and the yellow product was filtered and recrystallized from aqueous ethanol. The yield was 0.83 Gm. (31%); m.p. 198–199°.

N-4,5-Dichloro-2-nitrophenylamino Acid Ethyl Esters.—The following procedure is representative. N-4,5-Dichloro-2-nitrophenyl-DL-serine (3.0 Gm., 0.008 mole) was dissolved in 100 ml. of absolute ethanol. The solution was heated to 70°, and dry hydrogen chloride was introduced, with stirring, for a period of 2 hours. The resulting solution was evaporated to a small volume and cooled. Water was added until the ester precipitated, and the precipitate was collected. This precipitation from ethanol was repeated, and the product was dried over calcium chloride. The yield was 0.60 Gm. (19%); m.p. 127–128°.

REFERENCES

(1) Belleughi, M., and Wittgens, A., *Arzneimittel-Forsch.*, **8**, 263(1958).
(2) Ikegaki, K., *Arch. Antibiotics Japan*, **3**, 15(1950); Packchanian, A., *Antibiotics & Chemotherapy*, **7**, 13(1957).
(3) Sakai, S., Minoda, K., Akagi, S., and Okoti, N., *J. Sci. Research Inst. Tokyo*, **51**, 91(1957).
(4) Cooper, E. A., *Biochem. J.*, **7**, 175(1913).
(5) Glaser, E., and Prufer, H., *Biochem. Z.*, **137**, 429 (1923); Glaser, E., and Wulwek, W., *ibid.*, **145**, 514(1924).
(6) Ishiwara, F. Z., *Immunitatsforsch.*, **40**, 429(1924).
(7) Tien, N. B., Buu-Hoï, N. P., and Xuong, N. D., *J. Org. Chem.*, **23**, 186(1958).
(8) Feldmann, E. G., and Foye, W. O., This Journal, **48**, 419(1959).
(9) Abderhalden, E., and Blumberg, P., *Z. physiol. Chem.*, **65**, 318(1910).
(10) Sanger, F., *Biochem. J.*, **39**, 507(1945).
(11) Hartley, P., and Cohen, J. B., *J. Chem. Soc.*, **85**, 865 (1907).
(12) Rao, K. R., and Sober, H. A., *J. Am. Chem. Soc.*, **76**, 1328(1954).
(13) Green, F. C., and Kay, L. M., *Anal. Chem.*, **24**, 726 (1952).

Phytochemical Investigation of *Amphipterygium adstringens*

By EDWARD E. GONZALEZ† and JAIME N. DELGADO

A general phytochemical investigation was conducted on *Amphipterygium adstringens*, Schlecht. Numerous qualitative studies were conducted and several conventional techniques were employed in the detection, isolation, purification, and chemical characterization of constituents. Qualitative chemical tests showed the presence of phytosterols, glycosides, and tannins, and the absence of alkaloids and phloroglucides. An organic acid and a steroidal saponin were isolated from the diethyl ether and *n*-butanol extracts, respectively. The *p*-nitrobenzyl and *p*-phenylphenacyl derivatives of the carboxylic acid were prepared. The neutralization equivalent and infrared spectrum of the isolated acid were established. Chromatographic and infrared studies of the saponin have been reported.

This phytochemical study was initiated primarily because preliminary screening tests indicated that *Amphipterygium adstringens*, Schlecht (Fam. *Julianiaceae*) possessed a certain degree of anticancer activity (1). This observation, together with a survey of the available literature which revealed no apparent report on the exact chemistry of this botanical species, stimulated interest and prompted this investigation. Furthermore, since Martinez (2) briefly makes note of *Amphipterygium adstringens* in the second part of his book which deals only with medicinal plants lacking scientific investigation, it was felt that the project was worthy of exploration.

