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Dihydrosanguinarine, a Product of Sanguinarine Detoxification by *Verticillium dahliae*

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The alkaloid in commercial sanguinarine salts toxic to *Verticillium dahliae* was isolated and identified as sanguinarine. An isolate of *V. dahliae*, virulent to cotton, formed a blue fluorescent compound when grown in a medium amended with sanguinarine. This was accompanied by both a loss of sanguinarine and inhibitory activity from the culture media. Sanguinarine and inhibitory activity were unaffected and were recovered from nonfluorescing cultures inoculated with an avirulent strain of *V. dahliae*. The blue-fluorescing compound was isolated; its melting point and infrared and mass spectra were identical to dihydrosanguinarine, a compound formed by the addition of a single hydrogen to the C-6 of the sanguinarine molecule.

INTRODUCTION

Sanguinarine (SN) is an isoquinoline alkaloid found in the roots of *Sanguinaria canadensis* L. and other members of the Papaveraceae (1). It has been reported to be toxic to bacteria, human pathogenic fungi, and parasitic protozoa (2) and to have antiviral (3) and anticancer activity (4).

Greathouse and Rigler (5) found that SN was toxic to a number of soil-borne fungi including *Verticillium albo-atrum* Reinke and Berth. Presley (6) found that six defoliating strains of the fungus could be distinguished from three mild strains on media containing sanguinarine sulfate; defoliating strains had superior growth and exhibited blue fluorescence under ultraviolet light. We established that growth of, and development of blue fluorescence by, isolates of *V. albo-atrum* on sanguinarine nitrate medium was correlated with virulence to cotton (7). The above papers (5–7) were concerned almost exclusively with microsclerotial isolates of *V. albo-atrum* which we prefer to call *Verticillium dahliae* Kleb. according to Isaac (8).

The commercial sanguinarine salts used in previous studies (6, 7) consist of a mixture of closely related alkaloids, including SN, chelerythrine, protopine, and other minor constituents (9). In preliminary studies we have determined that the alkaloids present in the sulfate and nitrate salts are the same. Growth of virulent strains on SN together with the appearance of the blue fluorescent compound in the mycelium (7) suggests a detoxification of one or several of the alkaloids in the mixture.

The purpose of this work was to determine if SN was the toxic entity in commercial sanguinarine nitrate, to determine if the formation of blue fluorescence was a detoxification process, and to isolate and identify the detoxified product.

MATERIALS AND METHODS

Isolation of the Fungitoxic Alkaloid

One gram of commercial sanguinarine nitrate was dissolved in 400 ml of distilled water. The solution was adjusted to pH 3.0 with HCl and extracted three times with equal volumes of ethyl acetate. The aqueous

fraction then was made basic with NaOH and extracted three times with equal volumes of chloroform. The ethyl acetate, chloroform, and water fractions were dried *in vacuo* at 30°C, and the residues were dissolved in small volumes of chloroform. Aliquots were added to a medium containing sucrose (10 g/liter) and the salts from raw cotton media (10). The chloroform was removed under vacuum, 2% agar was added, and the medium was autoclaved at 15 psi for 10 min, since previous tests had shown that SN toxicity was not reduced by autoclaving. Final concentration of residues in the medium was 0.5 mg/ml. Media containing the various fractions were dispensed into petri dishes and inoculated in the center with hyphal tips from each of five virulent and five avirulent strains of *V. dahliae*. After 5 days at 23°C radial growth measurements were taken.

The active chloroform extract was chromatographed on thin-layer plates coated with silica gel 7GF with benzene–methanol (95:5). Each of the resolved zones was eluted with chloroform–methanol (80:20). Eluates were dried *in vacuo* at 30°C, dissolved in chloroform, and bioassayed as described above.

Compounds present in the active zone at R_f 74 taken from plates developed with benzene–methanol (95:5) were further separated on plates coated with silica gel 7GF with benzene–ethyl acetate–diethylamine (70:20:10). Zones were eluted and bioassayed as described previously, and the active fraction at R_f 75 was transferred to a column of silica gel CC4. The 15 × 2-cm column was washed with chloroform–formic acid (98:2) until all uv-detectable compounds that would move in the solvent were removed. The column was then washed with chloroform–methanol (90:10). The two fractions taken from the column were dried and bioassayed. The active fraction was recrystallized twice from chloroform.

