Isolation of Cantharidin from *Epicauta pestifera*

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Epicauta pestifera **Werner (*Meloidae*) is a species of blister beetle which is found in North America and contains a powerful irritant and vesicant. This paper reports the isolation, quantitative determination, and identification of the active principle responsible for this activity. Employing standard practices of analysis, which involved infrared and NMR spectroscopy, thin-layer chromatography, elemental analysis, and determination of physical constants, the substance was identified as cantharidin.**

THIS PAPER reports the isolation and quantitative determination of cantharidin from a species of blister beetle, *Epicauta pestifera* Werner (*Coloptera, Meloidae*) which is found in North America and occurs commonly in Mississippi. This particular specie of blister beetle has not been previously investigated for its vesicating principle. Cantharidin, as it was determined in this investigation, is the substance responsible for the strong irritating and vesicating effects. This compound has also been isolated from several other species of beetles of the genera *Epicauta* of Asia, Indonesia, and South America.

Investigators have reported previously that cantharidin occurs in *E. pilmus* Porter (1), which is found in Chile, in concentrations ranging from 1.7% to 3.5%; in *E. gorhami* Marseul (2), from 0.4% to 1.75%; in *E. waterhousei* (3), which is found in Formosa, from 1.02% to 1.26%; and *E. ruficeps* Ill. (4), which is found in Java, and *E. odspersa* (5), found in Brazil, in concentrations of 2.5%. Shimano *et al.* (2) showed that the substance is present in *E. gorhami* in the head (2.5%), thorax (1.2%), abdomen (94%), and legs (2%), which is contrary to general belief that it is confined principally to the abdomen where it may be secreted from between the segments of the underside. The cantharidin content varies considerably from one genera and species to another but is found usually in concentrations of approximately 1% and has been reported as high as 3.5% in *Epicauta gorhami* (2). In this investigation, cantharidin was found to be present in *E. pestifera* in the amount of 1.09% by weight.

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DISCUSSION

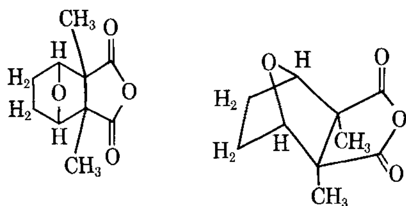
E. pestifera is, in the United States, often referred to as the common margined blister beetle of potatoes. It has been called *E. solani* Werner and *E. marginata* Fabr. The latter may sometimes be used in reference to *E. cinera* Forster. *E. pestifera* is widely scattered throughout North America and the U. S. but occurs principally from the Atlantic Coast to the Rocky Mountains. The seasonal range of the adult beetles is July to September. They are regarded as garden pests and are known to attack garden vegetables in addition to the potato plant. However, Werner (6) points out that the margined blister beetle of potatoes does have a grey form and that they occur on *Clematis* species of plants rather than potatoes, and furthermore, that they are actually an *Epicauta* species distinct from that found on potatoes. This and related species have been found on plants of the *Ranunculaceae, Solanaceae, Leguminosae,* and *Compositae* familes. One is referred to studies conducted by Werner (6–9) and Selander (10–13) on the *Meloidae*, genera *Epicauta*, for plants from which particular species were collected.

The *Meloidae*, or blister beetle family, are centered mostly in the arid and semiarid areas of the world, and at the present time, this family comprises approximately 75 genera and some 2000 described species (14). More than three-fourths of the North American entomological literature on the *Meloidae* deals with members of *Epicauta*. Werner (6–9) and Selander (15, 16) have made major contributions to the collection and entomological data of the genus *Epicauta* and the *Meloidae* in North America and Mexico, while MacSwain (14) and Blackwelder (17) have compiled comprehensive classification check lists on the Colepterous insects.

Because of its toxicity and irritation properties, cantharidin is little used in human medicine, although it was once used extensively as a vesicant. Today, its use is confined to veterinary medicine as an irritant and stimulant for hair growth. Among the uses that have been reported for this substance are: aphrodisiac, anticarcinogenic, antihypertensive, herbicide, insecticide, vesicant, hair growth stimulant, diagnostic agent in leprosy, and agent to remove warts. Several cases (18–21) of poisoning from cantharidin have been reported.

