of sedimentation studies that pepsin allowed to stand for brief periods in alkaline solutions remains surprisingly homogeneous, but that drastic changes in sedimentation constant take place when the material is subsequently taken to a lower *p*H. Unfortunately, it is possible to determine the activity of pepsin only in solutions of low *p*H, so that there is some ambiguity as to what *p*H range is pertinent to the observed inactivation process. It is perhaps not surprising that a substance as complicated as pepsin should undergo two or more different and unrelated reactions under similar experimental conditions.

NEW HAVEN, CONNECTICUT RECEIVED JULY 9, 1951

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC., AND THE NOYES CHEMICAL LABORATORY UNIVERSITY OF ILLINOIS]

Plant Insecticides. II.[1] The Alkaloids of *Haplophyton Cimicidum*

BY EDWARD F. ROGERS, H. R. SNYDER AND RUDOLPH F. FISCHER

Two insecticidal alkaloids, "haplophytine," $C_{27}H_{31}O_5N_3$, and cimicidine, $C_{23}H_{28}O_5N_2$, have been isolated from *Haplophyton cimicidum*.

Two crystalline insecticidal alkaloids have been isolated from the Mexican shrub *Haplophyton cimicidum* A.D.C. (*Apocynaceae*), thereby confirming predictions of earlier workers concerning the alkaloidal character of the plant's active principles.[2] The alkaloids, which have been named "haplophytine" and "cimicidine," were secured in yields of 0.007–0.03% and 0.003%, respectively. Haplophytine and cimicidine are amphoteric and this property was utilized in fractionation of the crude alkaloid obtained from the chloroform–methanol extractive of whole plant material.

On the basis of analytical results reported in this communication and corroborative unpublished data, the following empirical formulas are proposed: haplophytine, $C_{27}H_{31}O_5N_3$; cimicidine, $C_{23}H_{28}O_5N_2$. A close relation between the two compounds is indicated by similar ultraviolet and infrared absorption spectra. Of special interest in the infrared spectra (see Fig. 1) are: (a) the complete absence of absorption in the region 3700–3000 cm.$^{-1}$ (OH and NH) and (b) the complex absorption in the region 1850–1620 cm.$^{-1}$ (carbonyl); it is probable that two carbonyl groups are present and that OH and NH groupings are absent.[3]

Both alkaloids are toxic to German roaches on contact, ingestion and injection. The LD/50 dosage of haplophytine is 18 γ/g. (contact, 48 hr.) and for cimicidine is about 60 γ/g. It was observed that haplophytine caused prolonged paralysis at dosage levels far below the LD/50 value. The toxicity data on the various fractions (see Table I) indicate that approximately one-fourth of the toxic principles present in the crude alkaloid are recoverable as pure haplophytine and cimicidine. Some of the losses are due to incompleteness of precipitation and others are ascribable to degradation during separation; alkaline or acidic solutions of haplophytine darken more or less rapidly when exposed to the air. While not remarkably low, the LD/50 dosage of haplophytine compares favorably with that of several widely used insecticides and most of the toxicity of Haplophyton is probably due to this alkaloid. The total crude alkaloid has been found to be toxic to a wide range of insects including European corn borers, Mexican bean beetle larvae, Colorado potato beetle larvae and adults, grasshoppers, egg-plant lace bugs and codling moths.

Experimental[4]

Preliminary Treatment and Extraction of Plant.—Authentic *Haplophyton cimicidum*, dried whole plant, was ground in a fourteen-inch hammermill (30 H.P. motor) to pass through a one-half inch screen. Most of the material thus obtained was very finely divided and was extracted without further treatment. Samples of plant of two degrees of maturity were employed. The comparative extractions were run on young, relatively immature plants, but several extractions and much of the entomological work were conducted on rather mature plants (few leaves, many seeds, larger size).