Demonstration of Detoxification

Fifty-milliliter aliquots of a liquid-modified raw cotton medium were inoculated with 0.5-cm modified raw cotton agar plugs of strain 110 (virulent to cotton) or strain 277 (avirulent to cotton) of *V. dahliae*. After a 4-day incubation on a shaker at 23°C sterile, purified sanguinarine nitrate was added to each culture to obtain a final concentration of 350 μg/ml. After a further 4-day incubation the cultures were centrifuged and the pelleted fungus tissue extracted with 100% ethanol. Supernatant fluids were lyophilized and residues were extracted with 100% ethanol. Tissue and supernatant extracts were combined and evaporated to a small volume before incorporation into the modified raw cotton agar medium. Media containing the extract from cultures of 110 and media containing the extract from cultures of 277 were inoculated with hyphal tips from five avirulent and five virulent strains of *V. dahliae*; three replications were used for each strain and treatment. After incubation for 14 days at 23°C, radial growth measurements of the cultures were made. Growth in the presence of the extracts is expressed as a percentage of that on modified raw cotton medium.

Isolation and Identification of Detoxified Product

The SN used in this study was purified from a crude alkaloid mixture according to the method of Tin-Wa *et al.* (11), modified by following the migration of the orange fluorescent SN band down the column with uv light and collecting only the fraction that contained the band.

Purified SN (100 μg/ml) was added to 4-day-old shake cultures of isolate (110) of *V. dahliae* in modified raw cotton medium. Control cultures had no SN added. After 3-day incubation on the shaker, the cultures were centrifuged to remove fungus tissue. The supernatant extracts were acidified to

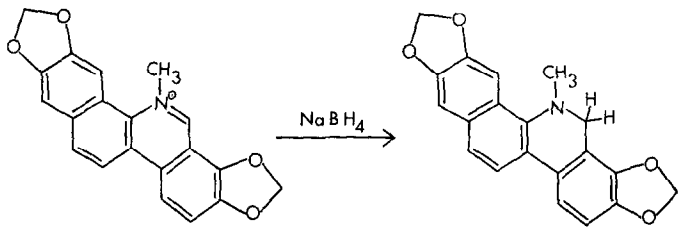

FIG. 1. *Sodium borohydride reduction of sanguinarine at C-6 to form dihydrosanguinarine.*

0.5 *N* with HCl and extracted with ethyl acetate. Mycelia and conidia were extracted with 70% methanol, which was subsequently diluted to 20% with 0.5 *N* HCl and extracted with ethyl acetate. The ethyl acetate extracts from culture filtrates and fungal tissue were combined and dried *in vacuo* at 30°C. The residue was dissolved in chloroform, washed thoroughly with deionized water, and concentrated *in vacuo* to a small volume.

The chloroform solutions were streaked on thin-layer plates of neutral alumina and developed in chloroform–formic acid, 98:2. A blue fluorescent zone at R_f 85, not present on the control plate, was eluted with chloroform, streaked on silica gel 7GF, and developed in petroleum ether–diethyl ether, 70:30. A blue fluorescent zone at R_f 63 was eluted with chloroform, and the solution was taken to dryness. The residue was dissolved in hexane and washed successively with 2% sodium bisulfite, 0.5 *N* NaOH, and deionized water. The hexane solution was taken to dryness and the residue was dissolved in benzene. Ethanol was added dropwise until a substance crystallized from the benzene solution.

Melting points were determined on a Kofler hot stage.[1] Infrared spectra in chloroform were obtained with a Beckman IR 18-A spectrophotometer and mass spectra were determined with a CEC 21-110 mass spectrometer.

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Dihydrosanguinarine (SNH) was prepared by sodium borohydride reduction of SN at C-6 (Fig. 1) according to the method of Brossi and Borer (9). It was cochromatographed with the detoxification product on neutral alumina developed with chloroform–formic acid and on silica gel 7GF developed with petroleum ether–diethyl ether. The melting point of a mixture of the two compounds was obtained, and their ir spectra were compared.

