BIOCHEMICAL SYSTEMATICS OF THE GENUS SOPHORA

DISSERTATION

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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December, 1973

Izaddoost, Mohamed, Biochemical Systematics of the Genus Sophora. Doctor of Philosophy (Molecular Biology), December, 1973, 78 pp., 9 tables, 35 illustrations, bibliography, 36 titles, appendix.

Three unusual amino acids, γ-amino-n-butyric acid, pipecolic acid, and 4-hydroxypipecolic acid, and an uncommon dipeptide, γ-glutamyltyrosine, have been isolated and characterized from the seeds of members of the genus Sophora. Structural proof of these compounds was carried out by paper chromatography, thin-layer chromatography, column chromatography on amino acid analyzer, infrared, nuclear magnetic resonance, mass spectrometry, and C, H, N analysis. The presence and absence of these compounds was used as a criterion for the classification of 23 species of the genus Sophora. A phylogenetic classification which seems to follow the morphological taxonomy of this genus was carried out on the basis of seeds that contained pipecolic acid, those which did not contain pipecolic acid, and plants which contained both pipecolic acid and 4-hydroxypipecolic acids. Another chemical classification was also introduced based on the presence and absence of γ-amino-n-butyric acid and γ-glutamyltyrosine.

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CHAPTER I

INTRODUCTION

The study of systematics in botany has classically been based on morphological features of the plants: flower, leaf, stem, etc. However, more recently, cytological, untrastructural, and biochemical information has been used to supplement the historical methods of classification. These latter techniques have proved to be a useful tool in obtaining taxonomical information.

Amino acid sequences and distributions of proteins, as well as hybridization studies on deoxyribonucleic acid (DNA), have been used to confirm morphological relationships at the generic level and above. The distribution and biosynthetic pathways of compounds of low molecular weight have also been used to approach some taxonomic problems at the generic level and lower. These compounds include flavonoids, terpenes, sesquiterpenes, alkaloids, phenolic compounds, volatile compounds, and unusual amino acids (amino acids which are not found in protein sequences).

The use of amino acids in taxonomic problems is aided by the fact that certain unusual amino acids are produced by different species of the same genus (2,33). These unusual amino acids indicate unique biochemical pathways which have evolved in the individual species.

Members of the genus Sophora (Family Leguminosae) are small trees and shrubs found in tropical and subtropical parts of the world. Some of the plants are toxic to man and most domestic animals and may even lead to the production of toxic milk (12,21). The seeds of some of these plants (better known as mescal beans, big-drunk bean, Frijollito and coral beans) contain an alkaloid, cytisine[i], which is thought to be hallucinogenic, although it has been pointed out that some members of the genus Sophora and other genera contain this alkaloid and are not hallucinogenic (25,32).

Traditionally, the members of the genus have been classified according to the shape of the ovary, presence and absence of stiples, arrangement and shape of the wings, etc. However, at this time, there is a disagreement as to the number of species found in this genus, as well as methods of classification of the genus, which lead to a variety of synonyms[ii] for the genus itself (7,16).

During the course of studies of the amino acid chemistry of Sophora secundiflora (mescal beans) I have found two unusual amino acids and one uncommon dipeptide, γ-glutamyltyrosine. We also have found in some species of Sophora another unusual amino acid, γ-amino-n-butyric acid. Therefore, it was of interest to carry out a systematic study on the distribution

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[i]Synonyms: Cytoxine, Laburnine, Ulexine, Baptitoxine, and Sophorine.

[ii]Some synonyms of Sophora are: Broussonetia Orteg, Edardsia Salisb, Patrinia Raf, Pseudosophora DC, etc.

of unusual amino acids in several species of the genus Sophora in an attempt to clarify and further delineate the classification of this genus.

CHAPTER II

MATERIALS AND METHODS

Materials

Species of Sophora to be used are as follows:

I—Plants obtained from Herbarium of the Department of Botany, University of Texas at Austin:

1-*Sophora angostifolia*
2-*Sophora allopecuroides*
3-*Sophora affinis*
4-*Sophora chrysophylla*
5-*Sophora griffithii*
6-*Sophora gypsophylla*
7-*Sophora japonica*
8-*Sophora microphylla*
9-*Sophora reticulata*
10-*Sophora serica nutteliana*
11-*Sophora secundiflora*
12-*Sophora tomentosa*

II—Plants obtained from the Herbarium of Southern Methodist University at Dallas, Texas:

1-*Sophora formosa*
2-*Sophora stenophylla*

III—Plants obtained from the Herbarium of the University of California, at Berkeley:

1-*Sophora leachiana*
2-*Sophora longipesman*
3-*Sophora macrocarpa*
4-*Sophora massafeurana*
5-*Sophora moorcroffiana*
6-*Sophora mollis*
7-*Sophora pachycarpa*
8-*Sophora tetrapetera*

IV—Plant obtained from the Herbarium of the University of Arizona at Tempe:

1-*Sophora arizonica*

Ion exchange resins, Amberlite CG 120 (used as ammonium and H^+ form respectively); Amberlite CG 400 (used as acetate form); Amberlite IR 50 (used as acetate form) were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. Ion exchangers for the automatic amino acid analyzer were obtained from Beckman Instruments, Fullerton, Calif. for system one and Bio Rad, Richmond, Calif. for system two (see section on methods for details). Cellulose NM 300 for thin layer chromatography was obtained from Sigma Chemical Company, St. Louis, Mo. Nonprotein amino acids and pipecolic acid were obtained from Biochemical Laboratories, Inc., Hawthorn, Calif. and γ-amino-n-butyric acid from Sigma Chemical Company, St. Louis, Mo. Standard amino acids and dipeptides were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio and Mann Research Laboratories, Inc., New York, N. Y. Solvents used were of reagent grade.

Methods

Paper chromatography. Ascending and descending chromatography were carried out on Whatman No. 1 paper using the following solvents: for descending chromatography;butanol:acetic acid: water (4:1:1 v/v); for ascending chromatography; (a) butanol: acetic acid:water (3:1:1 v/v) or (b) phenol:water (4:1 w/v) in the presence of ammonia vapor. Two dimensional chromatograms were prepared using solvents (a) and (b) respectively (see Fig. 2), (9).

Thin layer chromatography. Air dried layers of cellulose NM 300, (250 μm thickness) on 20 X 20 cm glass plates were used as the solid phase for ascending chromatography. 2-Propanol: butanone:1 N HCl (60:15:25 v/v) (a); and 2-methylpropanol-2: butanone:propanone:water:methanol:ammonium hydroxide (specific gravity 0.88) (40:20:20:14:1:5 v/v) were used as developing solvents. Two dimensional chromatograms were prepared using solvents (a) and (b) in the first and second dimension respectively. In this experiment it was found that only solvent (a) resulted in acceptable separation (4,13,14,24,31: Table II).

Electrophoresis. Ionophoresis was conducted on Whatman No. 1 or 3 mm papers using a water-cooled flat plate instrument (Savant Instruments, Inc., New York). A potential difference of 50 v/cm was applied for 30-45 minutes. The buffer solutions used were: (a) formic acid:acetic acid:water (3.2:146:1820 v/v) pH 1.9 and (b) pyridine:acetic acid:water (10:100:1900 v/v) pH 3.6 Fig. 3, (29).

Column chromatography on amino acid analyzer. Column chromatography was carried out in two separate systems:

(a) The Beckman 120 C amino acid analyzer was used with standard buffer systems and conditions for protein hydrolysates Fig. 4, (1,30).

(b) The Phoenix amino acid analyzer was used with standard buffer system with modifications as described below:

For the analysis of acidic and neutral amino acids, a column of 62 cm of Aminex A-4 obtained from Bio Rad Laboratories was used. The buffer was changed 150 minutes after

starting the automatic amino acid analyzer run. The flow rate of buffers was 80 ml/hour and pressures 100 and 40 mm Hg/cm^2 were applied for buffers and ninhydrin respectively (Fig. 5). The total run was 330 minutes.

Other methods. Nuclear magnetic resonance spectra were determined with a Varian A-60 analytical nuclear magnetic resonance spectrometer. Infrared absorption spectra were determined with a Perkin-Elmer 237 grating infrared spectrophotometer, using KBr discs. Optical rotations were determined using a Perkin-Elmer 141 polarimeter. Mass spectrometry was carried on a Hitachi, Model BMU-6E Mass spectrometer. Melting points of compounds and mixed melting points were determined with a Thomas Hoover capillary melting point apparatus.

CHAPTER III

RESULTS

Detailed extraction, separation and identification procedures were developed, with S. secundiflora being used as a reference for other species of Sophora.

Extraction and identification of water soluble, ninhydrin-reacting compounds from seeds of Sophora secundiflora. Mature seeds of S. secundiflora (200 g) were ground and defatted by repeated extraction using carbon tetrachloride and acetone. Ninhydrin-positive compounds were then isolated by repeated extraction with 50% ethanol. After evaporation of the alcohol (in vacuo), the aqueous solution was freed from salts, sugars, etc., using a column of Amberlite CG 120 (H^+ form) and eluted with ammonia. The ammonia was then removed from the eluate in vacuo (Fig. 1).

When the above extract was subjected to two dimensional paper chromatography and the chromatograms sprayed with ninhydrin, three ninhydrin-reacting compounds whose R_f values did not correspond to those of protein amino acids were observed (Figs. 2, 3, Table I). Hydrolysis of one of these spots suggested that it was a peptide (Table III). On paper electrophoresis at pH 3.6 this peptide behaved as an acidic compound (Fig. 3).

Fig. 1. Schematic representation of methods of extraction and isolation of free amino acids and peptide from seeds of Sophora secundiflora.

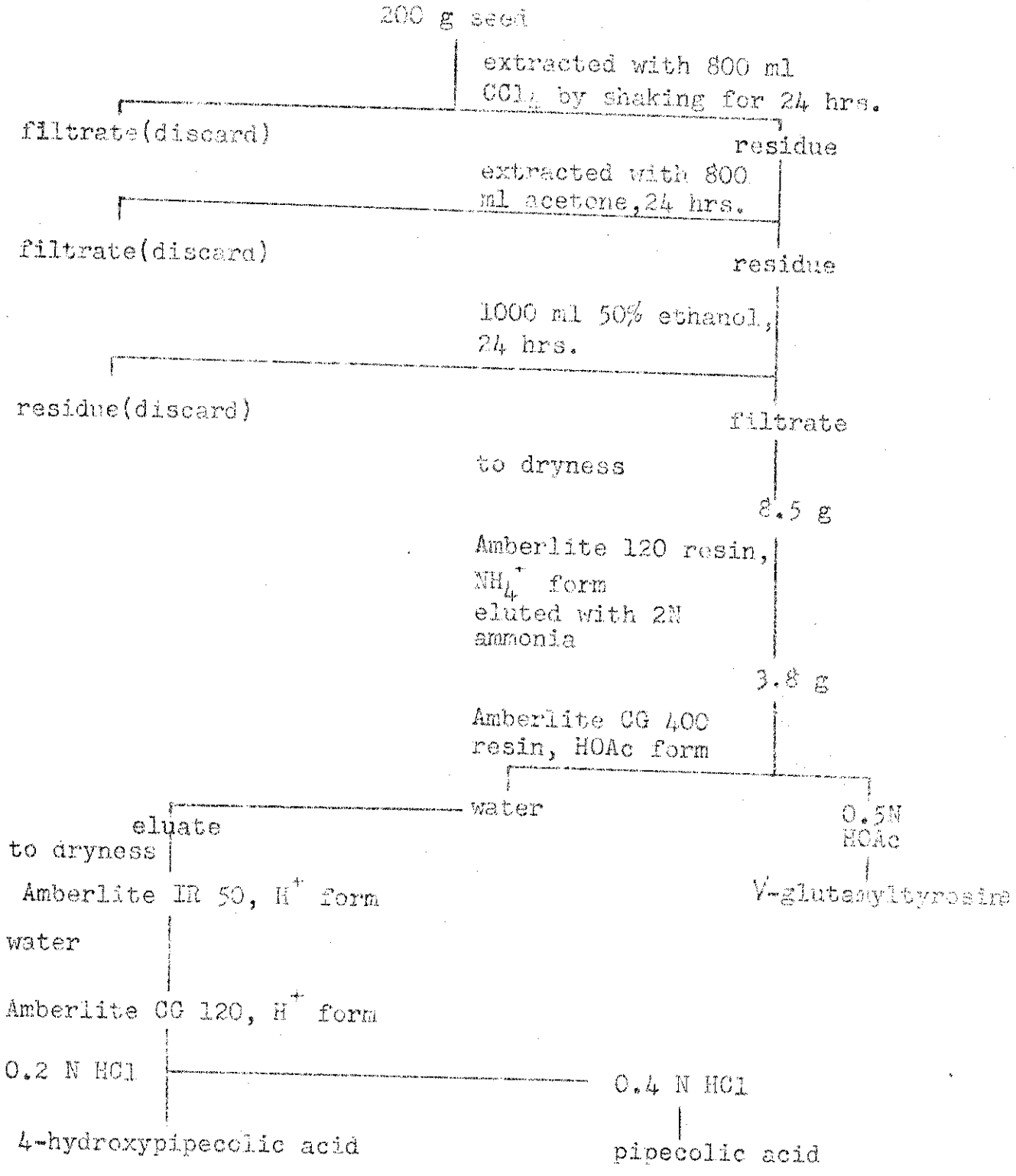

200 g seed
extracted with 800 ml CCl_4 by shaking for 24 hrs.
filtrate(discard)
residue
extracted with 800 ml acetone, 24 hrs.
filtrate(discard)
residue
1000 ml 50% ethanol, 24 hrs.
residue(discard)
filtrate
to dryness
8.5 g
Amberlite 120 resin, NH_4^+ form eluted with 2N ammonia
3.8 g
Amberlite CG 400 resin, HOAc form
water
0.5N HOAc
eluate
to dryness
γ-glutamyltyrosine
Amberlite IR 50, H^+ form
water
Amberlite CG 120, H^+ form
0.2 N HCl
0.4 N HCl
4-hydroxypipecolic acid
pipecolic acid

Fig. 2. A diagramatic representation of the resolution of ninhydrin-reacting compounds in extracts of the fresh seeds of Sophora secundiflora by two dimensional paper chromatography. Asp = asparatic acid, glu = glutamic acid, asm = asparagine, ser = serine, pro = proline, ala = alanine, unusual ninhydrin-reacting compounds are: unk 1 = γ-glutamyltyrosine, unk 2 = 4-hydroxypipecolic acid, and unk 3 = pipecolic acid.