An extraction container was prepared by cutting the bottom out of an ordinary five-gallon chloroform can. This was inverted, and the spout was connected to a 12-liter flask, which in turn was attached to a vacuum line. Seven to eight kilograms of ground plant was tamped into this container and covered with 12–13 liters of a mixture of 80–90% chloroform and 10–20% methanol. The can was covered and allowed to stand 2 or 3 days, after which it was drained, the vacuum line being used to complete the solvent removal. This process was repeated six times (only 5–6 liters came through each time), with concentration of the extracts as the volume reached 10–11 liters. The final solvent pass was pure chloroform, and the final volume of concentrate was about one liter of chloroform solution.

The above procedure represents the best of several tried on the younger plant material; chloroform and methanol were about equally effective as solvents, and a mixture of the two was somewhat better than either alone. Cold percolation with this mixture gave about the same yields as continuous hot extraction; water was about one-third as efficient as chloroform, and benzene was only one-tenth as effective a solvent.

Isolation of Haplophytine.—The concentrate was extracted four times with 200-ml. portions of 2 *N* hydrochloric acid, and the *p*H of the extracts was quickly adjusted to 8 with solid sodium carbonate; the resulting suspension was then extracted with four 200-ml. portions of chloroform. To determine the "total crude alkaloid" at this point, the

(1) For the preceding paper in this series see E. F. Rogers, F. R. Koniuszy, J. Shavel, Jr., and K. Folkers, THIS JOURNAL, **70**, 3085 (1948).

(2) (Mexico) Comision de Parisitologia Agricola 1.00-01; El Gusano de la Fruta (*Instrypetas ludens* I.D.B.); *Bol. Com. Parisitologia*, **1**, 21, 28–30, 45, 46, 49–51, 54, 55, 90, 108, 188; L. Flores, Datos para la Materia Medica Mexicana, Secretaria de Fomento (Mexico); *Instituto Mexico Nacional*, **4**, 93 (1907); C. C. Plummer, Toxicity of *Haplophyton cimicidum* to Fruit Flies, U.S.D.A. Circ. No. 455, April, 1938; R. E. Heal, E. F. Rogers, R. T. Wallace and O. Starnes, *Lloydia*, **13**, 89 (1950).

(3) L. Marion, D. A. Ramsay and R. N. Jones, THIS JOURNAL, **73**, 305 (1951).

(4) Unless otherwise noted, all decomposition points were determined on a calibrated Fisher block.

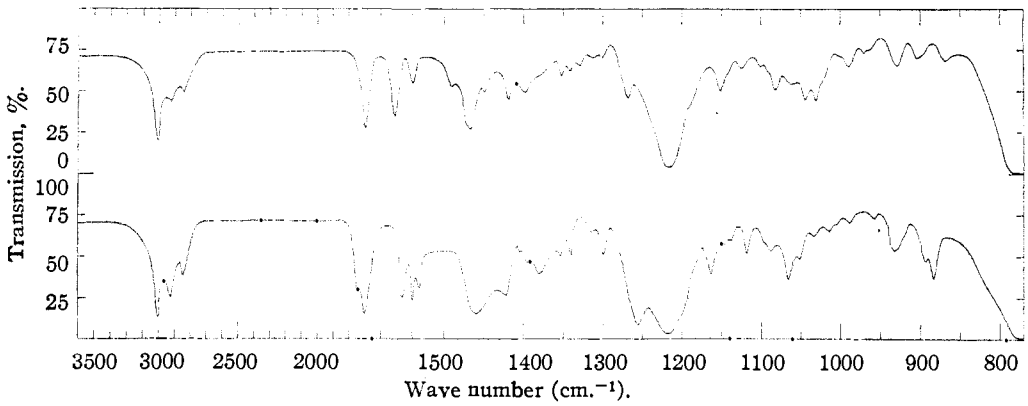

Fig. 1.—Infrared absorption spectra of haplophytine (upper curve) and cimicidine (lower curve) in chloroform solution (absorption at 3007, 1220–1213 and 927 cm.$^{-1}$ due to solvent).

