Dr. M. Aboul-Ela, Department of Biology, Texas Women's University. A voucher specimen (5-175) is available for inspection at the Herbarium of the Department of Biological Sciences, North Texas State University.

[2] Amberlite CG 120.

[3] Amberlite CG 400.

[4] Amberlite IR 50.

Table I—Amino Acid Analysis of *S. secundiflora* Seeds

Amino Acid	Elution Time, min	Macromoles of Amino Acid per Gram of Seeds	
		Unhydrolyzed	Hydrolyzed
Aspartic acid	75	5.0	12.5
Asparagine	85	Trace[a]	0
Serine	102	Trace	3.0
4-Hydroxypipecolic acid	110	32.0	26.0
Glutamic acid	122	31.0	80.0
Proline	132	3.5	3.0
Glycine	168	3.0	17.0
Alanine	176	6.0	7.0
Pipecolic acid	220	Trace	Trace
Unknown	225	90.0	Trace
Valine	231	Trace	Trace
Isoleucine	260	Trace	Trace
Leucine	272	Trace	Trace
Tyrosine	315	11.0	46.0
Phenylalanine	324	0	1.0
Cadaverine	14	Trace	0
Tryptophan	18	Trace	0
Lysine	24	Trace	Trace
Histidine	28	Trace	Trace
Arginine	48	Trace	Trace

[a] "Trace" indicates less than 0.5 μmole/g of seeds.

Amino acid analysis was carried out on an automatic amino acid analyzer[5], employing a buffer change from pH 3.28 to 4.25 at 150 min (flow rate = 80 ml/hr, temperature = 55°). Basic amino acids were analyzed by the standard procedure (10).

Fractionation and Analysis of Alkaloids—Samples of the alkaloid fraction were applied to thin-layer plates[6] (250 μm) and chromatographed in chloroform–methanol (75:25). Spots were visualized with the Dragendorff reagent, and the R_f values of the alkaloids were compared with those of authentic standards. The cytisine alkaloids were also identified by a modification of the specific color reaction of White (11). Upon spraying with a 1% (w/v) solution of ferric chloride in chloroform–acetone (3:1), the cytisine alkaloids developed an orange-red color. Subsequent spraying with a 3% solution of hydrogen peroxide caused the color to change to a light blue.

Cytisine was extracted and crystallized from acetone by the addition of ether; *N*-methylcytisine (caulophylline) was synthesized from cytisine according to the method of Cocklurn and Marion (12) for *N*-ethylcytisine. The physical and chemical properties of all these alkaloids were verified by comparison with published values (11–13).

Fractionation and Analysis of Lipids—The lipid extract was chromatographed on thin-layer plates[6] containing 1% ammonium sulfate. Chromatograms were developed in ether–benzene–ethanol–acetic acid (40:50:2:0.2) and visualized by charring at 200° (14). The steroid esters were isolated by preparative chromatography of 100 mg of the lipid extract on thin-layer plates, using a solvent of petroleum ether–ethanol (95:5). Under these conditions, most lipids remained at the origin, while the steroid esters migrated ($R_f \sim 0.45$).

The appropriate areas were scraped and eluted with chloroform–methanol (2:1). After solvent removal, the steroid esters were hydrolyzed under nitrogen by refluxing in methanol–benzene–sulfuric acid (90:5:5) for 3 hr. The methyl esters of the fatty acids were extracted with ether and subjected to GC at 250° on 15% ethylene glycol succinate[7] (80–120 mesh).

Pharmacology—Male Sprague–Dawley rats, 150–200 g, were used. Samples to be injected were taken to dryness and redissolved in physiological saline. Injections of 0.5 ml were performed subcutaneously, and controls received 0.5 ml of saline. For each experiment, five animals were used and received identical injections.

Spectral Studies—Proton NMR spectra were obtained on a 60 MHz spectrometer[8]. Deuterium oxide was used as the solvent, and all spectra are expressed relative to sodium 3-(trimethylsilyl)propinate-2,2,3,3-d_4. IR spectra were obtained using either potassium bromide or sodium chloride disks[9].