Early in our studies we discovered that the detoxification product of SN was rapidly photooxidized under the fluorescent lights of the laboratory. Therefore, all extraction procedures were carried out under reduced light, and chromatography was done under a photography darkroom lamp.

RESULTS

Inhibitor

Our method of purifying the fungitoxic component in commercial sanguinarine nitrate by solvent and chromatographic separation is shown in Fig. 2. None of the substances extracted with ethyl acetate from the dilute acid solution of sanguinarine nitrate were toxic to *V. dahliae*. The inhibitor was present in the chloroform extract of the dilute base solution. Chromatography (benzene–methanol, 95:5) and bioassay of the chloroform extract showed the inhibitor to be present in the uppermost colored zone at R_f 74. Chromatography (benzene–ethyl acetate-diethylamine, 70:20:10) and bioassay of compounds in this zone revealed that the inhibitor was again present in the

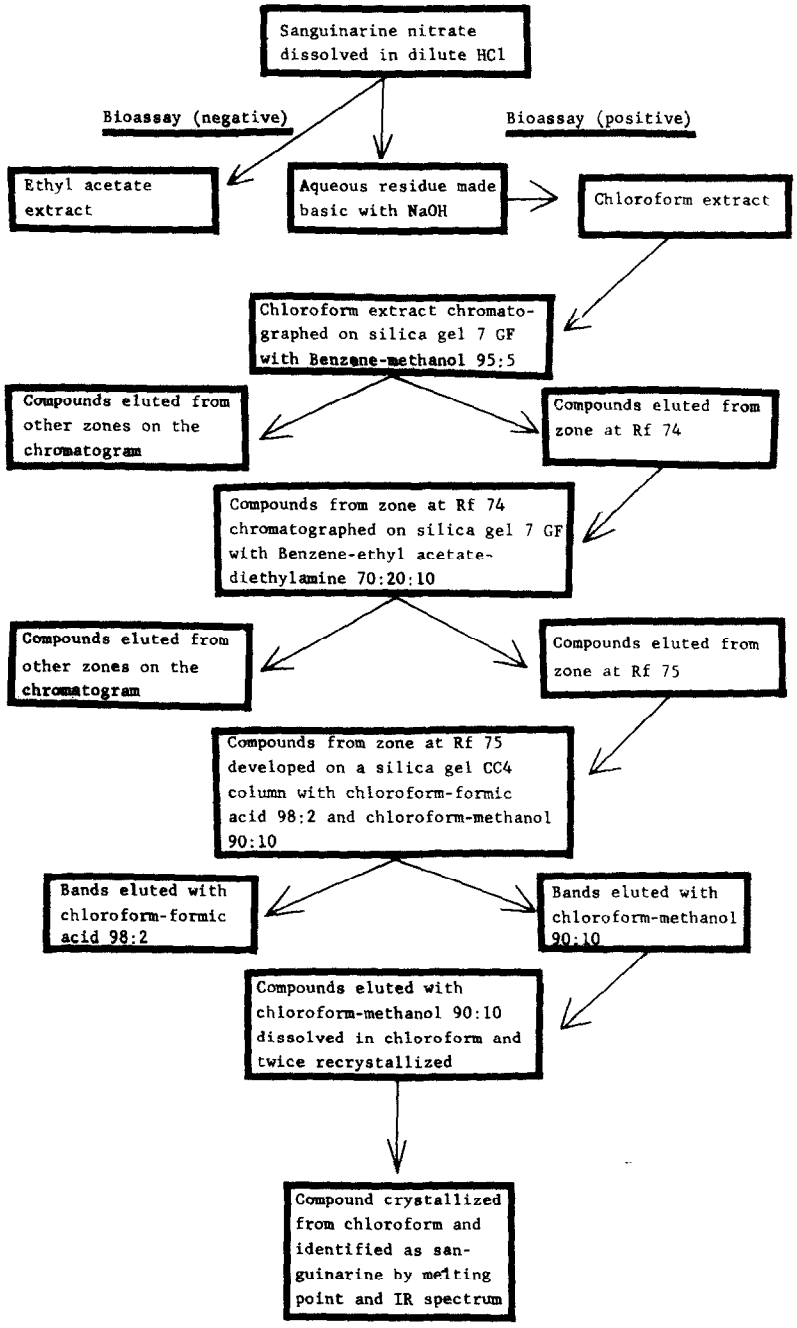

FIG. 2. *Purification procedure for the fungitoxic component in commercial sanguinarine nitrate.*