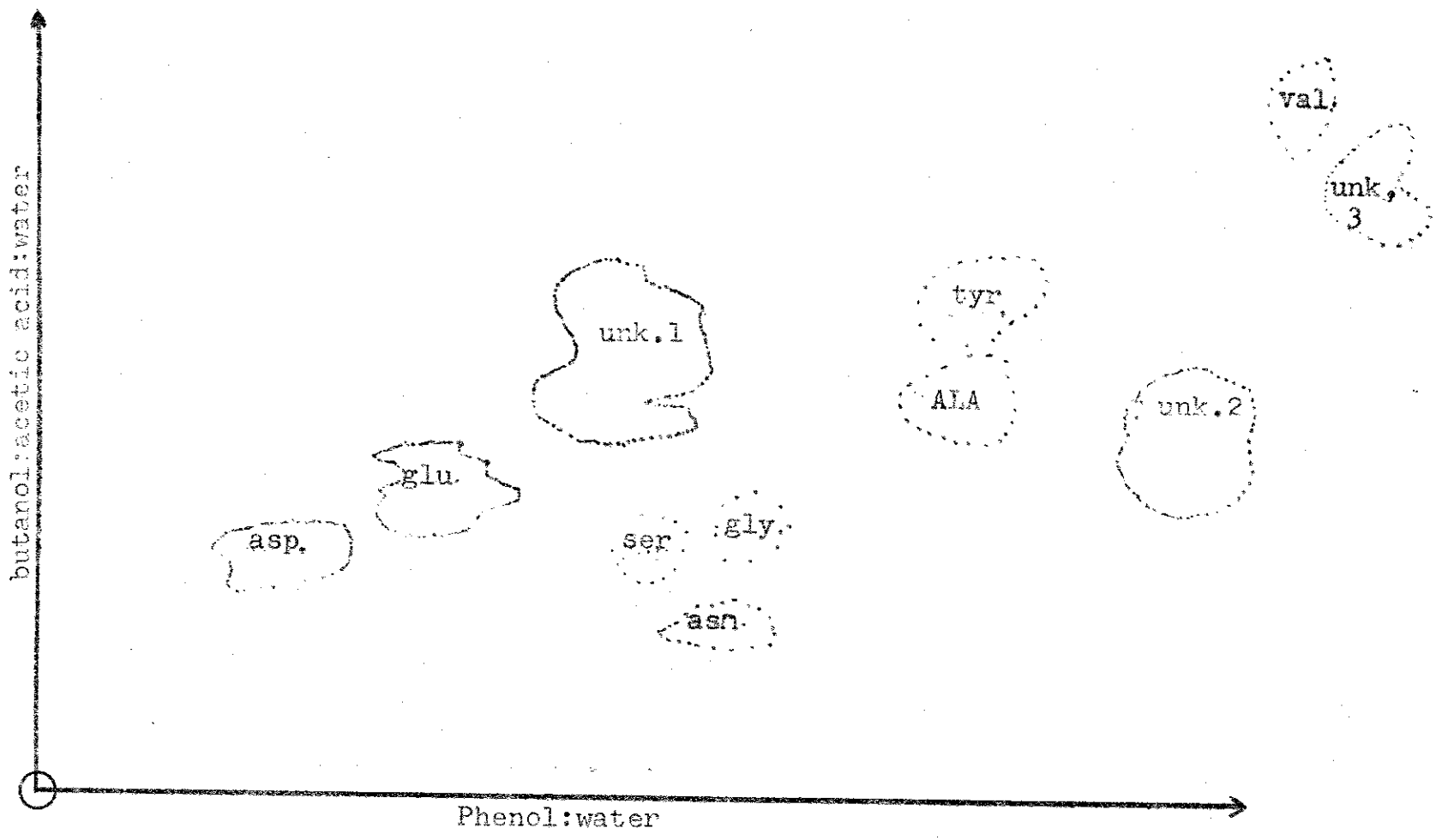

butanol:acetic acid:water
Phenol:water
val.
unk. 3
tyr
unk. 1
ALA
unk. 2
glu.
asp.
ser
gly.
asn

Fig. 3. A diagramatic representation of the resolution of ninhydrin-reacting compounds in the 50% ethanolic extract of the seeds of Sophora secundiflora by high voltage electrophoresis. Asp = asparatic acid, unk = unknown, glu = glutamic acid, lys = lysine, and arg = arginine. pH of buffer was 3.6, electrophoresis was run for 45 minutes at 5000 volts and 5-10 milliampers on 26 X 100 cm paper strips.

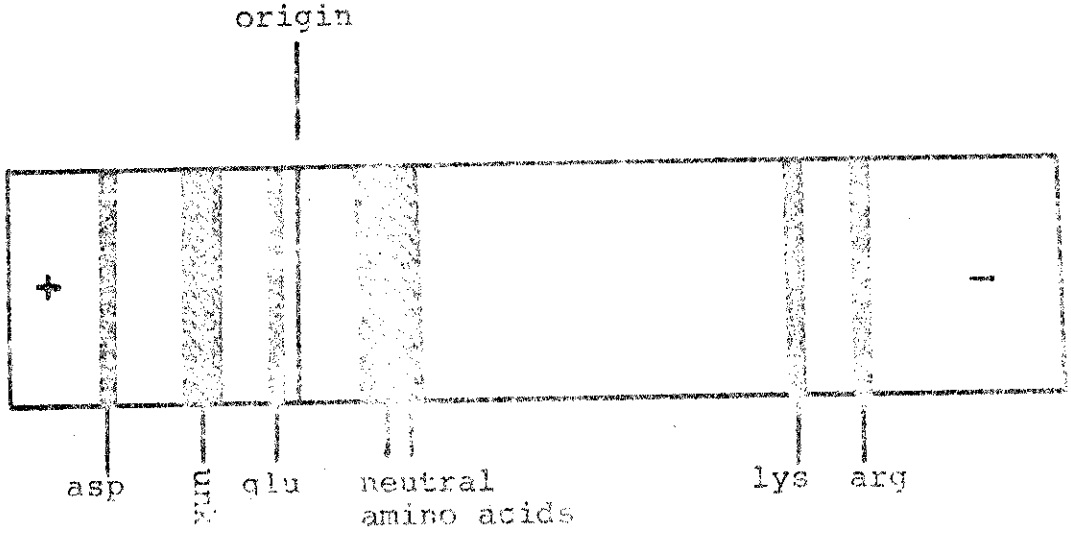

origin
+
-
asp
glu
neutral
amino acids
lys
arg

TABLE I

R_f VALUES OF NONPROTEIN, NINHYDRIN-REACTING COMPOUNDS FROM SOPHORA SECUNDIFLORA AND S. GYPSOPHYLLA ON PAPER CHROMATOGRAMS

	R_f values X 100			
	solvent 1[a]		solvent 2[b]	
	authentic samples	unknown	authentic samples	unknown
γ-amino-n-butyric acid	42	42	85	85
pipecolic acid	49	49	93	93
4-hydroxypipecolic acid		25*		83*
γ-glutamyltyrosine	29	29	21	21

a. Solvent 1 = butanol:acetic acid:water (3:1:1, v/v).

b. Solvent 2 = phenol:water (4:1 w/v) in the presence of NH_3 vapor.

* The compound was identified (see the text).

To isolate this peptide, an aliquot of the concentrated aqueous solution of ninhydrin-reacting compounds obtained from the above Amberlite CG 120 column was passed through a column of Amberlite CG 400 in acetate form. Only the unknown compound and the acidic amino acids were retained by the resin. The neutral and basic amino acids were eluted with water and retained for structural elucidation. The column was then eluted with 0.5 N acetic acid to elute aspartic acid, glutamic

and a small quantity of the unknown. The pure unknown was eluted in later fractions. The fractions containing this unknown were evaporated to dryness in vacuo and the residue was recrystallized from aqueous ethanol and aqueous acetone to yield 350 mg of the unknown.

Identification of the unknown peptide. The identification of this compound was carried out as described below.

Acid hydrolysis was carried out using 5 mg of the isolated crystalline compound in 6 N HCl in a sealed, evacuated tube for 17 hours at 100 C (35). The hydrolyzate yielded two ninhydrin-positive spots on a two-dimensional paper chromatogram whose R_f values corresponded to those of glutamic acid and tyrosine. The identity and 1:1 ratio of these two amino acids were confirmed with the amino acid analyzer. (The retention time of the peptide on column chromatography on automatic amino acid analysis on system I was 80 minutes and in system II was 225 minutes) (Figs. 4,5 and Table III).

Paper chromatography and electrophoresis resulted in the same R_f value and electrophoretic mobility as the synthetic γ-glutamyltyrosine obtained from Dr. John F. Thompson's laboratory (8,22) (see Figs. 2,3 and Table I,II).

Melting point of the isolated compound was 242-245 C. The mixed melting point of the unknown dipeptide with the authentic γ-glutamyltyrosine was also carried out. The mixture of the compounds melted at the same temperature (242 C) (17,22).

TABLE II

R_f VALUES OF NONPROTEIN, NINHYDRIN-REACTING COMPOUNDS FROM SOPHORA SECUNDIFLORA AND S. GYPSOPHYLLA ON THIN LAYER CHROMATOGRAMS

	R_f Values X 100			
	solvent 1[a]		solvent 2[b]	
	authentic samples	unknown	authentic samples	unknown
γ-amino-n-butyric acid	58	58	25	25
pipecolic acid	66	66	36	36
4-hydroxypipecolic acid		61*		27*
γ-glutamyltyrosine	59	59	3	3

[a]Solvent 1 = 2-propanol:butanone:1 N HCl (60:15:25, v/v).

[b]Solvent 2 = 2-methylpropanol-2:butanone:propanone:water: ammonium hydroxide (specific gravity 0.88):methanol (40:20: 14:5:1, v/v).

*The compound was identified (see the text).

The infrared spectrum of the isolated dipeptide was identical to that of synthetic compound (Fig. 6).

The optical rotation (17,22) was

$$[\alpha]_D^{25} = 26.00 \pm 0.4 \text{ (C 1, } H_2O\text{)}.$$

For N-terminal determination of the peptide, 2, 4-fluro-dinitrobenzene (DNP) was used. The reaction was carried out

according to Sanger's Method (27). The excess reagent was then extracted, the DNP-peptide was hydrolyzed, and the DNP-amino acid was isolated from the hydrolysis products by ether extraction. The R_f value of the DNP-amino acid was identical to that of N-dinitrophenyl-glutamic acid. This experiment indicated that the dipeptide has glutamic acid at its N-terminus (8).

TABLE III

A QUANTITATIVE ANALYSIS OF NINHYDRIN-REACTING COMPOUNDS IN A 50% ETHANOLIC EXTRACT OF SEEDS OF S. SECUNDIFLORA[1]

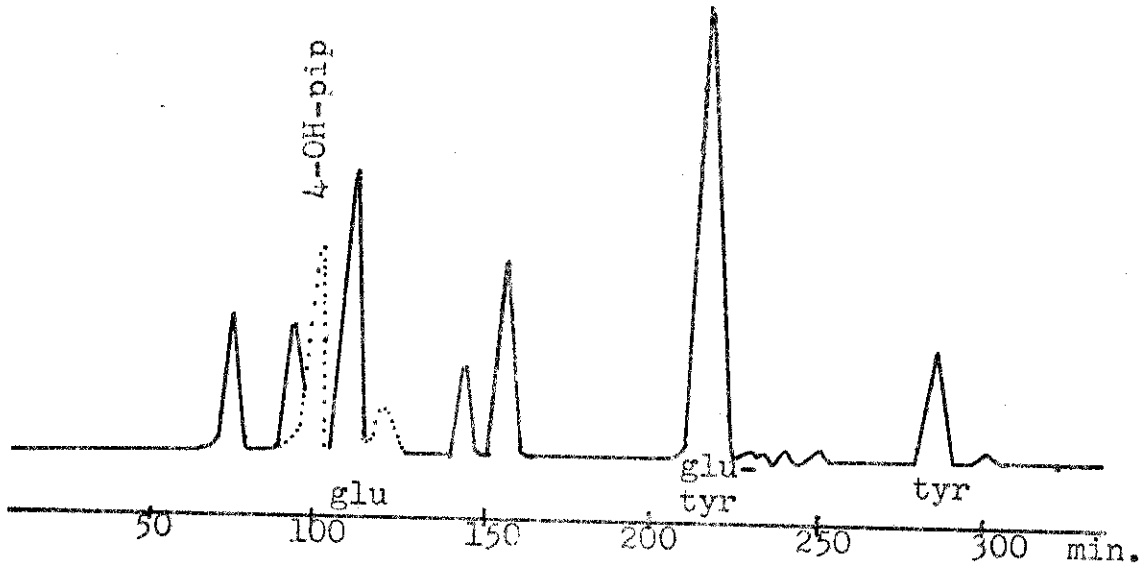
Amino Acid	50% EtOH extract	50% EtOH extract hydrolyzate
aspartic acid	5.0[2]	12.5
asparagine	t	
4-OH-pipecolic acid	32.0	26.0
glutamic acid	31.0	80.0
glycine	3.0	17.0
glulamyl tryosine	90.0	t
tyrosine	11.0	46.0
phenylalanine		1.0

[1] Obtained from Exposition Street, Austin, Texas.

[2] Values are in micromoles/gram of fresh seeds.

Fig. 4. Amino acid analysis on Beckman automatic analyzer (system I). (Top), amino acid analysis of the 50% ethanolic extract of the fresh seeds of Sophora secundiflora. (Bottom), same extract after hydrolysis with 6 N HCl (35).

Fig. 5. Amino acid analysis on Pheonix automatic analyzer (system II). (Top), amino acid analysis of the 50% ethanolic extract of the fresh seeds of Sophora secundiflora. (Bottom), same extract after hydrolysis with 6 N HCl (35).


4-OH-pip
glu
glu-
tyr
tyr
50
100
150
200
250
300 min.

