NEW INSECTICIDAL METABOLITES FROM SOIL ISOLATE W719

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In the course of screening soil organisms for new insecticidal metabolites, strain W719 was found to produce a group of metabolites active against the tobacco budworm *Heliothis virescens*. The active metabolites were purified by a combination of solvent partitioning and chromatographic steps, and the physico-chemical properties and insecticidal activity of the main components were determined. The two main components have MW's of 925 and 939, appear to belong to the macrocyclic lactam family of natural products, and possess significant insecticidal activity.

In a program for the discovery of novel natural products for use in crop protection, a tobacco budworm (*Heliothis virescens*) laboratory diet feeding assay was established to screen for insecticidal metabolite production by 5,000 soil microorganism isolates. Among the actives was strain W719, which produced a complex of novel lipophilic insecticidal metabolites. The crude culture extract and purified complex demonstrated moderate residual contact activity in foliar assays against tobacco budworm and beet armyworm, *Spodoptera exigua*. This paper outlines the purification, spectroscopic properties, and insecticidal activity of these metabolites.

Experimental

Strain Isolation and Storage

Strains used in the insecticidal screening program were isolated at the laboratory of Dr. JERRY ENSIGN, University of Wisconsin, Madison on colloidal chitin agar with 50 μg/ml cycloheximide from various soil and rhizosphere samples[1]. Strain W719 was not speciated, but is presumed to be a strain of *Streptomyces*. The strain was subcultured on Oatmeal agar (Difco) with cycloheximide at 50 μg/ml and passaged at 28°C. Freezer stock cultures were made by suspending whole cultures or spore preparations from agar slants in 10% glycerol, flash freezing, and storing at -70°C.

Cultivation

The 7 day growth of a 15-ml oatmeal agar slant (50 μg/ml cycloheximide) was suspended in 5 ml sterile deionized water, heat shocked at 45°C for 10 minutes[1], and used to inoculate 10 new 15 ml slants, one 100 ml slant, or one 500-ml tribaffled flask with a loose fitting plastic cap containing 150 ml of medium (glucose 10 g, soluble starch 20 g, Amber EHC (Amber Laboratories, Milwaukee, Wisconsin) 2 g, Bacto-casitone 2 g, Bacto-yeast extract 2 g and Bacto-beef extract 2 g, $CaCO_3$ 3 g, and deionized H_2O 1,000 ml). Flasks were incubated at 28°C with shaking at 150 rpm in a G25 New Brunswick platform shaker. For screening, 10 ml whole culture samples were taken at days 3.5, 5 and 7, lyophilized, and tested

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by diet incorporation bioassay against the tobacco budworm. Multi-liter productions were started by the inoculation of 100 ml oatmeal agar slants. Larger productions were grown in 500-ml production medium in 2.8-liter modified Fernback flask (tribaffled and curved bottom) with a Shin-etsu silicone closure and inoculated with 100 ml slants. A time course with analysis of production by bioassay and TLC showed day 3.5 to be the optimum harvest point. New Brunswick 10 liter fermenter productions were grown in the same medium, 28°C, 300 rpm agitation, 6 liters/minute air, pH control either with $CaCO_3$ or by the addition of 1.0 N NaOH to maintain the pH above 6.0, and a harvest time of 2.5 days.