Cantharidin (cantharidine) (exo-1,2-*cis*-dimethyl-3,6 - epoxyhexahydrophthalic anhydride) has been assigned structure I by Gadamer (22) and his collaborators (23). It is optically inactive,

nonresolvable, and exists naturally as the *cis*-isomer.



Further proof of this structure was made by Woodward and associates (24), who synthesized desoxycantharidine (cantharidin lacking the oxide bridge). Stork and his associates (25) reported the first total synthesis of cantharidin in 1952. However, other investigators also contributed significantly toward the chemistry and synthesis of this compound; worthy of mention, they are Schenck *et al.* (26), Paranjape (27), Pai *et al.* (28), Iyer *et al.* (29), and Ziegler *et al.* (30). Physical and chemical characteristics of cantharidin may be found in the literature (31, 32). In this investigation, 1 Gm. of cantharidin was found to be soluble in 50 ml. of dimethylsulfoxide at room temperature (28°).

The isolation of cantharidin from blister beetles has been studied extensively, particularly in an attempt to improve upon previous methods of separation. It occurs partly free and partly combined in the insect; the nature of the combination is unknown. This unknown complex adds to the difficulty of extraction and quantitative determination. In general, the methods employed for separation utilize continuous extraction with chloroform, acetone, or ethanol, alone or in the presence of an acid (HCl or HNO_3). The extract is concentrated *in vacuo* and treated with petroleum ether or benzene to remove lipid material, then crystallized from a mixture of petroleum ether and ethanol (1:9). Further purification may be obtained by repeated recrystallization or sublimation.

In this study, cantharidin was extracted from *E. pestifera* in a Soxhlet extractor using acetone, cooled, the insoluble material collected, the extract evaporated *in vacuo*, then submitted directly to sublimation. Pure cantharidin was obtained. The quantitative analysis was conducted using the procedure outlined in the "United States Pharmacopeia XI" (33). Infrared and NMR spectra, elemental analysis, physical constants, and thin-layer chromatography of isolated cantharidin when compared to the pure substance showed the two compounds to be identical.

EXPERIMENTAL

Approximately 200 Gm. of *E. pestifera* beetles were collected for this study during July and August of 1965 near Hattiesburg, Miss., and stored in a refrigerator (10°) in a closed jar to which a few drops of chloroform were added to prevent microbial deterioration. They were found on the ornamental vine, *Clemenatis paniculata*.

Isolation of Cantharidin.—The beetles (100 Gm.) were placed in a Waring blender and 150 ml. of acetone was added. The mixture, after being blended for 5 min., was decanted into a Soxhlet thimble and extracted for 18 hr. with acetone. The olive

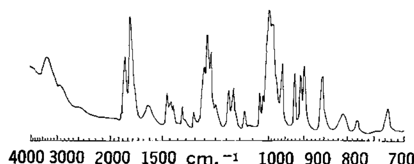


Fig. 1.—Infrared spectrum of cantharidin in KBr pellet.

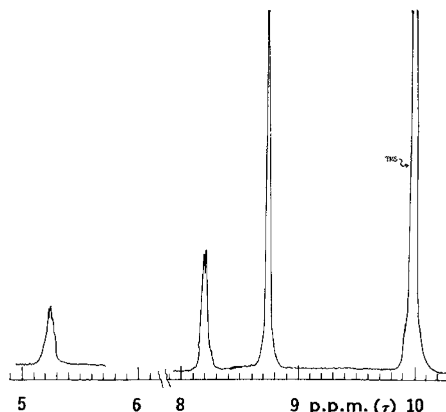


Fig. 2.—NMR spectrum of cantharidin in $CDCl_3$. Solvent, $CDCl_3$; temperature, 37°; filter bandwidth, 4 c.p.s.; R. F. field, 0.04 mg.; sweep time, 250 sec.; sweep width, 500 c.p.s.; sweep-offset, 0 c.p.s.; spectrum amp, 25; 10; integral amp, 625 →; remarks, 500 ←.

colored solution, on cooling, yielded a heavy dark brown precipitate and a white flocculant precipitate which settled on top of the brown syrupy material. The white substance (0.22 Gm.) was easily decanted from the heavy gummy material and sublimed directly at 110° with vacuum using a sublimation apparatus (McCarter, Nester Faust). The crystalline needle-like sublimate gave a melting point of 217–218° uncorrected. A mixed melting point with pure cantharidin showed no change. The infrared spectrum of the isolated cantharidin was obtained using a Perkin Elmer spectrophotometer in KBr pellets (Fig. 1) and was identical to the pure material.