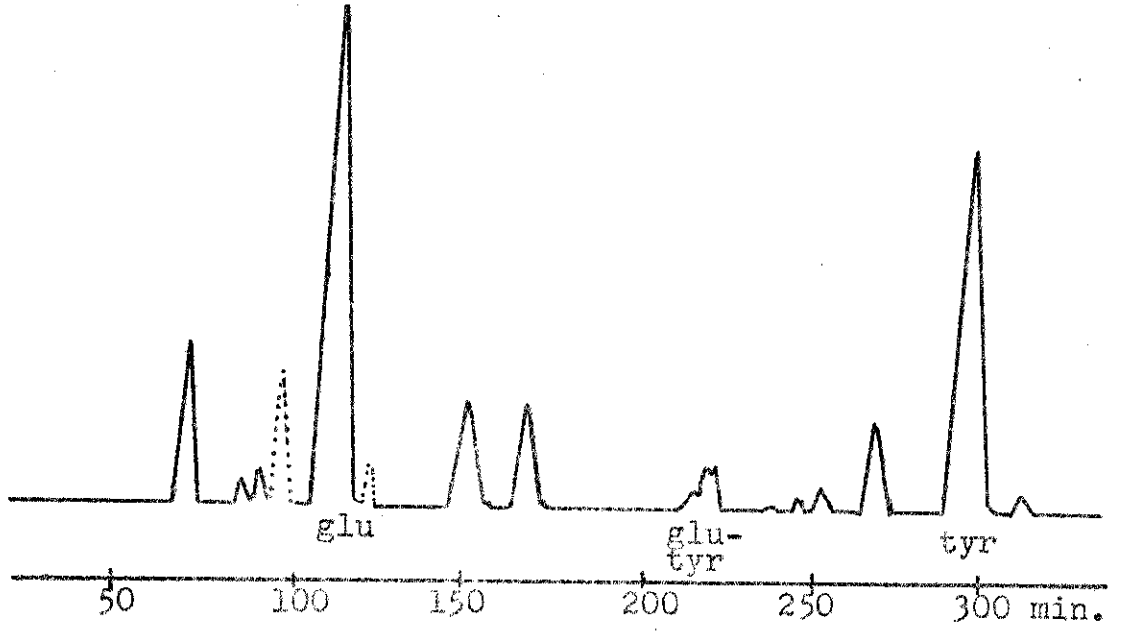
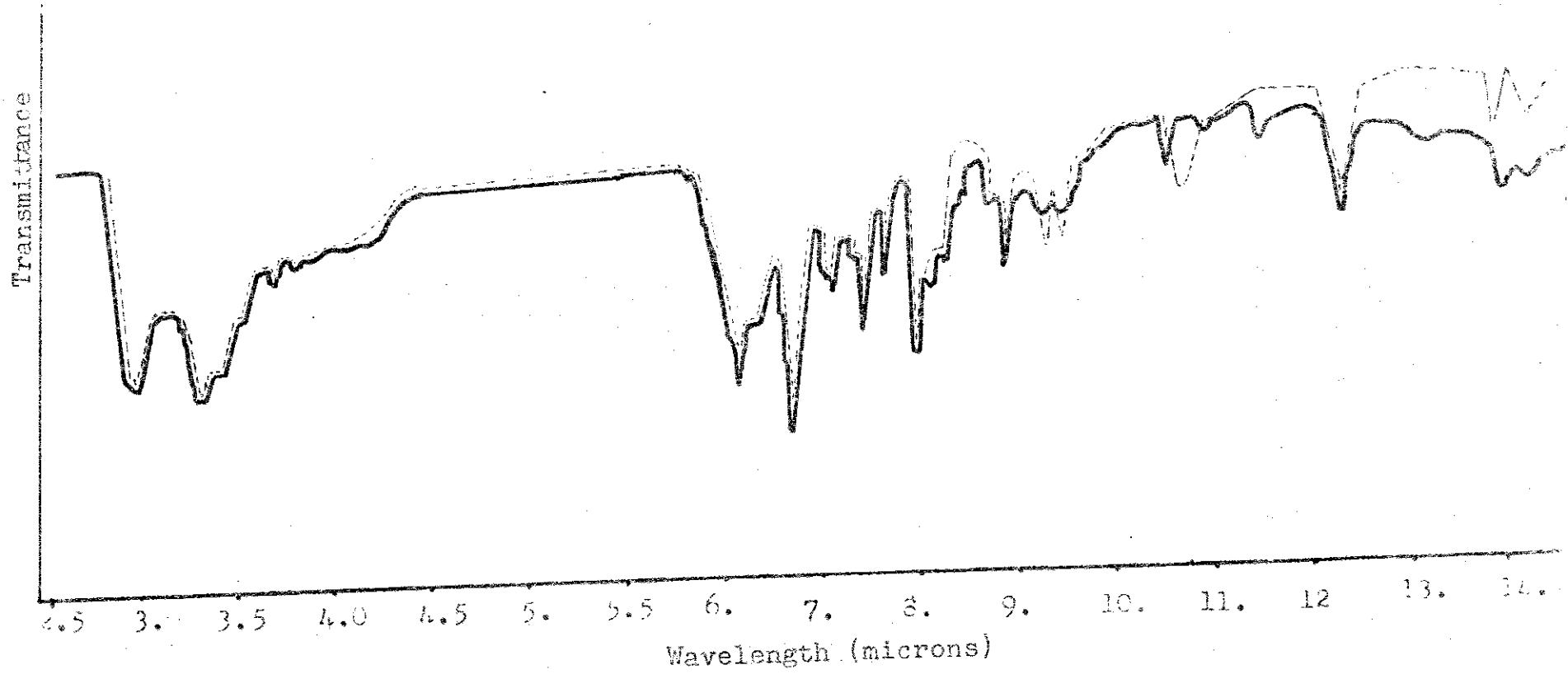

glu
glu-
tyr
tyr
50
100
150
200
250
300 min.

Fig. 6. Infrared spectrum of the dipeptide, γ-glutamyltyrosine. Synthetic compound --------, unknown compound ————————.


Transmittance
4.5
3.
3.5
4.0
4.5
5.
5.5
6.
7.
8.
9.
10.
11.
12
13.
Wavelength (microns)

Carbon, hydrogen, and nitrogen analysis indicated the presence of C 54.35; H, 5.88; N, 9.04 corresponding to a general formula of $C_{14}H_{18}O_6N_2$. According to this general formula the theoretical percentages are C, 54.19; H, 5.89; N, 9.07.

Determination of the gamma linkage of the dipeptide was done by the quantitative ninhydrin reaction of Van Slyke (34). In this reaction the ninhydrin reagent will react only with a free alpha-amino acid, resulting in the evolution of CO_2 from the free carboxylic acid portion of the amino acid. The CO_2 can be trapped in standard barium hydroxide and upon titration the amount of CO_2 can be determined. In this experiment 100 micromoles of standard amino acid or dipeptide reacted with ninhydrin (Table IV). The number of equivalents of CO_2 evolved from 100 micromoles of a free alpha-amino acid was the same as the number of equivalents of CO_2 evolved from 100 micromoles of the unknown dipeptide (Table IV). This measurement indicated the presence of only one free alpha-amino acid in the dipeptide. Thus the amino acid tyrosine of the dipeptide must be linked to the dicarboxylic amino acid, glutamic acid, via the gamma -COOH of the latter amino acid (18). Therefore the unknown dipeptide is gamma glutamyltyrosine (Table IV):

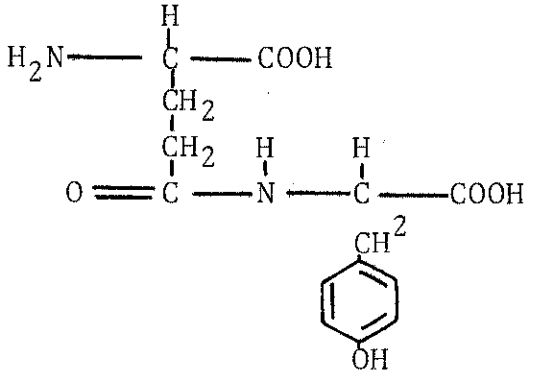


TABLE IV

TITRATION RESULTS FROM VAN SLYKE
NINHYDRIN DETERMINATION

	0.013 g asp	0.007 g gly	0.013 g gly-gly	0.019 g gly-asp	0.031 g gly-tyr
Control without ninhydrin-reacting comp.	7.5[1]	7.15	7.3	7.5	6.9
Experiment with ninhydrin-reacting comp.	5.10	5.15	7.1	7.4	4.7
Difference	2.40	2.00	0.20	0.10	2.2

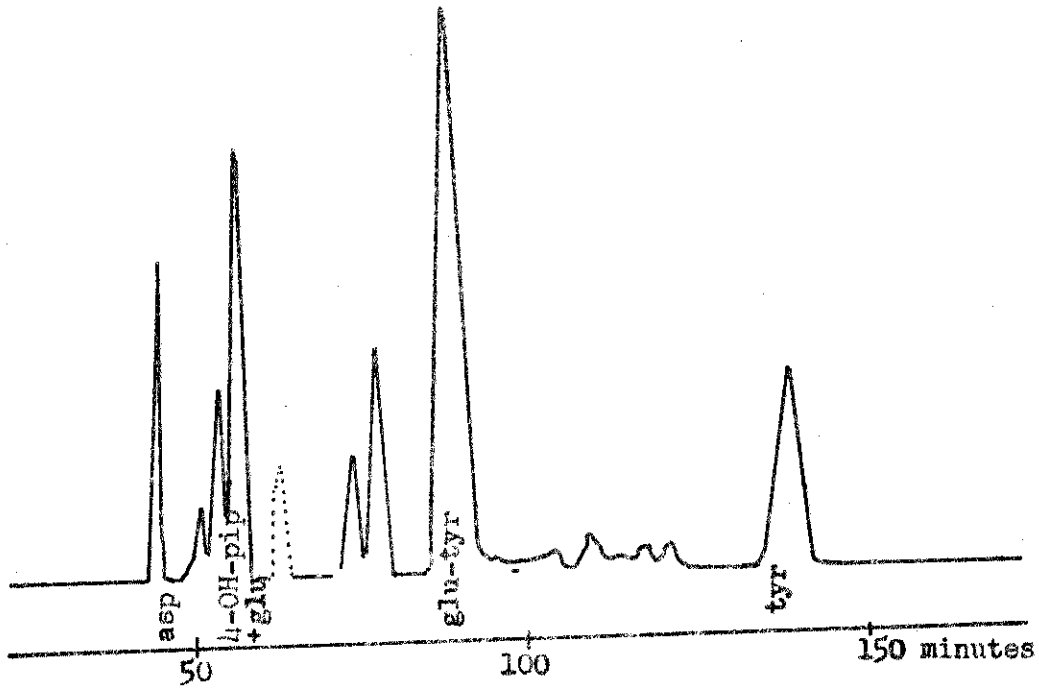
[1]Amounts of 0.1 N HCl in ml used to titrate the CO_2 evolved from 100 micromoles of amino acid or dipeptide upon reaction with ninhydrin.

Mass spectroscopy of the -glutamyltyrosine was carried out according to the procedure described in the literature (5) with modifications to obtain a volatile derivative for mass spectroscopy.

A few milligrams of the dipeptide were placed in a test tube and dried on a sand bath (110 C) while flooded with nitrogen gas. One ml of benzene was then added to the tube and evaporated to dryness. Later 1 ml of anhydrous HCl in methanol was added and the tube was covered loosely and heated to 110 C for 15 hours. The mixture was cooled, then 1 ml of trifluoroacetic anhydride in dichloromethane was added. The tube then was closed tightly, left 30 minutes at room temperature, then evaporated to dryness. The trifluoroacetic anhydride derivative was then dissolved in 1 ml chloroform and used as a sample for mass analysis. The mass spectrum indicated a small peak for the parent compound. This peak showed the expected molecular weight for glutamyltyrosine (31).

The nuclear magnetic resonance spectrum (60 MHZ, D_2)) 6.8-7.4 (quartet, 4H, J=8Hz, aromatic H), ca. 4.7 (partially hidden by water peak, ca. triplet, tyrosine CH), 4.06 (quartet, 1 H, J=1Hz, glutamic acid CH), 2.9-3.2 (ca. two doublet, 2H, Tyrosine CH_2, 1.9-2.8 (multiplet, 4H, glutamic acid CH_2-CH_2) (Fig. 7). The internal standard was Sodium 3-trimethyl silyl-propinate-2, 2, 3, 3-d4.

Isolation of amino acids from Sophora secundiflora. When the mixture of extracted amino acids was passed through a column


asp
4-OH-pip
+glu
glu-tyr
tyr
50
100
150 minutes

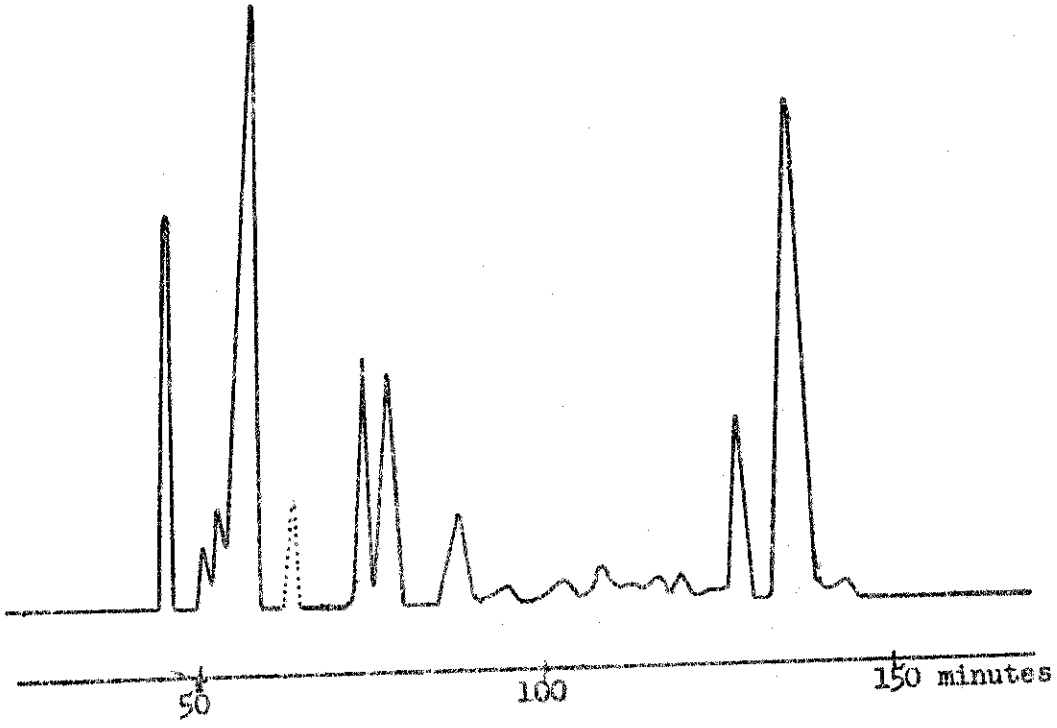
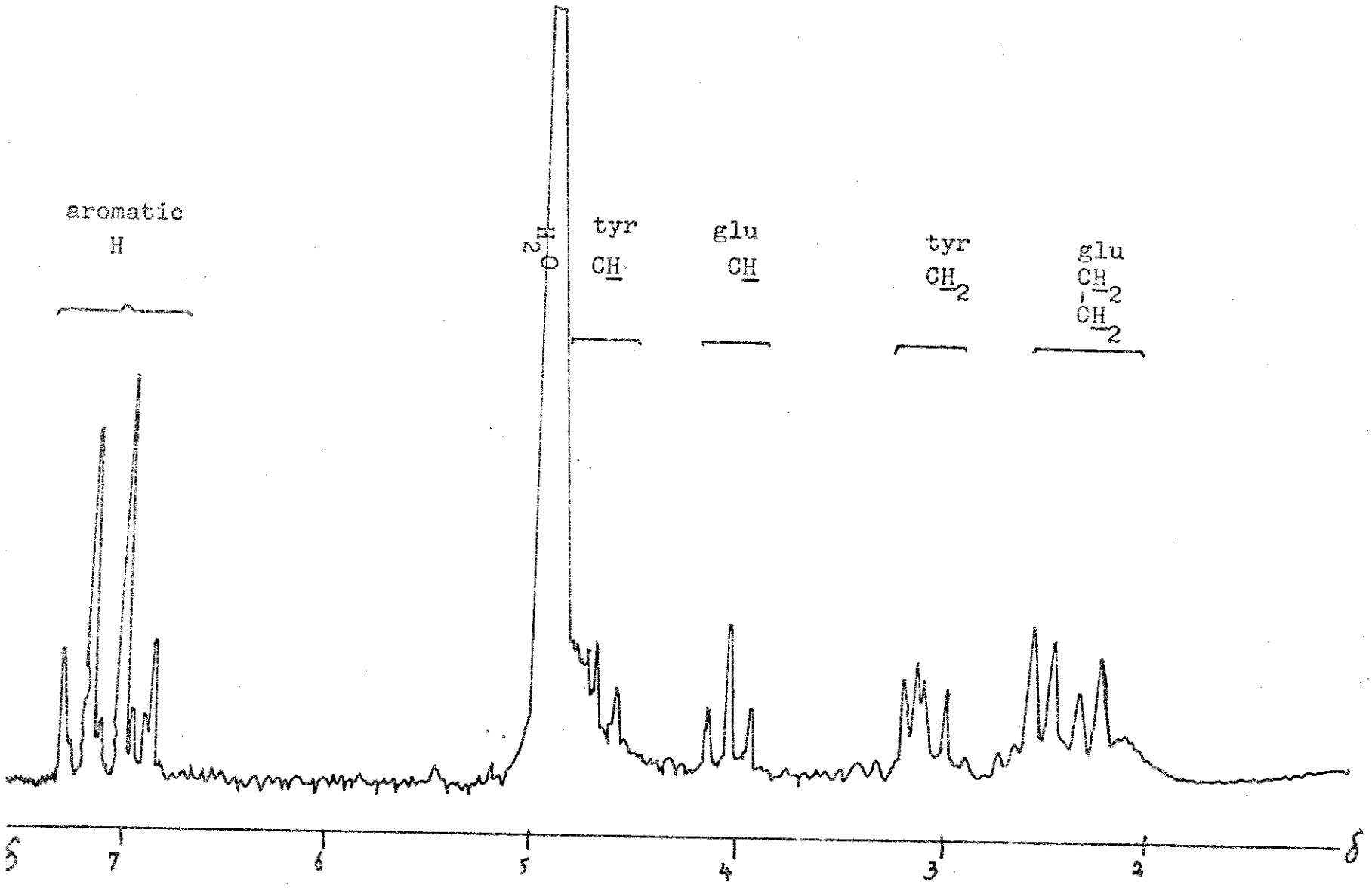