chloroform was replaced by ethanol, and the resulting suspension was evaporated to dryness *in vacuo*. The product was then powdered and thoroughly mixed. The yield was quite variable, depending on the amounts of resins, gums, etc., carried along in the various extractions. Ordinarily, the combined chloroform extracts were immediately extracted with four 125-ml. portions of 5% sodium hydroxide solution. The *p*H of the extracts was adjusted to 8 with Dry Ice and hydrochloric acid, and the suspension was extracted three times with 100-ml. portions of chloroform. The combined extracts were concentrated to about 5 ml. and transferred to a 50-ml. erlenmeyer flask. The solvent was replaced by 30–35 ml. of ethanol, 4–6 ml. of acetone was added, and the solution was placed in the desk for a month to ensure reasonably complete crystallization. It was always advantageous and sometimes necessary to seed with a small crystal of haplophytine. The crude yields varied from 1.9 to 2.4 g. (0.03%) when younger plant was employed, and a small, less pure crop was obtained from several combined mother liquors, ordinarily after three or four additional months. Minor variations in the above procedure did not materially affect the yield or quality of the pure alkaloid, but the quality of the crude, crystalline alkaloid was quite variable. When comparable quantities of the mature plant were treated as above, the separations were more difficult, and the final crude yield was only 0.4 to 0.6 g.

Two recrystallizations from ethanol–chloroform gave almost colorless crystals, m.p. 280–285° (dec.); $[\alpha]^{25}D$ +109.0° (1–3%, chloroform). Two more recrystallizations did not change the specific rotation, but the crystals became pure white, and the decomposition point rose to a constant value of 288–292° on the Fisher block or 290–293° in a capillary (rapid heating, starting at 250°). The actual range of decomposition depended on the rate of heating, the apparatus used, and the temperature at which the samples were placed on the hot stage; samples heated from room temperature invariably decomposed several degrees lower. Even with the purest samples, some preliminary darkening occurred at 270–275°, and with less pure samples, darkening occurred at 260–270°. In capillary tubes, the decomposition appeared to be accompanied by evolution of gas at about 290°.

Anal.[5] Calcd. for $C_{27}H_{31}O_5N_3$: C, 67.91; H, 6.54; N, 8.80; $2CH_3O$—, 13.00; $1CH_3$—N=, 3.15 ($2CH_3$—N=, 6.30); CH_3—C≡, 3.15; 2 active H, 0.42; mol. wt., 477. Found: C, 68.20, 68.17, 67.76, 68.09, 68.24[6]; H, 6.54, 6.37, 6.54, 6.23, 6.40; N, 8.70, 8.62, 9.05; CH_3O—, 11.01, 12.21; CH_3—N=, 2.79, 5.46; CH_3—C≡, 0.27; active H, 0.43; mol. wt., 498 (ebullioscopic, acetonitrile).

Haplophytine can be sublimed at bath temperatures of 240–246° at a pressure of about 10^{-3} mm.; at slightly higher temperatures, slow decomposition occurs. A sublimed sample, m.p. 285–288° (dec.) was crystallized from ethanol prior to analysis.

(5) All analytical samples were dried for several hours at 100° *in vacuo*; samples dried less vigorously gave values consistently low in carbon and hydrogen.

(6) Where more than one analysis is reported, the determinations were made on separate samples, each prepared from a different extraction batch.

Anal. Found: C, 68.00; H, 6.44.

Properties of Haplophytine.—The alkaloid is very soluble in chloroform, benzene, dioxane and ethyl acetate; it is moderately soluble in acetone, from which it may be crystallized as a micro-crystalline powder or as prisms. It is moderately soluble in methanol, and somewhat less soluble in 95% ethanol, from which it crystallizes in clusters of needles. It is practically insoluble in ether, petroleum ether or water, but readily soluble in dilute acid or alkali. Absorption maxima in the ultraviolet range are at 2200 Å. ($E_{1\%}^{1\,cm.}$, 580) and at 2650 Å. ($E_{1\%}^{1\,cm.}$, 165). Haplophytine is stable in the dry state over a period of years, but solutions in contact with air darken somewhat in several days. All samples of haplophytine examined have been identical in all respects (rotation, decomposition point, analyses and reactions) whether chloroform, methanol, water or benzene was used as the extraction solvent.