uppermost zone at R_f 75. Bioassay of the fractions eluted from the silica gel CC4 column showed that the toxin was present in the fraction eluted by chloroform–methanol, 90:10. The crystalline product from chloroform was found to be inhibitory and was identified as SN by its melting point and infrared spectrum (9, 12).

Inhibitor Detoxification

Addition of sanguinarine nitrate to the 4-day-old cultures of strains 110 and 277

turned the cultures bright orange. After a 4-day incubation the cultures of strain 277 were still bright orange, whereas in the cultures of strain 110 the bright orange SN had disappeared. Growth data for virulent and avirulent strains of *V. dahliae* on media containing ethanol extracts from strains 110 and 277 are given in Table 1. Growth of virulent strains was retarded, and growth of avirulent strains was inhibited by extracts from cultures of strain 277. On extracts from cultures of strain 110, growth of virulent strains was not appreciably affected and avirulent strains were retarded by only 40–50 %.

Detoxified Product

The detoxification product was readily separated from residual SN in the acid methanol solution by extracting with ethyl acetate. When partitioned between dilute acid (0.5 *N* HCl) and ethyl acetate, SN remained in the dilute acid while the detoxification product went into the ethyl acetate fraction.

TABLE 1

Radial Growth of Mild and Defoliating Isolates of Verticillium dahliae on Media Containing ETOH Extracts from Cultures of Mild or Defoliating Isolates Grown for 4 Days with 350 μg/ml Sanguinarine Nitrate

Strain	Source of ethanol extract added to medium			
	Virulent (No. 110) cultures		Avirulent (No. 277) cultures	
	Control (mm)	SN (% control)	Control (mm)	SN (% control)
Defoliating				
110	33[a]	82	32	72
118	35	86	32	69
121	35	86	32	72
128	33	91	32	72
138	30	100	34	71
Mild				
106	30	50	30	0
133	25	trace	26	0
187	28	61	27	0
276	35	57	34	0
277	36	57	30	0

[a] Data are averages of three replications.

Chromatography of the ethyl acetate extract on neutral alumina with chloroform–formic acid followed by the chromatography of a zone isolated from that system (R_f 85) on silica gel 7GF with petroleum ether–diethyl ether yielded a blue fluorescent substance not present in the extract from untreated cultures. The ir spectrum of the substance isolated from thin-layer plates showed the presence of long-chain compounds containing carbonyl groups. These contaminants were removed by extraction of a hexane solution with $Na_2S_2O_5$ and NaOH.

Recrystallization from benzene–ethanol produced a compound with the formula $C_{20}H_{15}NO_4$ ($M^+ \cdot 333.099784$) as determined by high resolution mass spectrometry. This was one mass unit higher than SN and indicated that SN might have been altered by the addition of a single hydrogen to form dihydrosanguinarine (SNH).

The melting point of the recrystallized compound was 188.5–189, that of synthesized SNH was 187–189. A mixed melting point of the two compounds was not depressed. Cochromatography of the detoxification product and SNH showed the R_f of both compounds on neutral alumina developed with chloroform–formic acid to be 85. Their R_f on silica gel 7GF developed with petroleum ether–diethyl ether was 63. In Fig. 3 a comparison of the infrared spectra of the detoxification product and SNH shows that they are identical. These data indicate that the detoxification product is SNH.