50
100
150 minutes

Fig. 7. Nuclear magnetic resonance of the dipeptide, γ-glutamyltyrosine. Courtesy of Dr. J. E. Johnson (Texas Woman's University).


aromatic
H
2H O
tyr
CH
glu
CH
tyr
CH2
glu
CH2
CH2
δ
7
6
5
4
3
2

of strongly basic resin (Amberlite CG 400, acetate form) and eluted with water, neutral and basic amino acids were obtained. This mixture was passed through a column of weakly acidic resin (Amberlite IR 50, H^+ form) using water as eluant (Fig. 1). The column retained only basic amino acids which were eluted with 0.5 N acetic acid and later identified as lysine and arginine using co-chromatography and co-electrophoresis in different solvents with standard samples, chemical analysis and analysis on the amino acid analyser (Figs. 2, 3 and Table III).

The aqueous eluant containing neutral amino acids was found to be slightly acidic. To remove this acidity the solution was evaporated to dryness using a rotary evaporator under reduced pressure. The residue was solubilized in water, and absorbed to a column of Amberlite CG 120, H^+ form washed with water and then eluted with a large volume of 0.2 N HCl. The first fractions containing ninhydrin-positive compounds were found to contain asparagine and serine. The second fraction contained a mixture of neutral amino acids. The amino acid content of these fractions was determined by methods described above. The third fraction contained an unknown neutral ninhydrin-reacting compound which developed a green color upon reaction with ninhydrin and also gave a red fluoresence under long ultraviolet wavelength Fig. 2. The column was then eluted with 0.4 N HCl and produced a fraction characterized by a sky blue color and red fluoresence under ultraviolet light when visualized with ninydrin.

Those fractions that developed a green color with ninhydrin were pooled and solvents evaporated. The residue was redissolved in water and purified by elution from a small column of Amberlite CG 400 in the acetate form by 0.5 N HCl. The eluate was concentrated and recrystallized from ethanol containing a small quantity of water to yield 120 mg of the unknown amino acid.

Identification of pipecolic acid. The amino acid responsible for the sky blue color was identified as pipecolic acid by the following methods:

Paper chromatography and electrophoresis resulted in the same R_f value and electrophoretic mobility as those found in literature (10,36), Table I.

Reaction with ninhydrin and isatin reagents also coincided with those of literature (26). Pipecolic acid develops a sky blue color with ninhydrin and a blue-green color with isatin.

Cochromatography and electrophoresis on paper and thin layer cochromatography on cellulose with authentic sample confirmed the above results.

The retention time of the amino acid on the amino acid analyzer coincided with that of the known pipecolic acid (Table III).

Identification of 4-hydroxypipecolic acid. The compound responsible for the green color was identified by following methods:

Paper chromatography of the isolated compound resulted in the same R_f value as reported in literature (26,36). This

chromatogram also developed a red fluroesence under ultra-violet light. This is one of the characteristics of pipecolic acid and its derivatives.

Reaction with ninhydrin and isatin agreed with those of the literature (11,26). The compound does not develop a color with isatin but it gives a variety of misleading colors with ninhydrin as described below.

When the isolated compound was subjected to chromatography and electrophoresis on paper, dried at 110 C, then dipped in ninhydrin reagent and heated at 110 C for 5 minutes a bright green color developed. On further heating (10 minutes), the color changed to a gray-green and eventually to a deep yellow color after remaining at room temperature for several days. According to the literature, 4-hydroxypipecolic acid gave first an orange-brown color upon ninhydrin spray then rapidly changed to purple (10); a characteristic green with ninhydrin that faded through khaki to gray (26); or first yellow, but turned sky-blue after heating gradually (6).

Melting point of isolated amino acid was 288-292 C. The compound changed to dark color at 170 C (6).

Optical rotation (11-36) also coincided with literature

$$[\alpha]_D^{27} = -12.5 \text{ (C 1, } H_2O\text{)}.$$

C, H, N, analysis indicated the ratio of C, 49.98; H, 7.58; N, 9.72 corresponding to general formula of $C_6H_{11}O_2N$.

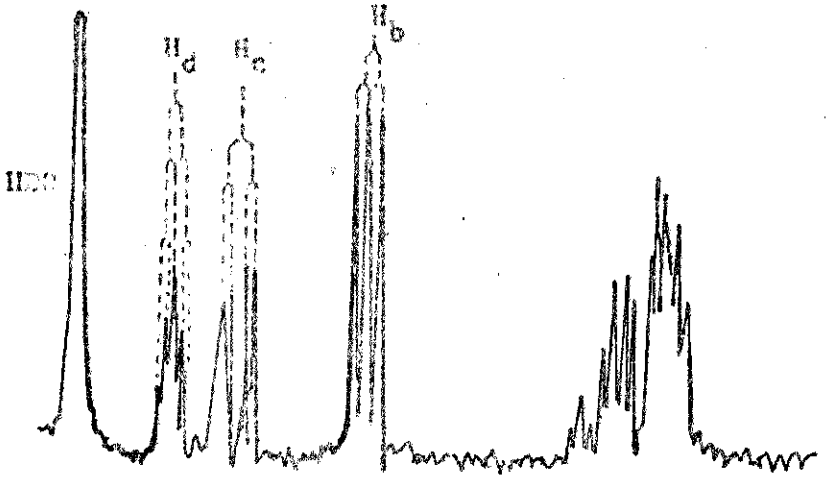
Nuclear magnetic resonance spectrum of the isolated compound in D_2O was identical to those given in literature (28), Fig. 8.

The retention time of the isolated compound on ion exchange chromatogrphy using the amino acid analyzer (system I) was 50 minutes which overlapped with glutamic acid. On system II however, the retention time was 110 minutes and the amino acid eluted prior to glutamic acid. The amino acid, 4-hydroxypipecolic acid, showed a high absorbance at 440 nanometers and much less at 570 nanometers (Figs. 5,6) (23).

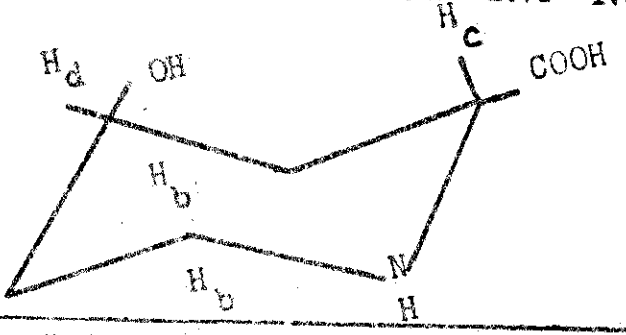
Oxidation products of organic compounds have been used as a key for their identification.

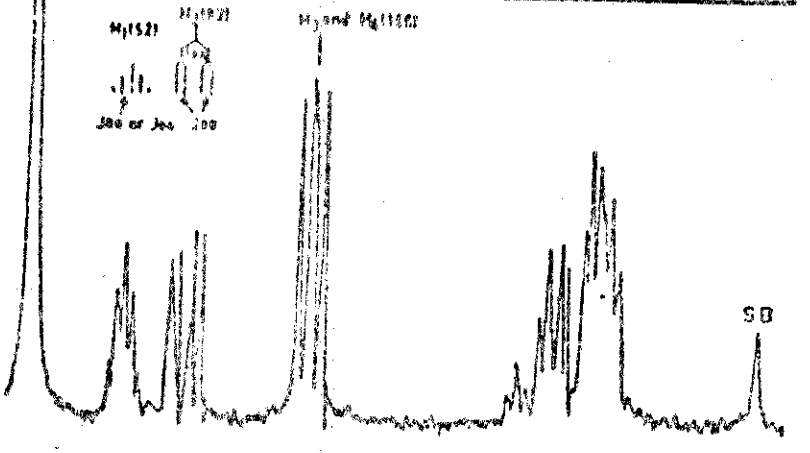
To study the oxidation products of the 4-hydroxypipecolic acid, a few milligrams of the unknown amino acid were oxidized with permanganate. The amino acid was first dissolved in 0.1 ml of sulfuric acid solution (10% v/v), then warmed to 60 C in a water bath. A 1% w/v solution of permanganate in 10% sulfuric acid was added dropwise to the above solution until a permanent pink color remained. The mixture was then diluted to about 4 ml and an excess of solid barium carbonate was added. When CO_2 evolution ceased, the mixture was centrifuged, and the clear supernatent removed and dried in vacuo. The residue was then dissolved in 3 ml of 10% isopropanol in water and subjected to two dimentional paper chromatography, paper co-chromatography and amino acid analysis on the amino acid analyzer. Beta alanine, glycine, γ-amino-n-butyric acid

Fig. 8. Nuclear magnetic resonance of 4-hydroxypipecolic acid. Top-obtained with the compound isolated from the seeds of Sophora secundiflora. Bottom-obtained by Schoolery and Virtanen (28).



(δ) 4.70 4.20 3.70 3.20 2.75 2.25 1.75 1.25





(δ) 4.70 4.20 3.70 3.20 2.75 2.25 1.75 1.25

and aspartic acid were the oxidation products of the isolated amino acid. These acids have been shown to be the oxidation products of 4-hydroxypipecolic acid under the same conditions (10).

Unusual amino acids in Sophora gypsophylla. Sophora gypsophylla contains in addition to gamma-glutamyltyrosine, pipecolic acid and 4-hydroxypipecolic acid, a fourth unusual compound γ-amino-n-butyric acid. The presence of this compound in S. gypsophylla was established by its R_f values on paper and thin layer chromatography and co-chromatography with the known sample (Table I, II).

Amino acid analysis of 23 species of Sophora. The amounts of different amino acids in the seeds of 23 species of Sophora as micromoles per gram of fresh uncoated seeds are recorded in Table V. According to these data, 10 of the 23 species under investigation had no detectable pipecolic acid. Therefore, these plants are classified as group I (shown in the bottom of the table). In the next 9 species, the unusual amino acid (pipecolic acid) was detectable, but only in trace amounts. Thus, these plants are classified as group II. In the extract of the group of four in the table, both pipecolic acid in trace amounts and a higher concentration of 4-hydroxypipecolic acid were detectable. This group is regarded as being more advanced and categorized in group III.

Each of the above categories can be further subdivided according to the presence or absence of the dipeptide,

TABLE V

THE AMINO ACID ANALYSIS OF 23 SPECIES OF SOPHORA IN SYSTEM II

Sophora species	*arizonica*	*formosa*	*gypsophylla*	*secundiflora*	*angustifolia*	*chrysophylla*	*leachiana*	*microphylla*
asp	21	70	15	17	6	4	9	6
asn	66	t	10	31		4		t
ser	t	85	27	t	18	12	34	t
4-OH-pip	26	97	141	228				
glu	60	186	64	114	3	63	t	
pro	t	28	t	t		t	t	34
gly	5	16	9	168	t	4	t	4
ala	10	51	21	51	t	3	13	6
pip	t	t	t	t	t	t	t	t
glu-tyr	105	102	178	330		52		106
val	t	t	t	t	t	t	t	
ile	t	t	t	t	t	t	t	
leu	t[1]	21	9	t	t	t	t	
tyr	22[2]	68	13	27	t	43	t	
phe[3]	t		t	t	t	t	t	

TABLE V--Continued

Sophora species	*mollis*	*nuttelliana*	*pachicarpa*	*stenophylla*	*tetrapetera*	*affinis*	*allopecuroides*	*griffithii*
asp	21	118	311	15	2	15	2	5
asn	t	24	21	61	4	15	4	
ser	t	t	t	t	4	145	3	28
4-OH-pip								
glu	t	8	12	6	17	65	29	13
pro	t	2	55	6	t	t	t	
gly	t	t	10	3	t	34	2	4
ala	t	2	42	2	t	49	2	7
pip	t	t	t	t	t			
glu-tyr					100	71	23	
val		22	14	15	t	t	t	12
ile	5	t	13	4	t	t		t
leu	t	t	5	5	t	t		t
tyr	t	t	t	t	4	28	t	t
phe	t	t	t	t	t	t		t

TABLE V--Continued

Sophora species	*japonica*	*longipesman*	*massafeurana*	*macrocarpa*	*reticulata*	*tomentosa*	*moorcroftiana*	Minutes
asp		31	14	5	13	48	5	75
asn		t	121			10	34	85
ser	21	4	t	5	17	15	t	102
4-OH-pip								110
glu	22	4	7	30	13	76	17	122
pro		t	t	t		t		132
gly	t	4	t	t	t	21	t	164
ala	5	6	14	11	t	27	9	275
pip								220
glu-tyr				99		2841	65	225
val	48	7	9		17			231
ile	t	3	t		t	t	t	264
leu	t	3	t		t	t	t	272
tyr	5	7	3	t	t	28	11	315
phe		3	6			t		324

[1]t = trace amount (less than 1 micromole/g seed).