Isolation of Cimicidine.—After standing 6 to 8 months to ensure reasonably complete crystallization of haplophytine, the combined, filtered mother liquors from this isolation were poured with stirring into ten volumes of commercial dry ether. The voluminous tan precipitate was separated by decantation and filtration and set aside for later examination, the straw-colored ether solution was concentrated, and the solvent was replaced by acetone. Cimicidine separated from this solution in large prisms; the original crystallization was very slow (10 months), but the seeding of succeeding runs with a small crystal of cimicidine reduced the time to about 2 months. From 116 pounds of dry plant, a total of 1.41 g. of cimicidine was obtained (0.003%); of this, about 0.7 g. was obtained as a first crop and 0.5 g. as a second crop from the mother liquors. An additional 0.2 g. was obtained by dissolving the ether–insoluble material, above, in ethanol and reprecipitating with ether. The ether-soluble fraction was treated as above, and cimicidine separated from solution in several months. All of the cimicidine isolated to the present time has been obtained from the immature plant material.

In all cases, the alkaloid crystallized in an almost pure state; one recrystallization from acetone–ethanol gave colorless prisms (or occasionally rods), m.p. 259–262° (dec.) (darkening from 235°); $[\alpha]^{25}D$ +123.0° (1–3%, chloroform). Two further recrystallizations did not change these properties. Samples obtained from aqueous and chloroform extractions were identical.

Anal. Calcd. for $C_{23}H_{28}O_5N_2$: C, 66.97; H, 6.84; N, 6.79; $1CH_3O$—, 7.52; $1CH_3$—N=, 3.64. Found: C, 67.33, 67.13; H, 6.58, 6.73; N, 7.10, 6.96; CH_3O—, 7.38; CH_3—N=, 0.34.

Cimicidine is similar to haplophytine in solubilities, except that it is less soluble in ethanol or acetone. It may be crystallized from either of these solvents, or best from a mixture of the two. Absorption peaks in the ultraviolet absorption spectrum are at 2275 Å. ($E_{1\%}^{1\,cm.}$, 510) and at 2600 Å. ($E_{1\%}^{1\,cm.}$, 155). This alkaloid is also remarkably stable in the dry state, and it darkens only slightly in solution over a period of days.

In one case, in an attempt to obtain an additional amount

TABLE I

Extraction solvent	Plant used	Crude alkaloid	Alkali-soluble	Alkali-insoluble	Haplophytine	Cimicidine
Water	6.8 kg.	5.8 g.	3.6 g.		0.2 g.	
		100 γ/g. (80%)	100 γ/g. (95%)	200 γ/g. (5%)	25 γ/g. (75%)	....
Chloroform	7.7 kg. mature	39.3 g. 300 γ/g. (50%)	.....	.....	0.6 g. 18 γ/g. (50%)	....
Chloroform	8.1 kg. immature	.....	.....	.....	2.2 g. 18 γ/g. (50%)	0.2 g. 75 γ/g. (63%)

of cimicidine, the mother liquors from the first crystallization were treated with a 1:1 mixture of acetone and absolute ethanol and allowed to evaporate slowly over a period of two months, with occasional addition of fresh solvent. A crystalline material separated, which proved to be pure haplophytine. One recrystallization from ethanol–acetone gave clusters of needles (0.39 g.), m.p. 280–285° (dec.); $[\alpha]^{25}D$ +109° (1%, chloroform). The identity of this material with haplophytine was confirmed by identical infrared spectra. When the mixed solvent was removed from the mother liquors and replaced by pure acetone, cimicidine again crystallized, contaminated by only minute amounts of haplophytine. Only a small portion of very impure haplophytine has been isolated from a variety of further fractionations of the haplophytine and cimicidine mother liquors.

Insecticidal Data.—These data are summarized in Table I. In this table, the upper value represents the yield of material, and the lower values are the levels of treatment (contact) followed by the per cent. mortality. The crude alkaloid is a heterogeneous mixture, completely insoluble in petroleum ether and almost completely insoluble in water; it is somewhat soluble in dry acetone or absolute alcohol and much more soluble if 10% water is present.