A survey of botanical literature revealed disagreements in the scientific nomenclature of *Amphipterygium adstringens*, Schlecht. Some of the early reports refer to the species as *Hypopterygium adstringens*. In the more recent literature the generic name is given as *Juliania*, family *Julianiaceae*. *Amphipterygium adstringens*, commonly known as "Cuachalalate," is a small tree indigenous to Mexico, growing from Michoacán to Morelos and Puebla to Oaxaca. The assumed medicinal effects of the bark are briefly described by Martinez (2). Concoctions of the bark are used extensively for alleviating numerous conditions, including malaria, intermittent fever, ulcers, and cancer of the gastrointestinal tract.

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EXPERIMENTAL

Source and Preparation of the Plant Material.—The chemical studies were conducted on authenticated samples.[1] The plant material used in this study consisted of the outer, dried bark of *Amphipterygium adstringens*, free of adulteration and adhering foreign matter. The hard, bulky pieces of bark were first reduced in size with a Wiley grinding laboratory mill, and then pulverized to a No. 20 mesh size in a W. J. Fitzpatrick, model D, comminuting machine. The ground material was then stored in tight metal containers to avoid contamination and possible deterioration.

Extraction Studies.—Suitable, accurately weighed samples of powdered material were extracted successively with a series of solvents in a Soxhlet continuous extraction apparatus by the Rosenthaler method (3). The solvents were used in the order of increasing polarity, and the extraction with a given solvent was continued until an aliquot of the colorless percolate left no residue when evaporated to dryness. The percentage of extractives were as follows: petroleum ether (b.p. range 30–60°), 6.11%; anhydrous ether, 4.62%; chloroform U.S.P., 0.82%; anhydrous ethanol, 9.61%; 70% ethanol, 4.07%.

Phytosterols.—In an attempt to determine the presence of phytosterols, effective application was made of the digitonin reaction according to the method described by Fieser and Fieser (4). The nonsaponifiable fraction of the petroleum ether[2] extract gave a positive reaction with digitonin, forming a white crystalline substance which melted at 139–143°. In order to substantiate fully the results of the digitonin reaction, other more general and classical qualitative tests were applied to fractions of the nonsaponifiable material. Positive results were obtained with Hesse's, Moleschott's, Liebermann's, and Hirschsohn's reactions.

Phloroglucides.—The Coward-Harris (5) procedure was employed as the basis for the qualitative analysis for phloroglucides. No precipitation was observed indicating their absence.

Alkaloids.—The chloroform extract of the whole drug (200 Gm.) was examined for alkaloids by the proximate assay method for alkaloids (6). Since the resulting extract gave a negative test with Mayer's reagent but produced slight opalescence with gold chloride T.S. and tannic acid T.S., an attempt to isolate alkaloids was made on a larger portion of crude drug following the procedure of Kleber and Gisvold (7). Negative results were obtained with Mayer's reagent, Lugol's solution, gold chloride T.S., picric acid T.S., and phosphomolybdic acid T.S., conclusively indicating the absence of alkaloidal substances in the plant material.

Glycosides.—A modified procedure of the Stas-Otto method was employed in the qualitative determination for glycosides. A mixture of plant material (300 Gm.) and ethanol containing tartaric acid (2%) was refluxed for approximately 12 hours. After the solvent was recovered by filtration, the marc was washed several times with hot ethanol and then pressed as free as possible of solvent. These filtrates were combined and evaporated to dryness in a Rinco rotatory evaporator under reduced pressure using steam as the source of heat. The residue was washed with hot distilled water (four 50-ml. portions) and the aqueous solution was extracted with diethyl ether (four 50-ml. portions) in a separator. The combined ethereal extracts were evaporated to dryness and the residue dissolved in ethanol. Aliquots of this solution were tested with Molisch reagent. Positive results were obtained, indicating the presence of carbohydrate substances and/or glycosides.