[2]Values represent micromoles/g of fresh seeds.

[3]Neither threonine, cysteine, methionine nor other neutral amino acids were detectable.

γ-glytamyltyrosine, was isolated and hydrolyzed, a 1;1 ratio of glutamic acid and tyrosine was determined using the amino acid analyzer.

Amino acid analysis of extracts of seeds of S. secundiflora was carried out in two different systems. The purpose of this was to distinguish the retention times of the amino acid, 4-hydroxypipecolic acid and the dipeptide γ-glutamyltyrosine. For example, in system one the amino acid overlaps with glutamic acid, but it can be separated in the second system employed. This method also confirmed the results obtained from two-dimensional paper chromatography on the separation and characterization of the various compounds. It was shown by these studies that although the amino acid patterns of a given species obtained from different locations are the same in most cases, there was a variation in the amounts of these compounds when the extracts were obtained from plants belonging to different locations (Tables VI, VII, VIII). This difference in level may be due to the effects of the environment and may explain the differences between wild and cultivated plants. For example, in Table VI, amino acid analysis indicates the variations in amounts of free amino acids in seeds of S. gypsophylla for one obtained from a population in Mexico and the other from Guadalupe Mountains in Texas. Furthermore, S. secundiflora, is native to Northern Mexico and Southern Texas, but when it is cultivated in Denton, Texas, the amounts of the amino acids stored in the seeds are reduced (Table VII). The

consistency of the amino acids and the difference of the amounts of these compounds are noteworthy. This is because they reveal that the changes in environment cannot have a drastic effect on the gene expression.

TABLE VI

QUANTITATIVE AMINO ACID ANALYSIS OF 50% ETHANOLIC EXTRACT OF FRESH SEEDS OF S. GYPSOPHYLLA OBTAINED FROM DIFFERENT LOCATIONS

Amino Acid	Mexico	Texas
asp	15[1]	t[2]
asn	9	
ser	28	2
4-OH-pip	141	103
glu	64	26
pro	t	t
gly	9	44
ala	21	12
pip	t	t
glu-tyr	178	57
val	t	t
ile	6	1
leu	9	2
tyr	13	8
phe	t	t

[1]Values represent micromoles/g of fresh seed.

[2]t = trace amount (less than 1 micromole/g seed).

TABLE VII

QUANTITATIVE AMINO ACID ANALYSIS OF 50% ETHANOLIC EXTRACT OF FRESH SEEDS OF S. SECUNDIFLORA OBTAINED FROM DIFFERENT LOCATIONS

Amino Acids	Austin, Texas	Denton, Texas
asp	17[1]	15
asn	31	12
ser	t[2]	t
4-OH-pip	228	99
glu	114	85
pro	t	t
gly	168	t
ala	51	10
pip	t	t
glu-tyr	330	141
val	t	t
ile	t	t
leu	t	t
tyr	27	11
phe	t	

[1]Values represent micromoles/g of fresh seeds.

[2]t = trace amount (less than 1 micromole/g seed).

Paper chromatography of 23 species of Sophora. Two-dimensional paper chromatography was carried out to further distinguish the differences in the amino acid composition and hence the

chemotaxonomic grouping within the genus Sophora. Each plant's extract was chromatographed in triplicate, using three different concentrations. The lowest concentration represented 0.002 g, the next 0.004 g, and the third 0.006 g of fresh uncoated seed. For example, when 50% ethanolic extract of seeds of Sophora affinis were chromatographed on paper using the above three concentrations, neither of the chromatograms indicated the presence of pipecolic acid. However, in plants containing pipecolic acid (Table IX), paper chromatography of the 0.002 g of seed extract revealed the presence of the amino acid. Paper chromatography was also used to determine whether or not the variations of leaf shapes within the same species reflect biochemical variations. For example, plants obtained from different herbaria and classified as Sophora secundiflora had variations in the appearance of their leaves (obovate and shiny, obovate and pubes pubscence, lanceolate and pubescence). It was shown by paper chromatography that the amino acid contents of these plants were the same and agreed with the name assigned for them (Fig. 2).

In these studies, 4 unusual amino acids were characterized within the genus Sophora. The R_f values of these compounds are given in Table I. The occurence of these amino acids in Sophora species are listed in Table IX. The tracings of the paper chromatograms of the 23 species of Sophora are given in the appendix.

TABLE VIII

QUANTITATIVE AMINO ACID ANALYSIS OF 50% ETHANOLIC EXTRACT OF FRESH SEEDS OF S. AFFINIS OBTAINED FROM DIFFERENT LOCATIONS

Amino Acids	Austin, Texas	Denton, Texas
asp	15[1]	5
asn	15	10
ser	145	80
glu	65	56
pro	t[2]	t
gly	35	10
ala	49	25
glu-tyr	71	63
val	t	t
ile	t	t
leu	t	t
tyr	13	15
phe	8	8

[1]Values represent micromoles/g of fresh seed.

[2]t = trace amount (less than 1 micromole/g seed).

TABLE IX

THE APPEARANCE OF FOUR CHEMOTAXONOMICALLY IMPORTANT NINHYDRIN-REACTING COMPOUNDS OBTAINED FROM 50% ETHANOLIC EXTRACT OF 23 SPECIES OF SOPHORA

Sophora species	4-hydroxy-pipecolic acid	pipecolic acid	γ-glutamyl-tyrosine	γ-amino-n-butyric acid
arizonica	+	+	+	+
gypsophylla	+	+	+	+
formosa	+	+	+	
secundiflora	+	+	+	
angostifolia		+	+	+
chrysophylla		+	+	
microphylla		+	+	
tetrapetera		+	+	
leachiana		+		+
nutteliana		+		+
pachicarpa		+		+
stenophylla		+		+
mollis		+		
griffithii			+	+
moorcroffiana			+	+
affinis			+	
allopecuroides			+	

TABLE IX--Continued

Sophora species	4-hydroxy-pipecolic acid	pipecolic acid	γ-glutamyl-tyrosine	γ-amino-n-butyric acid
macrocarpa			+	
tomentosa			+	
japonica				+
reticulata				+
longipesman				
massageurana				

CHAPTER IV

DISCUSSION AND CONCLUSIONS

In Leguminosae it has been frequently found that classification of species agreed, whether it was based on their morphology or on their chemotaxonomy (2). This finding suggests that mutations affecting amino acid biosynthesis, no less than mutations affecting morphological development, have contributed to the establishment of the individual species which we now recognize within the family. Indeed, in some cases, the evidence of evolutionary relationships provided by biochemical data may be less equivocal than that provided by more classical methods. If, for example, several species have a rare amino acid in common, it is most probable that they had descended from the same ancestral form in which the genome controlling the biosynthesis of that amino acid first arose. If species have more than one such amino acid in common, and these amino acids are of independent genetic origin, then the probability that the species are of common ancestry increases, since the likelihood of an identical combination of unusual genomes arising more than once is remote. Such biochemical data, however, may be used to re-evaluate the taxonomic significance of other features used in classification.

The use of unusual amino acids as phylogenetic "markers" in this sense is to use them as if they were single isolated

morphological characteristics. Such use may provide valuable information; but the full potential of the use of unusual amino acids in comparative studies will only be realized when their metabolic pathways, and the biochemical relationships which exist between them, have been elucidated. In fact each unusual amino acid carries with it some record of its own evolution in the form of its biosynthetic pathway and of the enzymes which control the pathway. By tracing biosynthetic pathways, relationships such as those between the γ-glutamyl-β-cyanoalanine and asparagine-accumulating species of Vicia and the homoarginine and lathyrine-accumulating species of Lathyrus have already been established (2,33). Similar studies concerned not only with the distribution and biosynthesis but also with the subsequent metabolism of unusual amino acids in these and other genera should increase our knowledge about the biochemistry and phylogeny of this plant family.

Although the amino acid distribution patterns of a given species of plant obtained from different locations may be the same in most cases, there could be a variation in the amounts of these compounds (Table VI, VII, VIII). The environmental influence on the quantitative variations in amino acids stored in seed is demonstrated in at least three species of the genus Sophora (Tables VI, VII, VIII). A possible cause of such a variation is the climate of the habitat. For example, Sophora secundiflora seed collected from Mount Bonnel, Austin, where the climate is warm, contained larger amounts of stored amino

acids when compared to the seed collected from Denton, Texas, where the climate is cooler. S. secundiflora is a native of Northern Mexico, a warm, humid region, and the Denton area is probably too cool for optimum metabolic acitivity.

Soil and nutritional factors could also influence plant growth and metabolism. Seeds of Sophora affinis were harvested at Austin, in a yard cultivated with grass which was fertilized twice a year. Others were obtained at Denton, Texas, from trees surrounded by other competing plants, and were located in an unattended and unfertilized lot. Although the seeds of the Austin plants were smaller than those from Denton, they stored more amino acids (Table VII). Variations in the amounts of amino acids stored in plants located in different environments were reported by Bell and Clark (6).

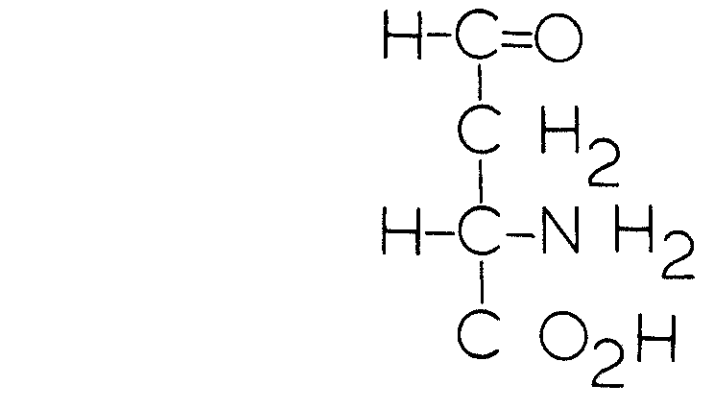
Possible biological origin of unusual amino acids. The higher plants, especially Leguminosae, have proved to be a rich source of unusual amino acids. The dormant seeds of some species in the family contain as much as five per cent by weight of a single nonprotein amino acid. Relatively little is known about the biosynthesis and fates of most of these amino acids, and we can only speculate as to their significance in the plants containing them. Fowden (18) has suggested that some of these compounds may arise because of a lack of specificity and flexibility in the enzymes which are primarily concerned with the synthesis of protein amino acids. For example, he has proposed that the biosynthesis of pipecolic acid, which is found in

some legumes, may be catalyzed by the enzymes whose primary role is in the synthesis of proline. This proposal also holds in the case of Mung bean (Phaseolos aureous) from which Fowden has isolated the lower homologue of proline, azetidine-2-caboxylic acid. Fowden, however, was not able to incorporate aspartic acid into the unusual amino acid, azetidine-2-carboxylic acid in laboratory, but still maintained that it could occur in nature.

In S. secundiflora the situation is different. In this plant a high concentration of aspartic acid is present, which is probably an indication of an aspartic semi-aldehyde pathway to lysine (Fig. 9) (20). Lysine has been shown to be a precursor of different alkaloids found in these plants(15). Therefore, one may reasonably speculate that pipecolic acid has evolved from one of the intermediates involved in the biosynthesis of lysine; namely, Δ1-piperidine-2, 6-dicarboxylic acid. The basis of this speculation is shown in Fig. 9. Successive hydrogenation and decarboxylation occurs naturally in amino acids (20) p. 342, and this could lead to the production of pipecolic acid via the aspartic semialdehyde pathway.

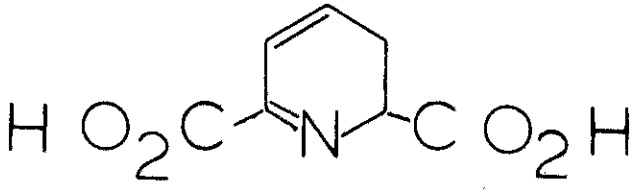
Biochemical systematics of the genus Sophora. In these studies it was found that some species of Sophora contain the unusual amino acid, pipecolic acid. Some contain both pipecolic acid and its derivative, 4-hydroxypipecolic acid (Fig. 10). However, 10 out of 23 species examined had no detectable pipecolic acid in their seeds. According to these observations, and based on

Fig. 9. Proposed pathway of pipecolic acid synthesis from aspartic semialdehyde. After Mohler and Cordes (20) page 674.