Acknowledgments.—Mr. B. A. Krukoff of Merck & Co., Inc., assisted in the procurement and identification of the plant material used in this study. Earlier entomological investigations were conducted in the laboratories of Merck & Co., Inc., and at the New Jersey State Agricultural Experiment Station and Rutgers University, New Brunswick, N. J., under the direction of Dr. R. E. Heal. Later insecticidal assays were conducted by Professor C. W. Kearns of the Entomology Department, University of Illinois. Infrared spectra were determined by Miss Elizabeth Petersen, using a Perkin–Elmer spectrophotometer. Ultraviolet spectra were determined by Miss Ella Richards, using a Carr Recording Spectrophotometer. Analytical data were determined by Misses Rachel Kopel and Emily Davis, and Mrs. Jean Fortney, University of Illinois, in the analytical laboratories of Merck & Co., Inc. and by the Clark Microanalytical Laboratories, Urbana, Illinois. Marie Fischer assisted in the preparation of the manuscript. The authors gratefully acknowledge the assistance rendered.

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Chemotherapeutic Dyes. IV. Phenoxazines and Benzo[a]phenoxazines[1]

BY RICHARD C. CLAPP, JOE H. CLARK, JACKSON P. ENGLISH, CATHERINE E. FELLOWS, RUTH E. GROTZ AND ROBERT G. SHEPHERD

Four variations of the structure of 5-arylimino-9-dialkylaminobenzo[a]phenoxazines, compounds active against mouse tuberculosis, have been made. These were: (a) the introduction of a 6-methyl, (b) the introduction of a 10-chloro, (c) the preparation of 5- and 9-monoarylimino and of 5-arylimino-9-arylamino derivatives and (d) removal of the benzo group to give analogously substituted phenoxazines. None of the compounds was as active as the unmodified model.

Certain benzo[a]phenoxazine dyes of the Nile Blue type have been found to have interesting tumor-staining[2] and antituberculous activity when administered orally to infected mice.[3] The effect of variations in structure on such activity has been studied in the three series, 9-dialkylamino-5-aralkyliminobenzo[a]phenoxazines,[4] 9-di-alkylamino-5-aryliminobenzo[a]phenoxazines[5] and 9-dialkylamino-5-heterocyclic-iminobenzo[a]phenoxazines.[6] Compounds of the second series were of the greatest interest, although those of the first series possessed activity, while those of the third series were without significant activity.

The present investigation was undertaken cooperatively with the above investigators to study still other variations in the structure of these dyes. This paper reports the preparation of compounds embodying four variations of the structure of the most active of the benzo[a]phenoxazines, namely, the 5-arylimino-9-dialkylaminobenzo[a]phenoxazines (I).

The first variation involves the substitution of a methyl group in the 6-position of the nucleus in order to study the effect of its possible interaction with substituents in the arylimino group. The

(1) Presented before the Division of Medicinal Chemistry of the American Chemical Society, Cleveland, Ohio, April, 1951. Paper III in this series, M. L. Crossley, C. M. Hofmann and P. F. Dreisbach, THIS JOURNAL, 74, 584 (1952).

(2) See M. L. Crossley, P. F. Dreisbach, C. M. Hofmann and R. P. Parker, *ibid.*, 74, 573 (1952), ref. 2.

(3) H. J. White, M. E. Schlosser and M. B. DiCenzo, paper presented at Meeting of Society of American Bacteriologists, Chicago, Ill., May, 1951.

(4) M. L. Crossley, P. F. Dreisbach, C. M. Hofmann and R. P. Parker, THIS JOURNAL, 74, 573 (1952).

(5) M. L. Crossley, R. J. Turner, C. M. Hofmann, P. F. Dreisbach and R. P. Parker, *ibid.*, 74, 578 (1952).

(6) M. L. Crossley, C. M. Hofmann and P. F. Dreisbach, *ibid.*, 74, 584 (1952).