[5] Beckman 120C, using Aminex 4A resin (BioRad).
[6] Adsorbosil-5.
[7] Gas Chrom P.
[8] Varian Mini-Mar.

Table II—Identification of the Unknown Peptide and 4-Hydroxypipecolic Acid

Method	Unknown Peptide	γ-Glutamyltyrosine[a]	4-Hydroxypipecolic Acid[b]
Paper chromatography			
Solvent 1[c]	0.29	0.29	0.25
Solvent 2	0.21	0.21	0.83
TLC			
Solvent 3	0.59	0.59	0.61
Solvent 4	0.03	0.03	0.27
Elemental analysis			
Carbon	54.35	(54.19)[d]	49.98 (50.00)
Hydrogen	5.88	(5.89)	7.96 (7.58)
Nitrogen	9.04	(9.07)	9.90 (9.72)
Amino terminal analysis [reaction with 1-fluoro-2,4-dinitrobenzene (12)]	Glutamic acid	Glutamic acid	—
Mass spectrum of trifluoroacetylated sample (13), daltons	310	310	—

[a] From Morris and Thompson (19). [b] Component that eluted at 100 min in amino acid analysis. [c] Solvent 1 = butanol–acetic acid–water (4:1:1 v/v); Solvent 2 = phenol–water (4:1) in the presence of ammonia vapor; Solvent 3 = 2-propanol–butanone–1 *N* HCl (60:15:25 v/v); Solvent 4 = 2-methylpropanol–butanone–methanol–propanone–water with 0.88% ammonia (40:20:1:20:14 v/v). [d] Calculated theoretical values are shown in parenthesis.

RESULTS

Amino Acids and Peptides—The results of amino acid analysis of the 50% ethanol-soluble extract of *S. secundiflora* seeds are presented in Table I. In addition to several L-α-amino acids, three unusual ninhydrin-positive compounds were resolved. 4-Hydroxypipecolic acid eluted at 100 min between serine and glutamic acid and comprised a major free amino acid. Acid hydrolysis of the extract resulted in a slight decrease in the concentration of 4-hydroxypipecolic acid, while its oxidation products, aspartic acid and glycine (aminoacetic acid), increased. Its proton NMR spectrum was found to be identical to that reported for authentic 4-hydroxypipecolic acid (15, 16). The structure of the compound was verified further by chromatography in several solvent systems and by carbon, hydrogen, and nitrogen analysis (Table II). A trace of ninhydrin-positive material eluted at 220 min coincident with standard pipecolic acid.

The major ninhydrin-positive material of the seeds eluted at 225 min. Upon acid hydrolysis, this component was destroyed with the stoichiometric formation of glutamic acid and tyrosine. These data suggested that the material was probably a peptide composed of tyrosine and glutamic acid or glutamine. This unknown peptide was isolated as described under *Experimental* and subjected to further analysis. Chromatographic properties in four solvent systems, carbon, hydrogen, and nitrogen analysis, amino terminal analysis, and mass and IR spectra were all consistent with an authentic sample of γ-glutamyltyrosine (Table II) (17–20).

The proton NMR spectrum of the isolated dipeptide was as follows: δ 6.8–7.4 (quartet, 4H, J = 8 Hz, aromatic H), ~4.7 (partially hidden by water peak, ~ triplet, tyrosine CH), 4.06 (quartet, 1H, J = 6 Hz, glutamic acid CH), 2.9–3.2 (~two doublets, 2H, tyrosine CH_2), and 1.9–2.8 (multiplet, 4H, glutamic acid CH_2CH_2).

The γ-linkage of the dipeptide was determined by quantitating the carbon dioxide released upon reaction with ninhydrin (21, 22) (Table III). Controls of the aspartic acid and glycine with one free

[9] Perkin-Elmer model 237.

Table III—Titration Results from Van Slyke Ninhydrin Determination

	0.013 g of Aspartic Acid	0.007 g of Glycine	0.013 g of Glycylglycine	0.019 g of Glycylaspartic Acid	0.031 g of γ-Glutamyltyrosine
Control (without sample)	7.5[a]	7.15	7.3	7.5	6.9
Experiment (with sample)	5.10	5.15	7.1	7.4	4.7
Difference	2.40	2.00	0.20	0.10	2.2

[a] Volume of 0.1 *N* HCl in milliliters used to titrate the carbon dioxide evolved from 100 μmoles of amino acid or dipeptide upon reaction with ninhydrin.