Tannins.—A hydroalcoholic (50% ethanol) extract of the plant material was prepared and aliquots of the solution were checked for tannins with various test solutions. A positive reaction was obtained with potassium dichromate, gelatin, ferric chloride, and uranyl acetate reagents. The tannins were isolated by the Feist and Bestehorn method (3) and an attempt was made to classify the tannins according to their specific chemical reactions with various reagents (8). In order to prevent one intense color from obscuring or masking another, several tannin solutions of ranging concentration were prepared. Positive results were obtained with fresh bromine water and ferric chloride T.S., while negative results were obtained with vanillin-hydrochloride and ammonium molybdate.

The production of a green color with ferric chloride T.S. indicated a catechol nucleus (two adjacent free phenolic groups) in the molecule, rather than a pyrogallol nucleus (three adjacent free phenolic) groups). The fact that the tannins were precipitated with bromine water reveals that they are of the nonhydrolyzable type since the hydrolyzable tannins do not react in this manner. Negative results obtained with ammonium molybdate and vanillin-hydrochloride indicate the absence of gallic acid and phloroglucin tannins, respectively.

Examination of Diethyl Ether Fraction.—The marc which had been previously exhausted with petroleum ether was extracted with diethyl ether by cold percolation. The ethereal extract was concentrated under reduced pressure to 50% of its original volume and placed in the deep freezer at −10° for several days. A substantial amount of pyramidal shaped crystals precipitated from the ethereal solution. The yellowish crystals which formed were removed by filtration and washed several times with cold acetone and methanol. These washings removed most of the colored impurities. The crude crystalline substance was recrystallized from ethanol and dried, *in vacuo*, at 80° with potassium hydroxide in an Abderhalden drying pistol. The crystalline material melted at 165–166° and was acidic to both litmus paper and phenolphthalein T.S. The crystals proved to be insoluble in water, methanol, acetone, concentrated hydrochloric acid, and sulfuric acid; sparingly soluble in hot methanol, cold ethanol, chloroform, and carbon tetrachloride. On the other hand, the substance exhibited a high degree of solubility in hot ethanol, sodium hydroxide solution, alcoholic potassium hydroxide, and sodium bicarbonate solution. Some of the qualitative tests that were applied for the classification and partial chemical characterization are summarized as follows: (*a*) ferric chloride test for phenols, negative; (*b*) Molisch test for carbohydrates, negative; (*c*) ceric ammonium nitrate test for alcohols, negative; (*d*)

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[1] Kindly furnished by Dr. H. R. Schmidt, research pharmacognosist, Department of Research, S. B. Penick and Co., New York, N. Y.

[2] Unless otherwise specified, petroleum ether refers to technical grade petroleum benzin, boiling range 30–60°.

Liebermann-Burchard test for sterols, negative; (*e*) Fuchsin-aldehyde test, negative; (*f*) lead acetate test, positive; (*g*) picric acid test, negative; (*h*) cupric sulfate test, positive; (*i*) iodine test, negative; (*j*) potassium permanganate test, positive (ppt.); (*k*) sodium fusion test for nitrogen, phosphorus, and sulfur, negative. The preceding data eliminated several possibilities and indicated that the isolated substance was possibly an organic acid.

Anal.—Found: C, 78.26; H, 10.99. The neutralization equivalent of the acidic crystalline substance was determined according to the standard analytical method described by Niederl and Niederl (9). The average neutralization equivalent was determined to be 550.20.

Esterification.—The *p*-nitrobenzyl and *p*-phenylphenacyl derivatives of the organic acid were prepared from the corresponding bromides according to the procedures of Kelly (10) and Drake (11), respectively. These two particular derivatives were chosen because there is usually a considerable difference in the melting point of the *p*-nitrobenzyl and *p*-phenylphenacyl ester of any one acid. During the esterification process, it was essential to maintain the reaction mixture slightly acidic since alkalies could hydrolyze the parent halides to the corresponding alcohols. The prepared *p*-nitrobenzyl ester melted at 72–73° and the *p*-phenylphenacyl ester at 127–128°.