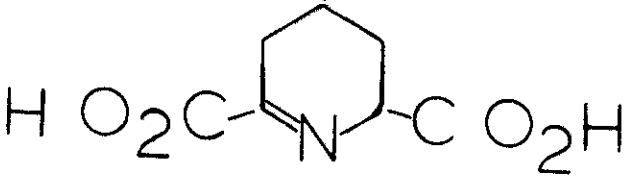


* Pyruvate

aspartic semialdehyde

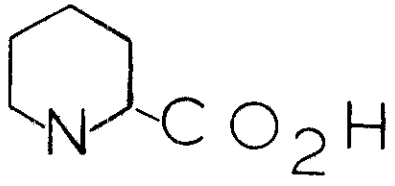


1-piperidine-2,6-dicarboxylic acid



2,3-dihydrodipicolinic acid

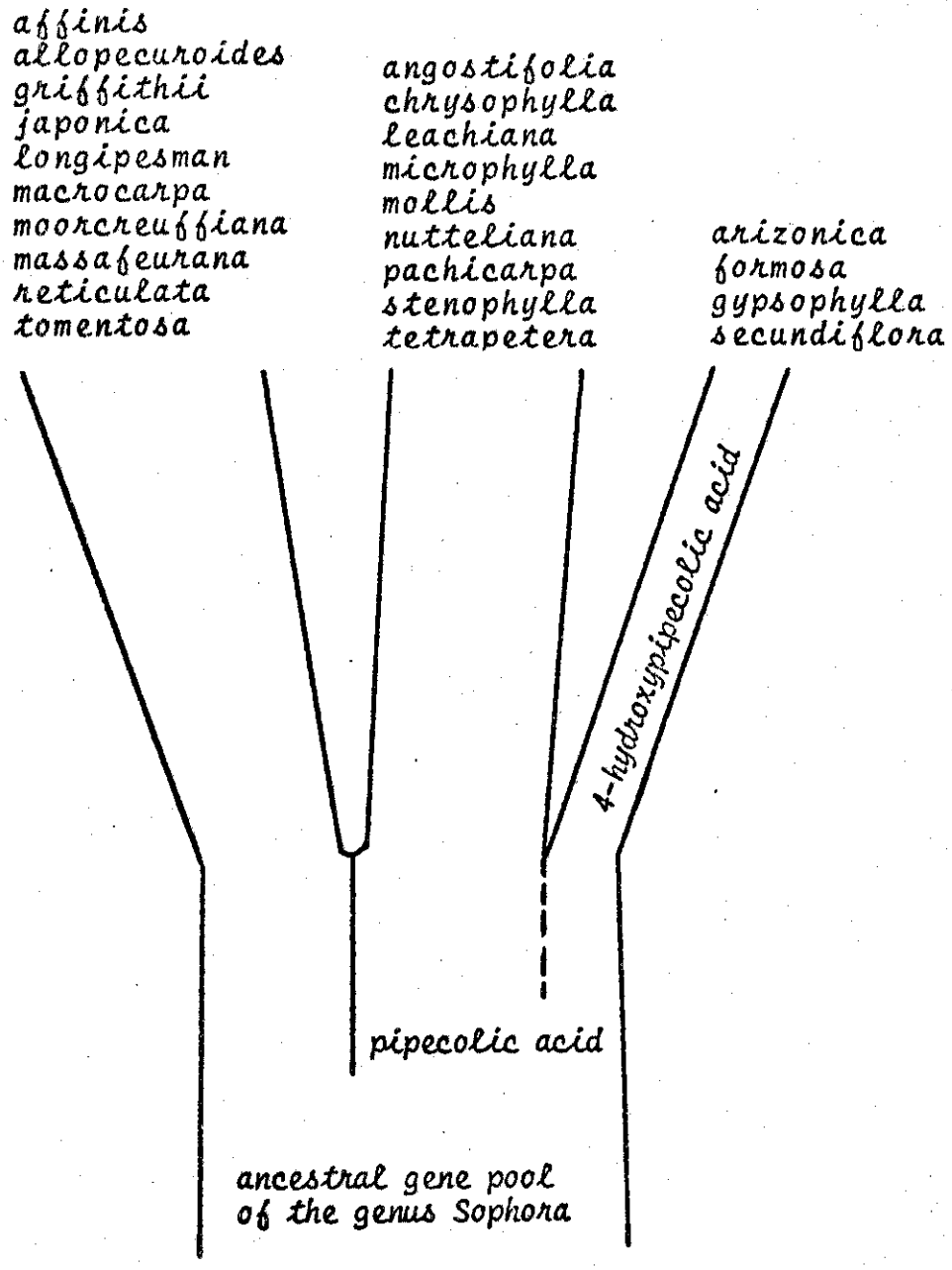
?



pipecolic acid

LYSINE

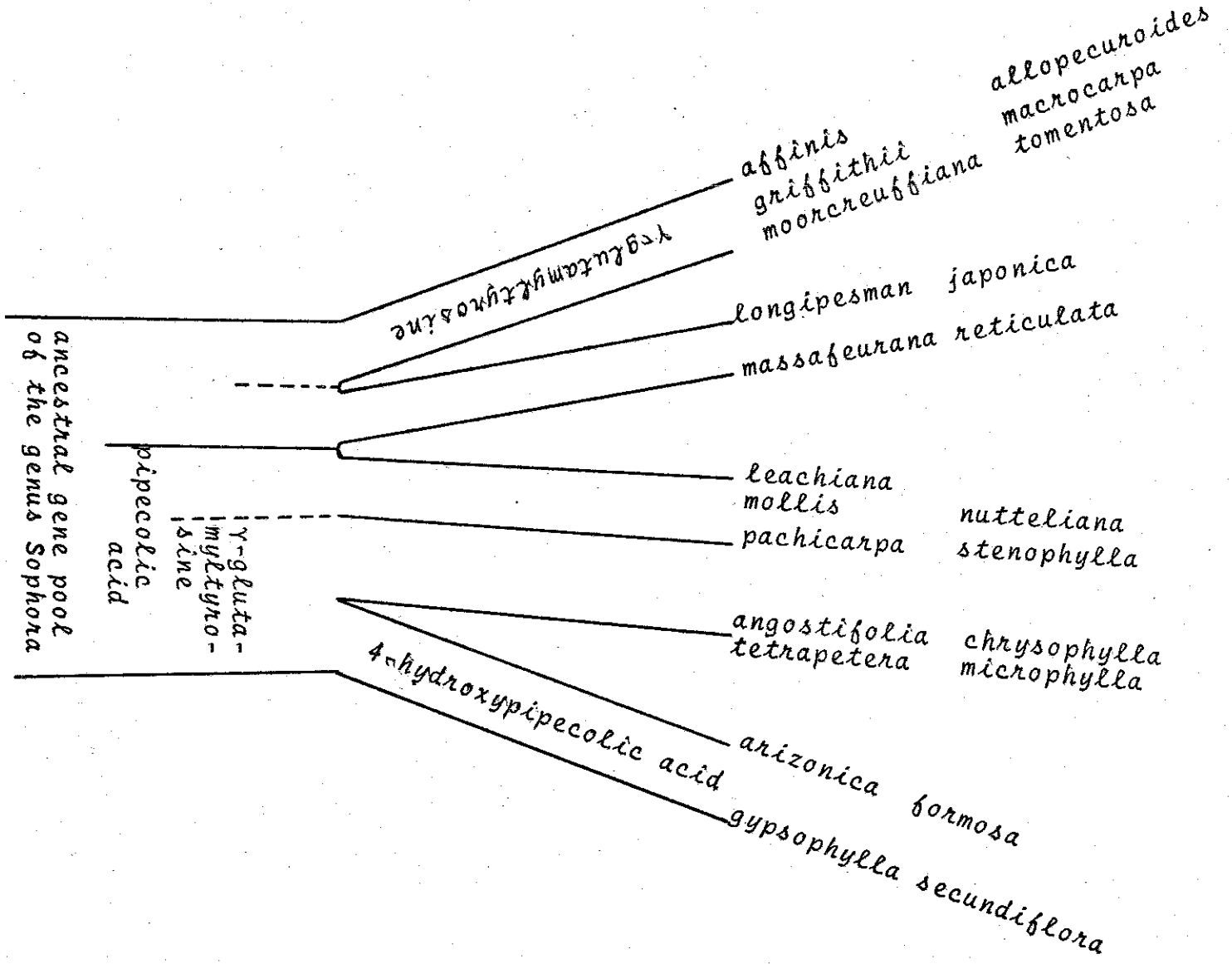
Fig. 10. Proposed phylogenetic tree of twenty-three species of the genus Sophora according to their pipecolic acid and 4-hydroxypipecolic acid content.


affinis
allopecuroides
griffithii
japonica
longipesman
macrocarpa
moorcreuffiana
massafeurana
reticulata
tomentosa
angostifolia
chrysophylla
leachiana
microphylla
mollis
nutteliana
pachicarpa
stenophylla
tetrapetera
arizonica
formosa
gypsophylla
secundiflora
4-hydroxypipecolic acid
pipecolic acid
ancestral gene pool
of the genus Sophora

the assumption that pipecolic acid is the precursor of 4-hydroxypipecolic acid, the genus Sophora may be divided into three subgeneric groups. Those species which contain no pipecolic acid are regarded as more primitive of the genus and are called group I or Persian group, because most of the members of the group are found in the Persian region of the world. Those species which contain pipecolic acid were categorized in group II or Hawaiian group, because most members of the group are found in Southeast Asia and Hawaii. Those plants which contain both pipecolic acid and its derivative, 4-hydroxypipecolic acid, were named the Texan group or group III. All members of this latter group belong to the Southwestern region of North America.

The dipeptide, γ-glutamyltyrosine, was found in some members of each group but absent in others. Accordingly the first two groups (I, II) were subdivided into two subgroups, one of which contained the dipeptide and the other without (Fig. 11). It should be emphasized here that this branching is not of any significance regarding evolution of the Sophora species. The presence of the dipeptide merely indicates the presence of a series of enzymes which evolved independently of those involved in biosynthesis of pipecolic acid (Fig. 11), Table IX). However, if the dipeptide is regarded as a marker for classification of the genus, plants containing the compound should be considered more advanced when compared with those which do not. In other words, the classification here may be

Fig. 11. Proposed subgrouping of the main groups in Fig. 10 according to γ-glutamyltyrosine content.


ancestral gene pool of the genus Sophora
γ-glutamylhydroxamine
pipecolic acid
γ-glutamyltyrosine
4-hydroxypipecolic acid
affinis
griffithii
mooreroubfiana
allopecuroides
macrocarpa
tomentosa
longipesman
japonica
massafeurana
reticulata
leachiana
mollis
pachicarpa
nutteliana
stenophylla
angostifolia
tetrapetera
chrysophylla
microphylla
arizonica
formosa
gypsophylla
secundiflora

based on the pipecolic acid content or the dipeptide content since the two compounds are not related (8).

According to further studies in the amino acid chemistry of these plants, there appeared to be another mutation, independent of those mentioned above. This mutation has led to the appearance of another unusual amino acid, γ-aminobutyric acid, in some Sophora species. Accordingly, further biochemical classification of the genus was arranged in Fig. 12 and Table IX.

As mentioned previously, if the amino acid γ-aminobutyric was used as a reference for the evalution of the evolution of the genus Sophora, then the genus could be divided into two branches. Sophora plants which contain the amino acid, γ-aminobutyric acid would be regarded as the more advanced group of the genus.

It should be emphasized here that more information about the amino acid biosynthetic pathway is required to establish a sequence of evolutionary events leading to the relationships between the species. In light of these findings, it could be concluded that the chemical analysis confirms the present classification based on morphological concepts. Accordingly, an Adensonian or numerical concept of taxonomy can be employed to establish a single phylogenetic tree representing the genus. This unusual amino acid basis of classification, however, contributes to the refinement of the morphological system by subdividing the genera into subgenera. Since the genus is

Fig. 12. Proposed chemosystematics tree of 23 species of Sophora according to their unusual amino acid content. Small branches show the presence of γ-amino-n-butyric acid.

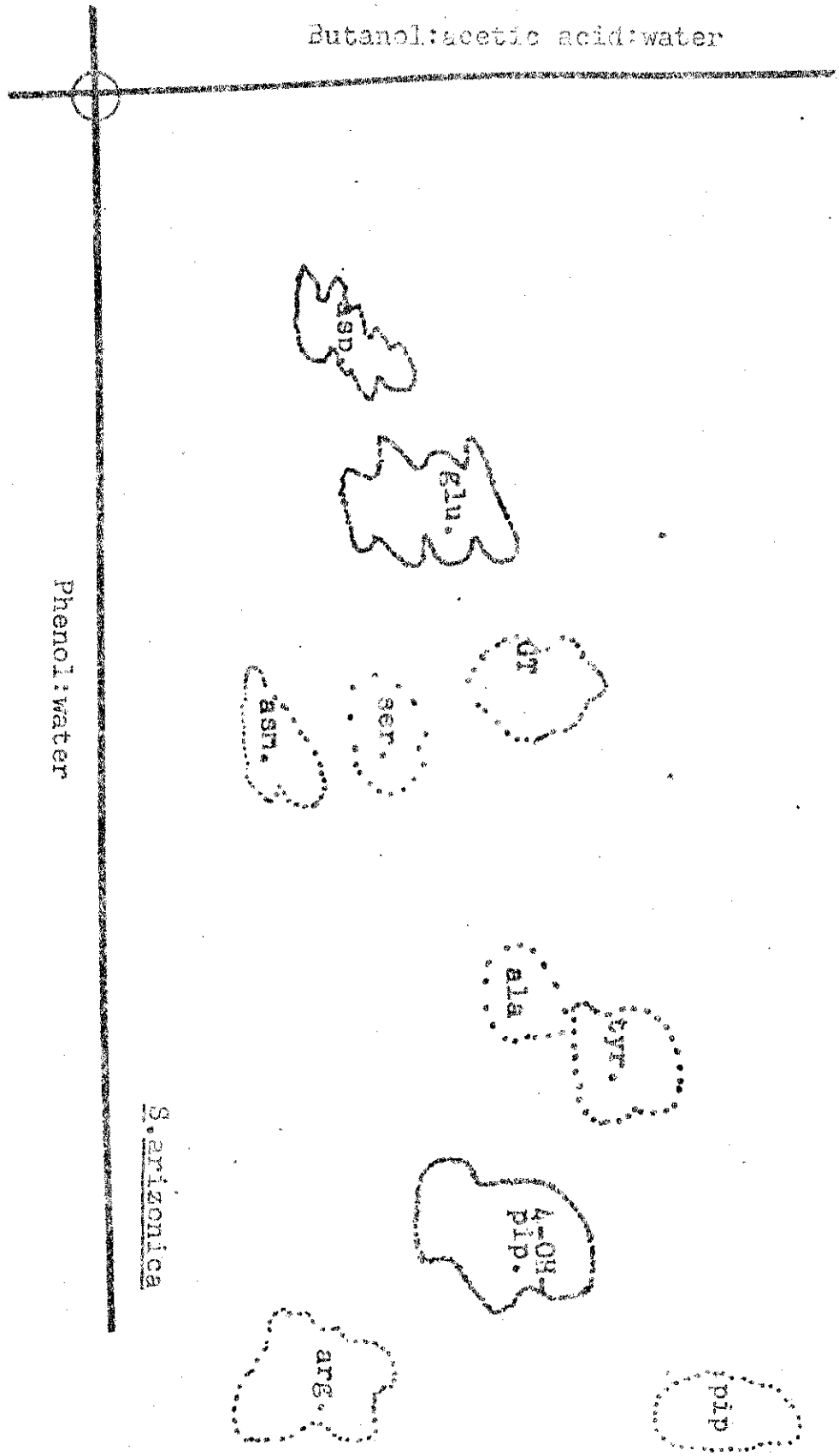
albinia
tomentosa
alopecuroides
macrocarpa
γ-glutamylglycine
gnidiithii
moorcroftiana
longipesman
massaiensis
japonica
reticulata
mollis
leachiana
pachycarpa
nuttelliana
stenophylla
angustifolia
chrysophylla
microphylla
tetraptera
formosa
secundiflora
arizonica
gypsophylla
4-hydroxypipecolic acid
γ-glutamyl
tyrosine
pipecolic
acid
ancestral gene pool
of the genus Sophora

quite heterogeneous, more data about alkaloids, phenolic compounds, flavonoids and lipids, etc. (19) would be necessary to make further refinements in the present classification and in tracing the evolution of the genus Sophora.