DISCUSSION

SN is the compound in commercial sanguinarine nitrate active against *V. albo-atrum* and *V. dahliae*. The formation of a blue fluorescent substance in virulent cultures of *V. dahliae* is accompanied by a loss of SN from the culture and a loss of inhibitory activity. We postulate this is a detoxi-

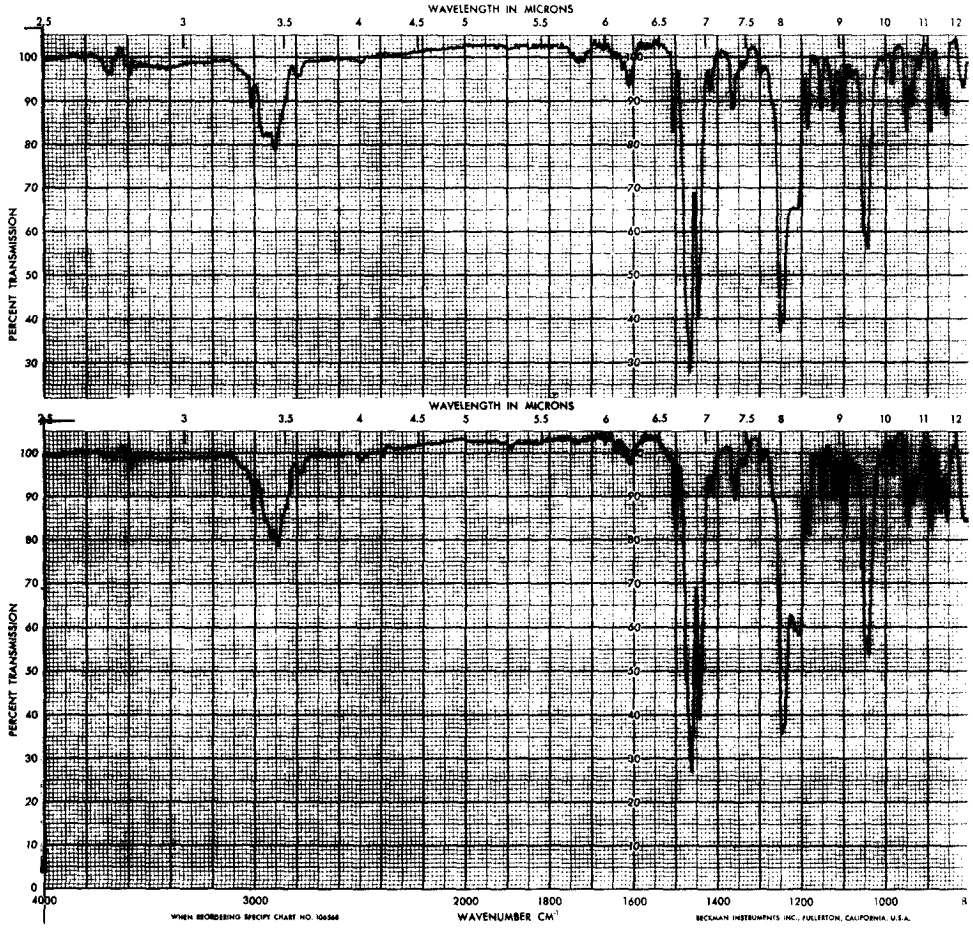

FIG. 3. *Infrared spectra of the detoxification product (top) and dihydrosanguinarine (bottom) dissolved in chloroform.*

fication reaction whereby virulent strains of *V. dahliae* reduce SN to SNH. Sakar (13) has presented evidence to show that at least one of the toxic mechanisms of SN is its reaction with the sulfhydryl groups of enzymes. This is probably an addition reaction at C-6 of SN, a position known to undergo attack by nucleophiles (CN^-, OCH_3^-, $OC_2H_5^-$, H^-) (14). The dihydro derivative being reduced at this position is incapable of this reaction and therefore effectively detoxified. The exact mechanism of SN detoxification remains to be elucidated, and that aspect is now under investigation.

Although there is no SN in cotton, the results of this and another study (7) indicate that the capacity to detoxify SN, by reducing it, is strongly correlated with *V. dahliae* virulence to cotton. What role this reaction plays in the host–parasite interaction of *V. dahliae* and cotton remains to be elucidated.

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