The neutralization equivalent value and the melting points of the prepared derivatives did not compare with any values reported in the literature.

Infrared Analysis.—A potassium bromide wafer of the crystalline substance was prepared for the infrared spectrum analysis since it was not sufficiently soluble in chloroform. The infrared spectrum was determined in an infrared recording spectrophotometer, model 4-55, Baird Associates, Inc. The absorption spectrum of the isolated acid is shown in Fig. 1.

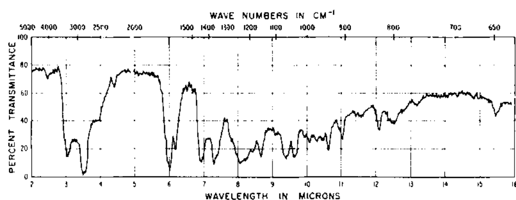

Fig. 1.—Infrared spectrum of the unknown acid in potassium bromide wafer.

The broad absorption bands between 3.0 and 3.6 μ (3310–2860 cm.$^{-1}$) fall within a frequency range which is normally assigned to carboxylic acids; however, the specific band observed under 3.5 μ (2850–2860 cm.$^{-1}$) may be conferred also by $—CH_2—$ vibrations (12). Inspection of the spectrum for the carboxyl function disclosed a stretching band with a shoulder near 6.0 μ (1700–1650 cm.$^{-1}$). The absorption band at 6.9 μ (1465–1400 cm.$^{-1}$) could be manifested by the covalent carbon-oxygen bond of the carboxyl group. Some indications of the presence of an isopropyl group is found in the absorption band observed in the 7.3–7.4 μ (1370–1350 cm.$^{-1}$) region (12). Furthermore, the bands appearing near 8.1 μ (1250 cm.$^{-1}$) and 8.7 μ (1150 cm.$^{-1}$) give reason to suspect its presence. The other weak peaks noted above 9.0 μ may possibly be deformation frequencies associated with either propyl or carboxyl group vibrations.

Examination of Aqueous Fraction.—During the preliminary chemical studies, it was noted that the aqueous extract produced vigorous foaming when agitated. This observation, and the fact that the Stas-Otto test for glycosides was positive, suggested the possibility of existing saponins. In order to provide more convincing evidence, the aqueous extract of the plant material was extracted with hot *n*-butanol by means of a separator and the residue obtained after evaporation of the butanol was tested with antimony trichloride T.S. (13). A positive reaction was observed with the production of a yellow color.

In order to isolate an adequate amount of saponin, 10 Kg. of powdered bark were extracted by means of cold percolation in a large metal percolator (36 in. high and 14 in. inside diameter). The percolation was continued at a moderate rate over a period of several days until 15 gallons of liquid were collected. Extreme care was exercised to avoid fermentation during the long period of slow maceration and percolation. The aqueous percolate was lyophilized to dryness in a F. J. Stokes Lyophilyzer, model 2004L X 3, and the combined residues washed and refluxed in *n*-butanol.[3]

After filtration, the *n*-butanol extract was concentrated by distillation, *in vacuo*, at 55–60° to a small volume. Cold anhydrous acetone (200 ml.) was added to the contents of the flask causing the precipitation of a light amorphous substance. A total of 13.4 Gm. of crude saponin was recovered by the aforementioned process. The solid amorphous substance was hygroscopic and soluble in water, with considerable foaming and opalescence. It was soluble in methanol and ethanol, insoluble in petroleum ether, diethyl ether, chloroform, and acetone. Some of the basic qualitative tests that were applied for the chemical classification are summarized as follows: (*a*) Molisch test, positive; (*b*) ferric chloride test, negative; (*c*) Liebermann-Burchard test, negative; (*d*) Seliwanoff test, negative; (*e*) antimony trichloride test, positive; (*f*) basic lead acetate test, positive; (*g*) the addition of ether to an aqueous solution caused the formation of a gelatinous precipitate; (*h*) the addition of sulfuric acid (1 ml.) to a concentrated aqueous solution produced a crimson color. The preceding chemical data substantiated that the amorphous substance under consideration was a saponin.