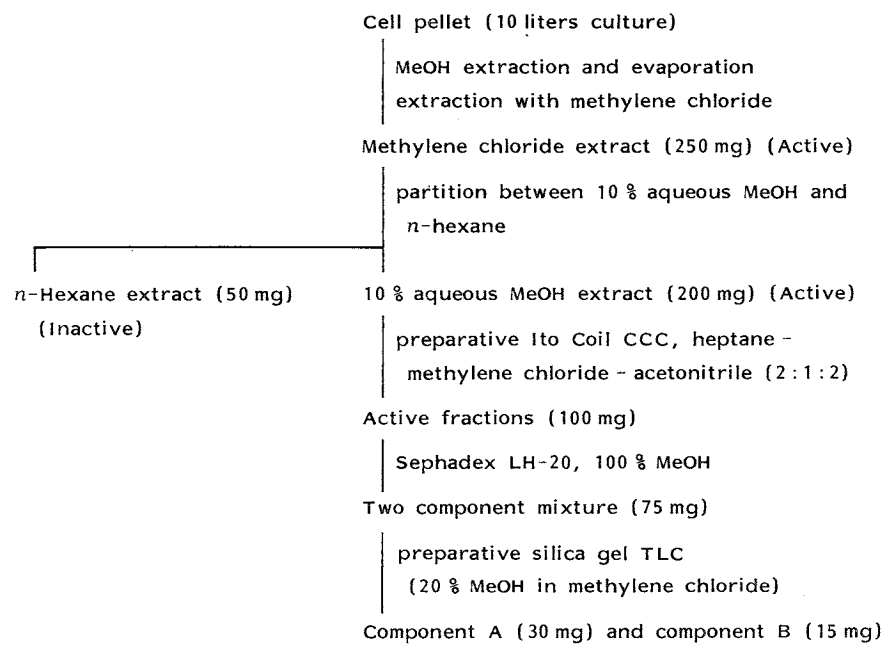
Instrumental Methods

Mass spectra were obtained on a VG ZAB mass spectrometer operating in the FAB ionization mode. Positive and negative ion mass spectra were scanned from 1,100 to 100 daltons at 10 seconds/decade. Magic bullet and glycerol were used as dispersant agents. Accurate mass analysis were performed at 6,000 resolution by manual calibration of linear voltage scans across the molecular ion regions for the samples to which has been added a similar amount of PEG-600 as an internal mass marker. TLC was performed with Merck silica gel 60 F_{254} precoated glass plates and the spots were visualized with 10% isopropanolic solution of phosphomolybdic acid. UV spectra were recorded on a Cary 15 Spectrophotometer, IR spectra were recorded on Perkin-Elmer 983 Grating Spectrophotometer. ^1H NMR was recorded on IBM 300 AF (300 MHz) spectrometer and ^{13}C NMR spectrum was recorded on the IBM 300 AF spectrometer operating at 75 MHz.

Isolation and Purification

The isolation and purification procedure of the active metabolites is shown in Scheme 1. The activity in a fermentation of strain W719 was found to be on the cell mass. The culture broth (10 liters) was centrifuged in 1 liter polypropylene bottles at $2,000 \times g$ to separate the mycelium from the broth. The mycelium was extracted with methanol, and the extract was evaporated to a dark green aqueous slurry *in vacuo*. Analytical samples were taken and found to be active against *Heliothis virescens* (see Table 1). The remaining extract was diluted with water and extracted three times with dichloromethane. The combined dichloromethane extract was dried over sodium sulfate and evaporated *in vacuo* to give a dark green residue (250 mg). The dichloromethane extract was found to be active and was a very complex mixture

Scheme 1. Isolation and purification of active components from culture W719.



both on TLC and HPLC. The dichloromethane extract was partitioned between *n*-hexane and 10% aqueous methanol. The active aqueous methanolic extract (200 mg) was subjected to liquid-liquid partition chromatography using an Ito Multi-Layer Coil Apparatus (P.C. Inc., centrifugal counter current chromatography), using the lower phase of heptane - dichloromethane - acetonitrile (2 : 1 : 2) mixture as a mobile phase. The active fractions obtained were combined and concentrated to dryness affording a pale green residue (100 mg). This residue was chromatographed over a 3.2 × 120 cm Sephadex LH-20 column using methanol as the eluting solvent. The active fractions from the Sephadex LH-20 column were combined and showed one major spot and one minor spot on silica gel TLC. Silica gel preparative TLC (20% methanol in dichloromethane) gave two pure compounds, component A (30 mg) and component B (15 mg).