APPENDIX

Tracings of the two dimensional paper chromatograms of 23 species of the genus Sophora. The solid boundries are indicative of the higher concentration of the ninhydrin-reacting compounds relative to the others.

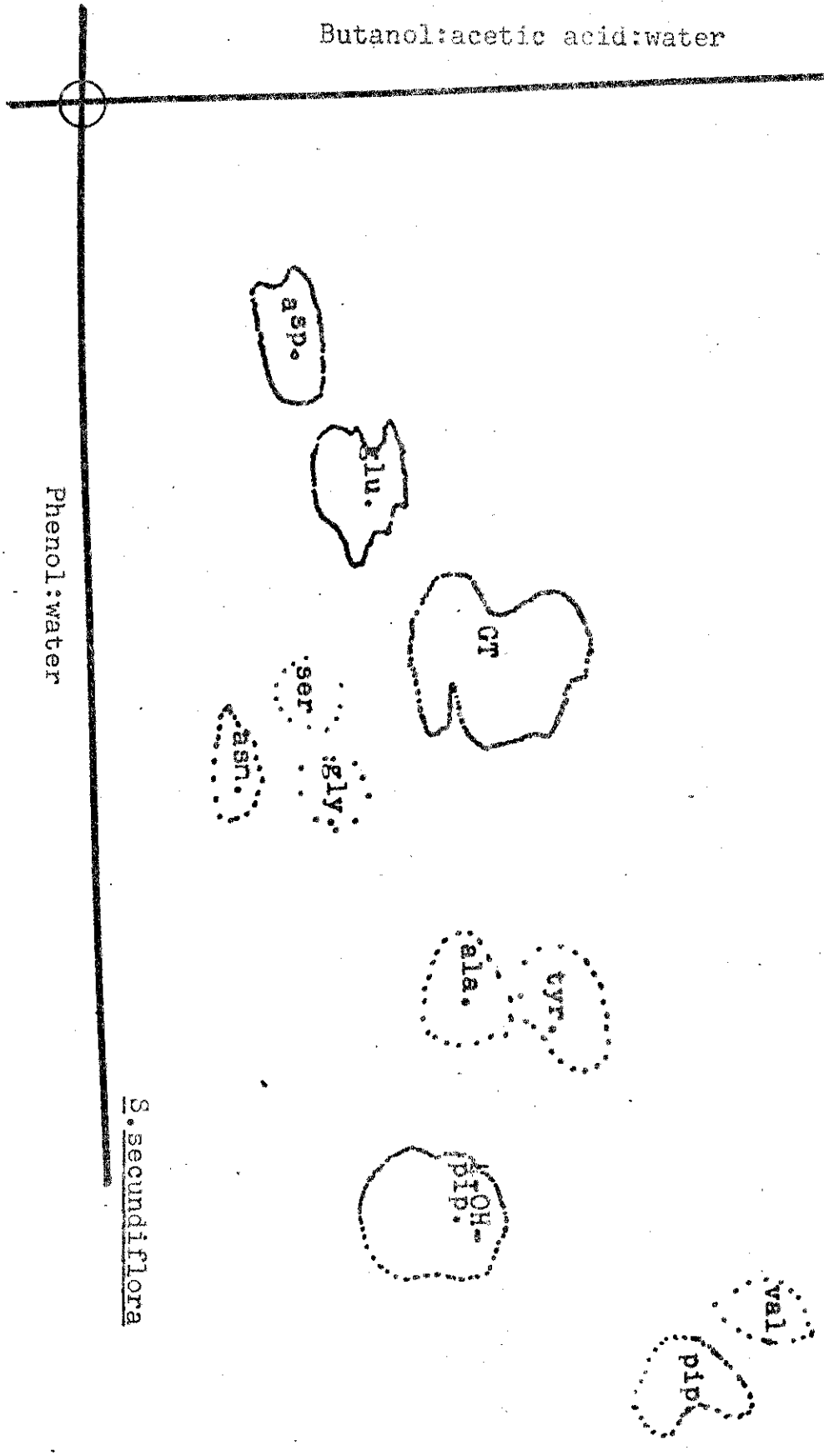
Ala = alanine; asp = aspartic acid; asn = asparagine; glu = glutamic acid; glu-tyr = γ-glutamyltyrosine; GT = γ-glutamyltyrosine; gly = glycine; ile = isoleucine; leu = leucine; 4-OH-pip = 4-hydroxypipecolic acid; phe = phenylalanine; pip = pipecolic acid; pro = proline; thr = threonine; tyr = tyrosine; ab = γ-aminobutyric acid.

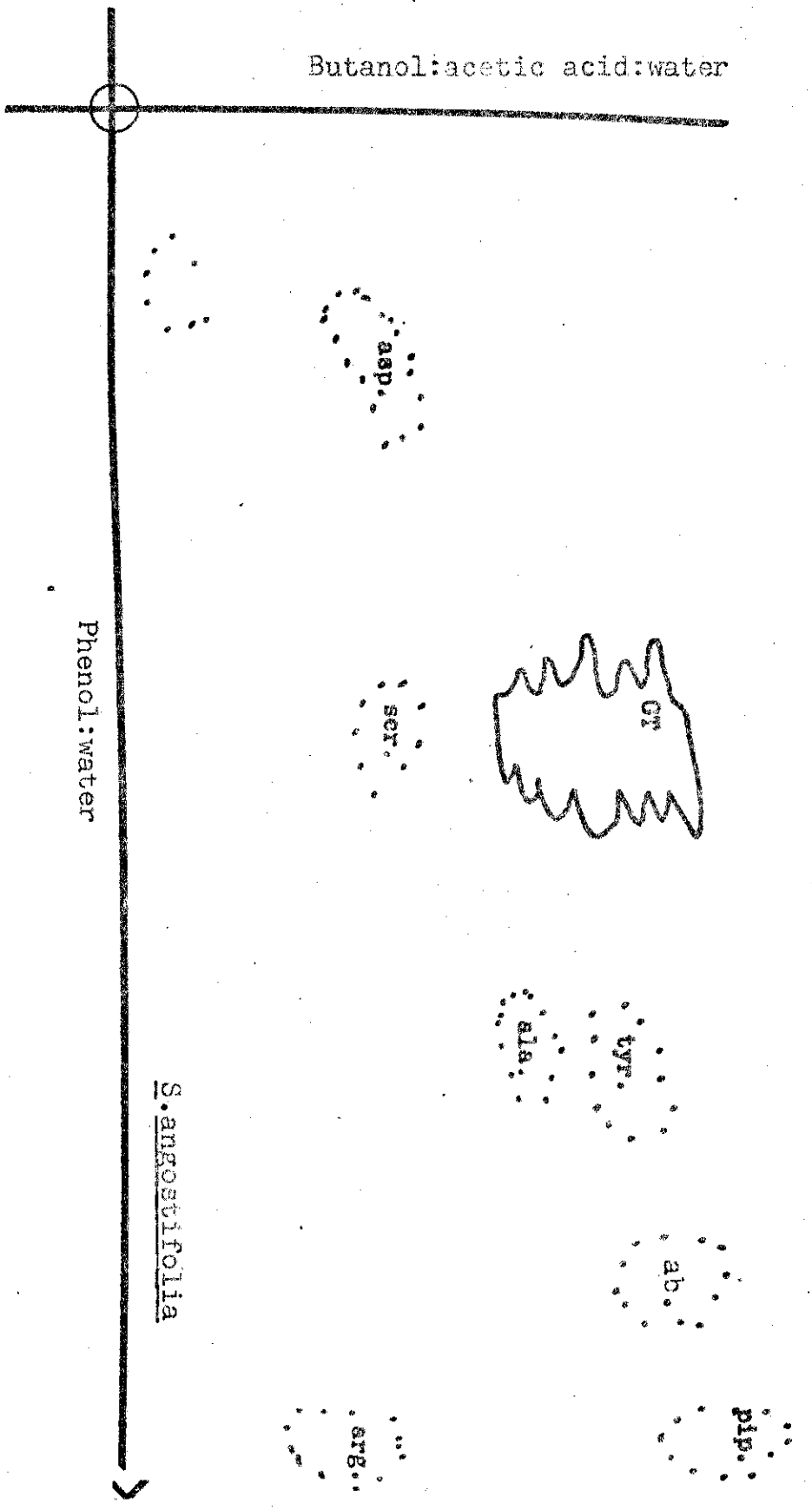

Butanol:acetic acid:water
Phenol:water
S. arizonica
asp.
glu.
gly.
asn.
ser.
ala.
tyr.
4-OH pip.
arg.
pro.

Butanol:acetic acid:water
Phenol:water
asp.
glu.
ser.
gly.
GT
thr.
ala.
tyr.
4-OH-
pip.
val.
ab.
pip.
leu.
ile.
lys.
arg.

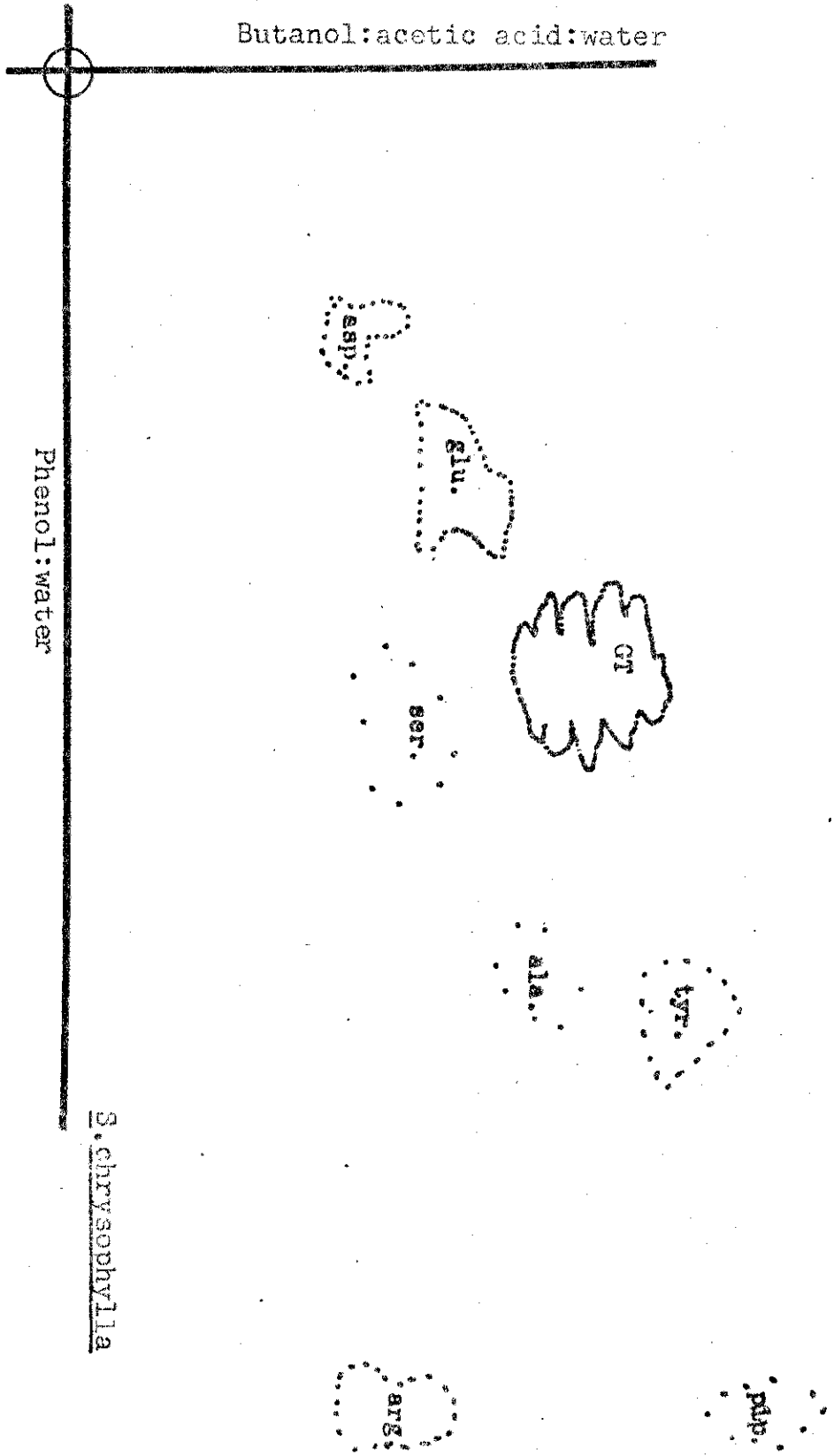
S. formosa

S. gypsophylla


Butanol:acetic acid:water
Phenol:water
S. secundiflora
asp.
glu.
GT
ser.
asn.
gly.
ala.
tyr.
pro-OH-
pro.
val
pro



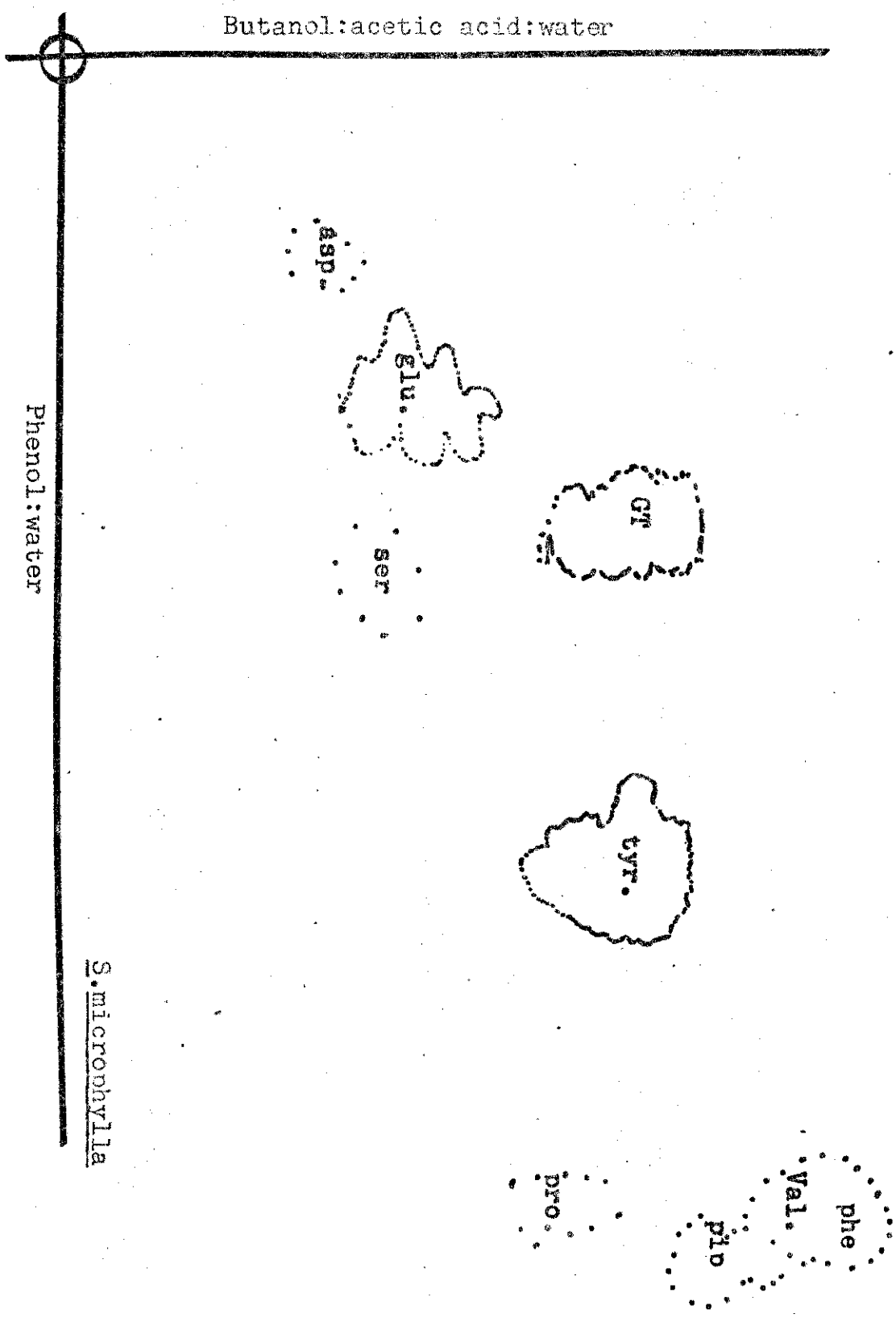
S.angostifolia



S. chrysophylla

Butanol:acetic acid:water
Phenol:water
asp.
glu
ser.
ala
ab.
val
leu.
ile
pip.