Hydrolysis of Saponin.—Hydrolysis of the above described glycosidal compound was accomplished by means of strong mineral acid in alcohol and water mixtures. A saponin sample (4 Gm.) was dissolved in a hydroalcoholic mixture composed of distilled water (50 ml.) and ethanol (25 ml.), to which was added concentrated sulfuric acid (8.6 ml.). The acidic mixture was refluxed vigorously on a steam bath for 6.5 hours. The foaming that occurred on heating was controlled by the addition of *n*-amyl alcohol (5 ml.). The reaction mixture was allowed to cool and the contents poured into a separator and extracted with warm chloroform (three 50-ml. portions). The combined chloroform extracts were con-

[3] Grateful acknowledgment is made to Dr. F. P. Cosgrove for his technical assistance in this phase of our study.

Table I.—Solvent Systems and R_f Values for Unknown and Reference Sapogenins[a]

Solvent System	a	b	c	d	e
Petroleum ether, ml.	400	400	200	100	0
Toluene, ml.	0	50	100	200	400
Ethanol, ml.	10	10	10	30	10
Water, ml.	90	90	90	70	90
Chlorogenin	...	...	...	0.310	0.290
Gitogenin	...	...	...	0.522	0.496
Hecogenin	...	0.227	0.430	0.851	0.878
Tigogenin	0.396	0.641	0.778	0.911	0.960
Diosgenin	0.433	0.645	0.832	0.956	0.988
Sarsasapogenin	0.701	0.862	0.755	1.000	1.000
Smilagenin	0.728	0.859	0.931	1.000	1.000
Unknown genin	0.698	0.865	0.750	1.000	1.000
Unknown genin plus sarsasapogenin	0.695	0.864	0.755	1.000	1.000

[a] Chemically pure (A.R.) reagents were employed in preparing all solvent systems used for chromatographic studies.

centrated to a small volume under reduced pressure in a Rinco rotatory evaporator; the sapogenin was then precipitated by the addition of methanol A.C.S. (10 ml.). The crude genin was recrystallized from methanol and used for chromatographic and infrared studies.

Paper Chromatographic Analysis of Sapogenins.—The apparatus and technique used for the chromatographic analysis of the sapogenins was essentially that described by Heftmann and Hayden (14). Whatman No. 1 filter paper was employed throughout the chromatographic studies and all chromatographic work was carried out in an air-conditioned room at 23°. After resolution was complete, the sapogenin chromatograms were sprayed with a freshly prepared solution of trichloroacetic acid in chloroform (25%) in the manner of Svendsen and Jensen (15). The sheets were heated in a chromatography oven at 90–100° for approximately 15 minutes and, thereafter, the sapogenins were detected by their varying fluorescence under ultraviolet light.

The R_f values of the unknown genin and reference compounds which were chromatographed individually and as mixtures with themselves and with the unknown sample are listed in Table I.

Reference Sapogenins.—Pure authenticated sapogenin samples for experimental study were kindly supplied by Dr. O. Mancera, Syntex Laboratories, Mexico City; and Dr. E. Heftmann, National Institutes of Health, Bethesda, Md. Comparative chromatographic and infrared studies of the unknown genin and reference sapogenins were made. All of the sapogenins were used in a concentration of 1 mg. per ml. of chloroform U.S.P.

Identification of Sugar Moieties of the Saponin.—After removal of the aglycon from the hydrolyzed mixture, the filtrate was neutralized with barium carbonate and filtered. The filtrate was concentrated to a syrupy consistency, dissolved in ethanol, and filtered to give a yellow solution. All of the solutions that were prepared were stored in the refrigerator and warmed to room temperature before using for chromatographic analysis.