α-amino-α-carboxylic acid showed evolution of 1 mole of carbon dioxide/mole of amino acid upon reaction with ninhydrin. Control dipeptides (glycylglycine and glycylaspartic acid) containing no free α-amino-α-carboxylic group showed essentially no carbon dioxide evolution. The unknown dipeptide, upon reaction with ninhydrin, evolved 1 mole equivalent of carbon dioxide/mole of peptide, thus confirming the presence of an α-amino-α-carboxylic group. These data are only consistent with the γ-glutamyltyrosine linkage.

Alkaloids—Cytisine was isolated in crystalline form from the alkaloid fraction. *N*-Methylcytisine was synthesized from cytisine according to the literature (11) and used as a reference to confirm the presence of this alkaloid in the plant. Anagyrine and termopsine were also isolated but in lower quantities. The IR and UV spectra and the chromatographic behavior of these alkaloids corresponded to reported values (11–13).

Lipids—The lipid fraction comprised 20% of the dry weight of the seeds of *S. secundiflora*. When this lipid fraction was analyzed by TLC, only small amounts of phospholipids, free fatty acids, and *mono-, di-, and triglycerides were observed.* Most of the lipid materials migrated at the solvent front and thus were presumed to be steroid esters (14). After hydrolysis of these esters, the fatty acid components were determined by GC. The number of carbons, degree of unsaturation, and relative amounts of each fatty acid were determined as follows: 16:0 (13.1%), 16:1 (0.7%), 18:0 (3.6%), 18:1 (47.0%), 18:2 (34.5%), 18:3 (0.5%), 20:0 (0.5%), and 20:1 (0.1%).

Toxicity—To assess the toxic nature of the various fractions, a series of pharmacological experiments was conducted. When the 50% ethanol-soluble extracts from 250 mg of seeds (containing both amino acid and alkaloid fractions) were injected simultaneously into rats, death occurred within 10 min. But if the alkaloids were first removed from this extract by alkaline chloroform, the alkaloid-free material (amino acid fraction) was no longer lethal to the animals. However, the animals displayed an obvious hypersensitivity to sound for at least 1 hr following the injection.

When the alkaloids alone (from 250 mg of seeds) were injected, the animals exhibited drowsiness and complete closure of the eyes. However, the alkaloid extract alone was not lethal, and complete recovery was apparent by 2 hr postinjection. When mixtures of the alkaloid extract and the alkaloid-free amino acid fraction were injected together at the same concentration as before, death resulted to all animals within 10 min.

Lipid extracts from 250 mg of seeds administered either as the crude extract or the isolated steroid esters caused no observable effects on the animals. Mixtures of either the lipid extract and alkaloid fraction or the lipid extract plus the amino acid fraction gave the same response as the extracts without the added lipid fractions.

DISCUSSION

Although the presence of pipecolic acid, 4-hydroxypipecolic acid, and γ-glutamyltyrosine was observed previously in plants, this study is the first demonstration of a combination of these amino acids and the dipeptide in a single plant. While the physiological function of γ-glutamyltyrosine remains obscure, high concentrations of this peptide were reported in other plants (19, 20, 23).

The described pharmacological studies demonstrate that a 50% ethanolic extract of 250 mg of the seeds containing both amino acid and alkaloid fractions is lethal to animals. However, neither the alkaloid nor amino acid fractions alone are lethal; therefore, both classes of compounds apparently are involved in the toxicity of these seeds. While it is known that homologs of proline (such as pipecolic acid and 4-hydroxypipecolic acid) can be incorporated into peptides leading to nonfunctional proteins (24), the rapid death of the animals does not seem to be consistent with the synthesis of such nonfunctional proteins.

Although a variety of possible explanations could be proposed to account for the synergistic toxicity of the alkaloids and amino acid fractions, more detailed studies are required to establish the precise mechanisms involved.

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