S. leachiana



S. microphylla

Butanol:acetic acid:water
Phenol:water
asp.
glu.
ser.
gly.
asn.
tyr.
leu.
ile.
val.
pip.
S. mollis

S. serica nuttelliana

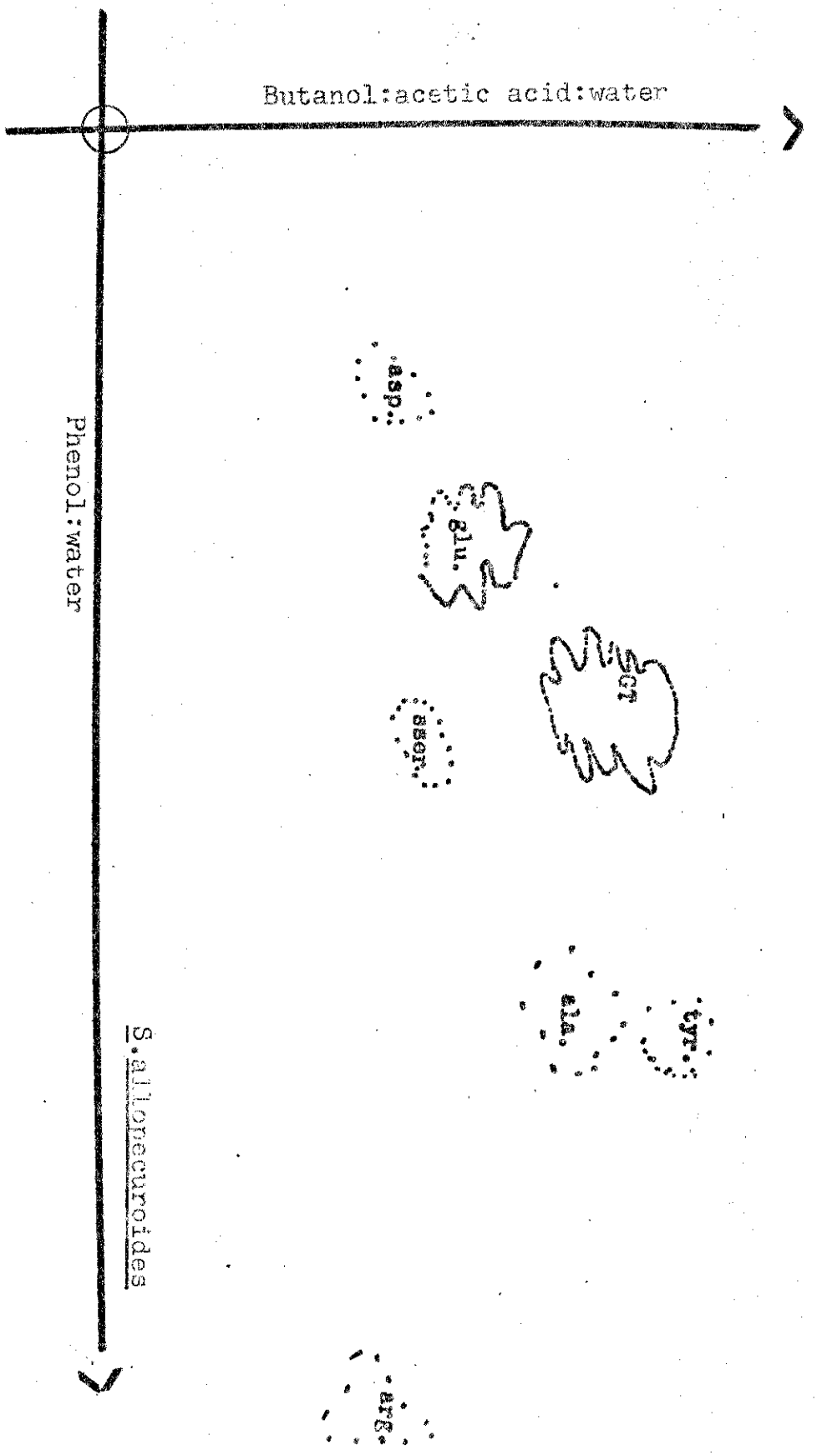
S. pachycarpa

S. stenophylla

Butanol:acetic acid:water
Phenol:water
S. tetrapetera
asp.
glu.
gr.
cys.
dip.

Butanol:acetic acid:water
Phenol:water
asp.
glu.
ser.
GLY
ala.
tyr.
arg.

S. affinis

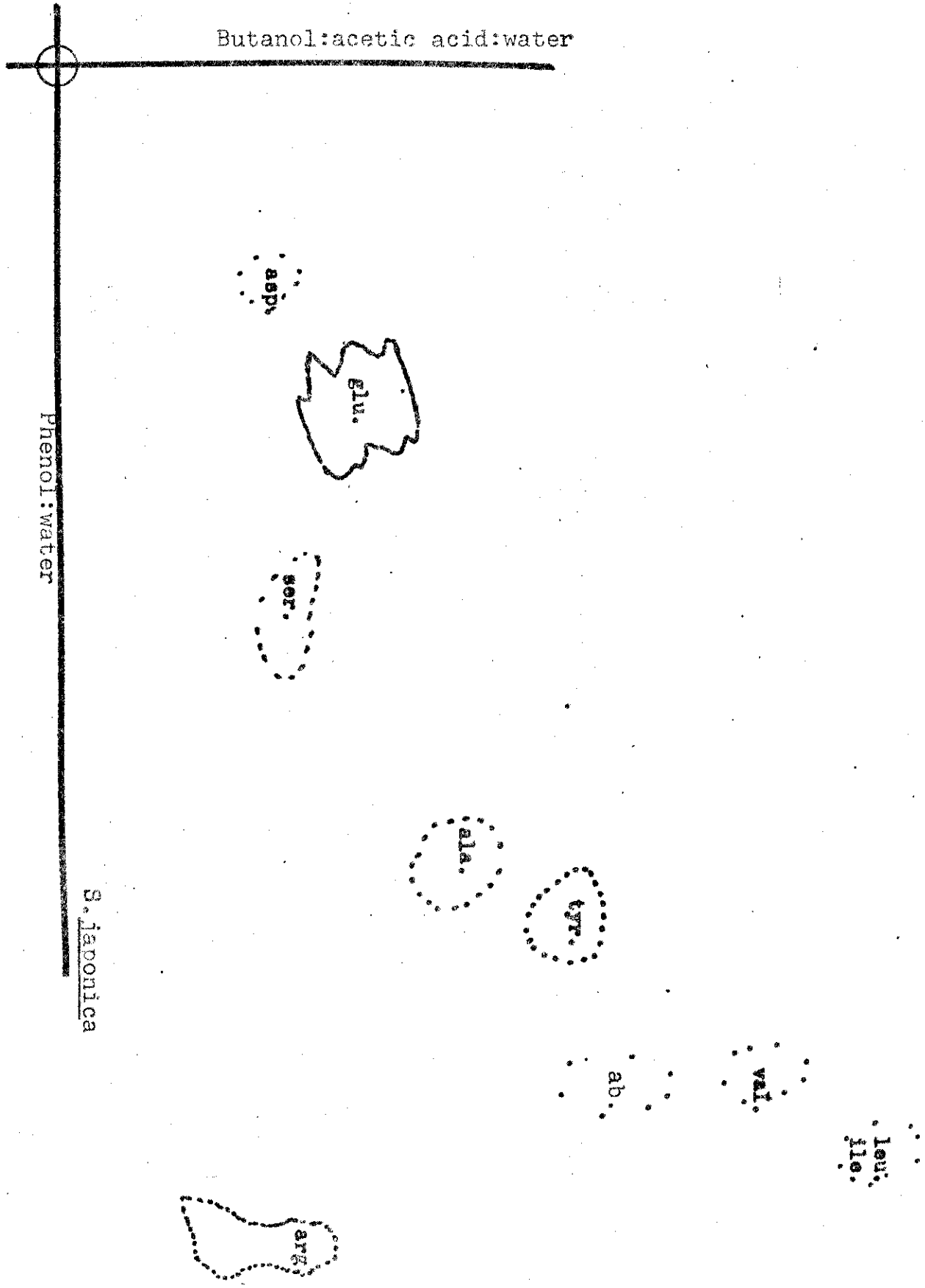

Butanol:acetic acid:water
Phenol:water
S. allomeuroides
asp.
glu.
ser.
ala.
tyr.
arg.

Butanol:acetic acid:water

Phenol:water

S. griffithii

asp.
glu.
GT
ser
asn.
ala.
tyr.
ab.
val.
lys
arg
ile
leu

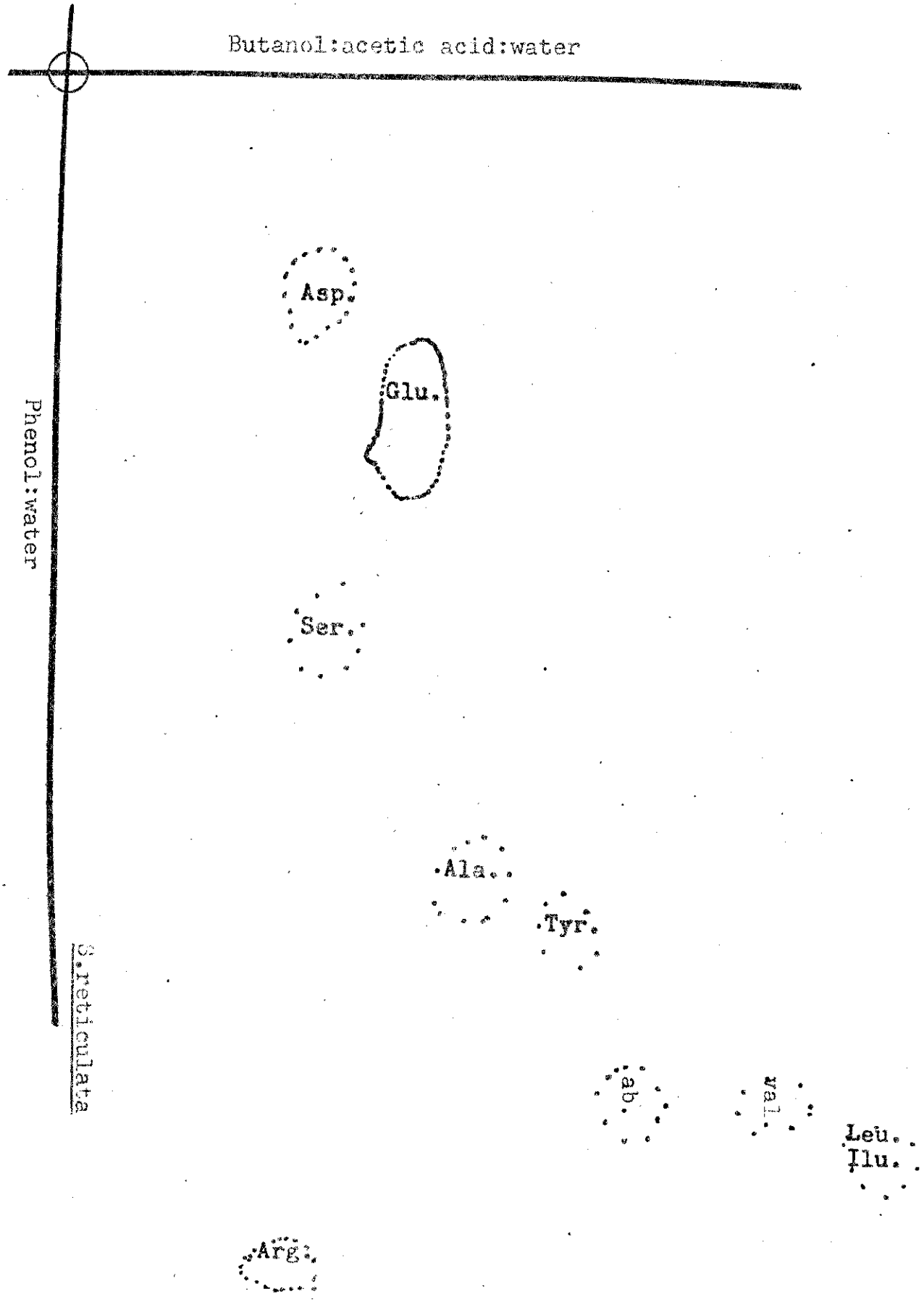

Butanol:acetic acid:water
Phenol:water
S. japonica
asp.
glu.
ser.
ala.
tyr.
ab.
val.
leu.
ile.
arg.

S. longipesmen

Butanol:acetic acid:water
Phenol:water
S. macrocarpa
asp.
glu.
ser.
gly.
Cys
tyr.
val
leu.
ile.

S. moorcroftiana

S. massafeurana


Butanol:acetic acid:water
Phenol:water
Asp.
Glu.
Ser.
Ala.
Tyr.
ab.
val.
Leu.
Ilu.
Arg.
S.reticulata

Butanol:acetic acid:water
Phenol:water
S.tomentosa
asp.
glu.
GT
ser.
gly
tyr.
arg.

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