Two different solvent systems were employed for the chromatographic resolution of the carbohydrates. One solvent system (S-1) was composed of *n*-butanol (4 parts), distilled water (5 parts), and glacial acetic acid (1 part) (16). The other solvent system (S-2) consisted of 2,6-lutidine (13 parts), and distilled water (7 parts) (17). Because solvent system S-1 was subject to esterification, it was always freshly prepared and used only once. Solvent system S-2 never was used more than twice in the experiments.

An ascending chromatographic technique was used for the separation and identification of the sugar residues. The chromatograms were developed with the mobile phase for approximately 16 hours. After resolution was complete, the dried chromatograms were sprayed with a solution of aniline acid phthalate in water-saturated *n*-butanol and dried for 5 minutes in a chromatography oven at 100°. On sheets developed with this reagent, aldopentoses appeared as red spots and hexoses as green to brown spots. Extreme care was exercised to prevent any destruction of the paper when heating.

Several known sugars of analytical purity were chromatographed individually, and as mixtures with themselves and with the unknown sample. The R_f values obtained with the two different solvent systems are given in Table II.

Table II.—Summary of R_f Values for Carbohydrates

Compound	R_f Values S-1	R_f Values S-2
D-Xylose	0.353	0.554
D-Glucose	0.295	0.534
D-Galactose	0.306	0.496
D-Rhamnose	0.526	0.641
Unknown sugar mixture	0.297 0.523	0.536 0.644

Infrared Spectra as Proof of Identity.—Since the unknown genin behaved chromatographically like sarsasapogenin, application of infrared spectra was made as a further check in establishing its identity. Chloroform solutions (30%) of the unknown genin and sarsasapogenin were prepared for infrared analysis. The infrared absorption spectrum of each sample was determined in an infrared Beckman spectrophotometer, model IR-5, using 0.059 mm. sodium chloride cells. Chloroform U.S.P. was used as the standard reference sample. The infrared absorption spectra of the isolated sapogenin and sarsasapogenin are shown in Figs. 2 and 3, respectively.

It will be noted that the infrared spectrum of the unknown sapogenin bears great resemblance to that of sarsasapogenin. Each of the two steroidal compounds produced curves with distinctive bands in the

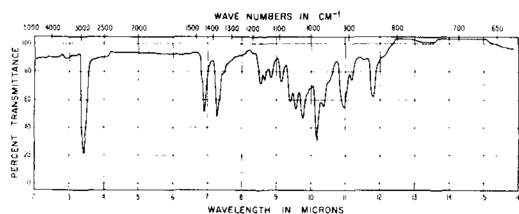

Fig. 2.—Infrared spectrum of the unknown sapogenin in chloroform U.S.P.

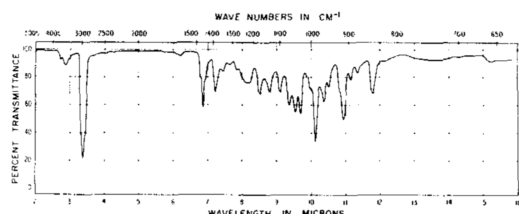

Fig. 3.—Infrared spectrum of sarsasapogenin in chloroform U.S.P.

"finger-print region" which is characteristic of sapogenins. Between 850 and 1350 cm.$^{-1}$ intense absorption bands conferred by the spiroketal side chain are observed (18). The bands of maximum absorption in this highly specific region are located near 987 and 908 cm.$^{-1}$. Those absorption bands observed in the region between 1350 and 1500 cm.$^{-1}$ seem to be associated with vibrations localized in the methyl groups (19). The notable peak located near 2940 cm.$^{-1}$ could be assigned to the $—CH_2—$ vibrations of the molecule. Inspection of the spectra for the 3-hydroxyl group disclosed two bands of medium intensity between 1000 and 1050 cm.$^{-1}$ (20). The absorption bands below 850 and above 1800 cm.$^{-1}$ have not been extensively studied since these regions do not show any prominent features of reasonable consistency.