NMR Spectroscopy.[1]—Nuclear magnetic resonance (NMR) spectra were obtained with a Varian A-60A spectrometer and were in agreement with the expected chemical shifts, multiplicity, and intensities. Samples were prepared as 10% solutions in $CDCl_3$ and were not degassed. Tetramethylsilane (TMS) was used as internal reference standard. The NMR spectra of pure cantharidin and the isolated substance (Fig. 2) consisted of the following: (*a*) a sharp singlet at $\tau = 8.74$, relative intensity 3, assigned to the methyl protons of the methyl groups *cis* to the oxide bridge; (*b*) a doublet, $J = 2$ c.p.s., centered near $\tau = 8.21$ with relative intensity of 2 assigned to the adjacent pairs of protons on the cyclohexane ring; (*c*) a broad, low field absorption

[1] Acknowledgment is made to Mr. J. L. McClanahan, and Mrs. J. M. Settine, Chemistry Department, University of Mississippi, for their assistance in measuring and interpreting the spectra.

centered near $\tau = 5.25$, relative intensity 1, assignable to tertiary protons of the type at the oxide bridge. The absorption peaks at $\tau = 2.70$ are assigned to a $CHCl_3$ impurity in $CDCl_3$ and at $\tau = 10$ to the reference standard, TMS.

Anal.—Calcd. for $C_{10}H_{12}O_4$: C, 61.21; H, 6.17; O, 32.62. Found: C, 61.29; H, 6.27; O, 33.52.

Thin-Layer Chromatography.—Thin-layer chromatography was conducted on acetone solutions of cantharidin prepared from *E. pestifera* and of the pure substance. Chromagram sheets (Eastman, K301R2) and prepared Silica Gel G plates were used in the tests with comparable results. Shimano (2) has reported the use of paper chromatography for analyzing mixtures containing cantharidin.

Several detection systems were tested with satisfactory results: iodine; bromocresol green, 0.1% in isopropanol with NaOH added until appearance of blue color; 50% aqueous sulfuric acid solution with a trace of chromium trioxide. In the case of iodine the chromatogram was placed in a closed container containing iodine crystals. Cantharidin with bromocresol green gave a yellow coloration. A contrasting white spot revealed the presence of cantharidin when sprayed with the sulfuric acid solution.

Development of the chromatogram can be made with either acetone, chloroform, or an acetone–benzene (1:9) mixture. All systems showed relative movement of cantharidin: acetone (R_f = 0.74–0.75); chloroform (R_f = 0.61 to 0.59); acetone–benzene mixture (R_f = 0.50).

The R_f values and chemical response to the indicators for pure cantharidin and isolated cantharidin were sufficiently close to conclude these data as further confirmation that the isolated substance was indeed cantharidin.

Quantitative Determination.—A quantitative estimation of cantharidin and *E. pestifera* was determined by the method outlined in the U.S.P. XI (33). The dried beetles (15 Gm.) were ground to a coarse powder and placed in a round-bottom flask. A mixture (150 ml.) of benzene and petroleum ether (2:1) which contained 2 ml. of HCl was added. The flask was stoppered, shaken, and allowed to stand for 24 hr. at room temperature (28°). It was then heated to 40° and maintained at this temperature for 3 hr. with frequent shaking. After cooling, a portion of the mixture, 100 ml. representing 10 Gm. of beetles, was decanted and filtered into a tared beaker. The solution was evaporated to a volume of approximately 5 ml. using a stream of nitrogen gas and 5 ml. of chloroform added. The solution was evaporated to dryness. Ten milliliters of a wash mixture of equal volumes of petroleum ether and absolute ethanol which had previously been saturated with cantharidin was added to the residue to remove coloring and lipid materials. This mixture was allowed to stand for 15 min., then decanted through a pledget of purified cotton. This step was repeated until approximately 40 ml. of the wash solution had been used. The crystals which had collected on the cotton were then washed in the same manner with 25 ml. of petroleum ether which had been saturated with cantharidin. The crystals were white and free of coloring and lipid materials. Chloroform (10-ml. portions) was passed over the cotton to remove any cantharidin adhering to the cotton. This washing was returned to the tared beaker. The solvent was evaporated, and the crystalline residue dried for 0.5 hr. at 60°. The 100 ml. of original extract, representing 10 Gm. of beetles, yielded 0.1092 Gm. of cantharidin or approximately 1.09% by weight of dried beetles.