SUMMARY

A general phytochemical investigation was conducted on *Amphipterygium adstringens*, primarily on the basis of its promising anticancer activity. Numerous chemical qualitative studies were conducted and several conventional techniques were employed in the detection, isolation, purification, and characterization of constituents.

The results of this phytochemical study may be summarized as follows:

1. The qualitative tests for the presence of phytosterols were positive.

2. The determinations for the presence of phloroglucides and alkaloids were negative.

3. The tannins were found to be members of the nonhydrolyzable group containing a catechol type of nucleus.

4. A crystalline substance (m.p. 165–166°) was isolated from the diethyl ether extract of the marc previously exhausted with petroleum ether.

5. On the basis of chemical qualitative tests, solubility properties, and infrared studies, the crystalline substance was characterized as an organic acid. The prepared *p*-nitrobenzyl ester melted at 72–73°, and the *p*-phenylphenacyl ester at 127–128°.

6. The Stas-Otto analysis for the presence of glycosides was positive.

7. A steroidal saponin was isolated from the *n*-butanol extract of the aqueous fraction.

8. Paper chromatographic and infrared analysis of the isolated sapogenin showed that its physical-chemical properties were similar and in direct agreement with those of sarsasapogenin.

9. Hydrolysis of the saponin and subsequent paper chromatographic analysis of the carbohydrate residues showed the presence of D-glucose and D-rhamnose.

REFERENCES

(1) Gonzalez, E. E., McKenna, G. F., and Delgado, J. N., THIS JOURNAL, in press.
(2) Martinez, M., "Las Plantas Medicinales de Mexico," 4th ed., Ediciones Botas, Mexico, D. F., 1959, p. 404.
(3) Rosenthaler, L., "The Chemical Investigation of Plants," 3rd ed., G. Bell and Sons Ltd., London, 1930, pp. 36–112.
(4) Fieser, L. F., and Fieser, M., "Natural Products Related to Phenanthrene," 3rd ed., Reinhold Publishing Co., New York, N. Y., 1949, p. 102.
(5) Coward, R. H., and Harris, L. E., THIS JOURNAL, **45,** 325(1956).
(6) "The National Formulary," 11th ed., J. B. Lippincott Co., Philadelphia, Pa., 1960, p. 405.
(7) Kleber, J. W., and Gisvold, O., THIS JOURNAL, **41,** 218 (1952).
(8) Gisvold, O., and Rogers, C. H., "The Chemistry of Plant Constituents," rev. ed., Burgess Publishing Co., Minneapolis, Minn., 1943, pp. 311–329.
(9) Niederl, J. B., and Niederl, V., "Micromethods of Quantitative Organic Analysis," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1948, p. 66.
(10) Kelly, T. L., and Segura, M., *J. Am. Chem. Soc.*, **56,** 2497(1934).
(11) Drake, N. L., and Bronitsky, J., *ibid.*, **52,** 3715(1930).
(12) Gilman, H., "Organic Chemistry," vol. 3, John Wiley & Sons, Inc., New York, N. Y., 1953, pp. 140–150.
(13) Fiedler, K. R., and Gisvold, O., THIS JOURNAL, **46,** 317 (1957).
(14) Heftmann, E., and Hayden, A. L., *J. Biol. Chem.*, **197,** 47(1952).
(15) Svendsen, A. B., and Jensen, K. B., *Pharm. Acta Helv.*, **25,** 241(1950).
(16) Schwartz, S. M., and Gisvold, O., THIS JOURNAL, **46,** 324(1957).
(17) Berry, H. K., *et al.*, *Univ. Tex. Publ.*, 5109, 25(1951).
(18) Scheer, I., *et al.*, *Anal. Chem.*, **26,** 550(1954).
(19) Cole, A. R., *et al.*, *J. Am. Chem. Soc.*, **74,** 5662(1952).
(20) Jones, R. N., *et al.*, *ibid.*, **75,** 158(1953).