Further work is being conducted on the structure–activity relationship of the cantharidin molecule and the specific structure of certain derivatives of which past investigators have expressed some reservation.

REFERENCES

(1) Pfister, A., *Anales Quim. Farm.* (*Chile*), **1940,** 26; through *Chem. Abstr.*, **35,** 6061(1941).
(2) Shimano, T., Nizuno, M., and Bato, T., *Ann. Proc. Gifu. Coll. Pharm.*, **3,** 44(1953).
(3) Chen, M.-Y., and Liang, T.-T., *J. Agr. Assoc. China* (*Taipei*) (*N. S.*), **19,** 30(1957).
(4) Zyp, Van C., *Pharm. Weekblad*, **54,** 295(1917).
(5) Berwatzik-Vogl, *Lehrb. J. Arzneimittellehre, 3 aufl*, **1910,** 542.
(6) Werner, F. G., *Bull. Mus. Compar. Zool.*, **95,** 421 (1945).
(7) Werner, F. G., *Psyche*, **50,** 65(1943).
(8) *Ibid.*, **48,** 75(1945).
(9) *Ibid.*, **60,** 105(1953).
(10) Selander, R. B., *J. Kansas Entomol. Soc.*, **27** (3), 84(1954).
(11) Selander, R. B., *Entomol. Soc. Washington*, **61** (5), 205(1959).
(12) Selander, R. B., *The Coleopterists Bulletin, VIII*, 1954, p. 1.
(13) Selander, R. B., *Ann. Entomol. Soc. America*, **50,** 88 (1957).
(14) MacSwain, J. W., *Angeles*, **1956,** 182.
(15) Selander, R. B., *Illinois Biol. Monog.*, No. 28, 1960.
(16) Selander, R. B., *Proc. U. S. National Museum, III*, (No. 3428), 197(1960).
(17) Blackwelder, F. E., *U. S. National Museum Bull. 185*, **I–IV,** 343(1945).
(18) Wyosocki, K., *Wienr Klin. Wochschr.*, **45,** 964(1932).
(19) Stary, Z., *Samml. Vergiftungsfallen 7, Abstr. A*, **1936,** 117.
(20) Teare, D., "What's New," Abbott Laboratories, North Chicago, Ill., 1962, No. 229.
(21) Kusche, E., *Med. Monatsschr.*, **7,** 252(1953).
(22) Gadamer, J., *Pharm. Post.*, **46,** 821(1914).
(23) Danckwortt, P. W., *Arch. Pharm.*, **252,** 632(1914); Gadamer, J., *ibid.*, **252,** 636(1914); Rudolph, V. W., *ibid.*, **254,** 454(1916).
(24) Woodward, R. B., and Loftfield, R. B., *J. Am. Chem. Soc.*, **63,** 3167(1941).
(25) Stork, G., Van Tamelen, E. E., Friedman, L. J., and Burgstahler, A. W., *ibid.*, **75,** 384(1953).
(26) Schenck, G. O., and Wirtz, R., *Naturwissenschaften*, **40,** 581(1953).
(27) Paranjape, K. D., *et al.*, *Proc. Indian Acad. Sci.*, **19A,** 385(1944).
(28) Pai, V. N., and Guha, P. C., *J. Indian Chem. Soc.*, **11,** 231(1934).
(29) Iyer, B. H., and Guha, P. C., *J. Indian Inst. Sci.*, **23A,** 159(1941).
(30) Ziegler, K., *Ann.*, **551,** 1(1942).
(31) "The Merck Index," 7th ed., Merck and Co., Inc., Rahway, N. J., 1960, p. 204.
(32) Garrido, J., *Acta Cryst.*, **1,** 159(1948).
(33) "United States Pharmacopeia," 11th rev., Mack Publishing Co., Easton, Pa., 1936, p. 105.