Component A

Colorless waxy solid, UV (1% solution in MeOH), λ_{max} nm 215; IR (KBr) cm^{-1} 3455 (–OH), 2970 ~ 2920 (br), 2700, 1716 (–C = O), 1668 (α,β-unsaturated carbonyl), 1200 ~ 1000 (–C–O–C), 970 (–CH = CH–); ^{13}C NMR ($CDCl_3$) δ 148.7, 133.5, 129.2, 119.3, 106.1, 98.4, 98.2, 85.8, 81.6, 79.5, 74.7, 74.6, 74.4, 72.2, 69.6, 68.1, 67.8, 67.1, 61.8, 54.2, 43.2, 41.4, 39.9, 38.3, 35.6, 35.3, 34.5, 30.6, 30.3, 30.2, 29.6, 29.1, 29.0, 28.7, 28.6, 26.8, 23.5, 22.1, 19.9, 13.4, 11.3, 10.0, 6.0, 5.5; Mass spectrum (*m/z*) (FAB, glycerol): 927 (M + 1), 909 (M – H_2O), 893, 879, 829, (M^+ measured 926.6159, $C_{50}H_{88}NO_{14}$ calcd 926.6205).

Component B is structurally related to component A as indicated by its very similar UV and NMR spectra, but its HR mass spectrum indicated that it has a MW 14 amu higher than component A, suggesting that it may be a methyl ether of component A. Structure elucidation of these two new insecticides will be published elsewhere.

Insect Assays

The diet incorporation assay for activity against the tobacco budworm was used for screening broths and following activity during isolation procedures. The sample was dissolved in water or acetone and mixed with 3 ml of laboratory diet in a 20-ml vial. One or two second instar larvae were placed in each vial and mortality was recorded after 5 days. Topical activity against tobacco budworm was measured by applying 1 μl of an acetone solution of the sample to the anterior one-third of five 50-mg larvae. Injection activity against tobacco budworm was measured by injecting 0.5 μl of an acetone solution of the sample laterally into the posterior one-third of five 35-mg larvae. For both topical and injection assays, larvae were maintained separately in 35 mm plastic Petri dishes containing laboratory diet, and mortality was determined at 24 hours after treatment and confirmed 3 days after treatment. Foliar tests were performed by dipping the well-expanded first or second true leaves of cotton plants into an aqueous solution or detergent suspension of the test material, and allowing the leaves to dry. For tobacco budworm assays, ten 33-mm discs were cut from the leaves and placed in 35 mm Petri dishes with one second instar larva per dish. For beet armyworm, two whole leaves from which the edges had been trimmed were placed in 85 mm Petri dishes with five second instar larvae. For two-spotted spider mite (*Tetranychus urticae*), 33 mm leaf discs were placed in the center of moist 55 mm Whatman No.1 filter paper discs on top of 125 ml plastic specimen containers filled with water, and 10 to 15 insects were applied to each disc. Mortality was determined at 24 hours and confirmed at 3 days. Cotton aphid (*Aphis gossypii*) activity was measured by infesting cotton plants at the first true leaf stage before dipping the leaves into the test material. The infested and treated plants were maintained in the greenhouse and mortality was measured 3 or 4 days after treatment. Test material applications for residual contact activity against tobacco budworm and beet armyworm were as described above. The plants then were incubated in the greenhouse and were placed outside in direct sunlight for 4 hours each day. Leaves were removed from plants for infestation at times after treament as indicated in Table 3. Mortality in all experiments was corrected for mortality in the controls by application of ABBOTT's formula[2].

Results and Discussion

The methanolic extract of the cell pellets of *Streptomyces* W719 showed significant insecticidal activity when tested against *H. virescens*. As shown in Scheme 1, the isolation of two active components was guided

Fig. 1. IR spectrum of component A (KBr).

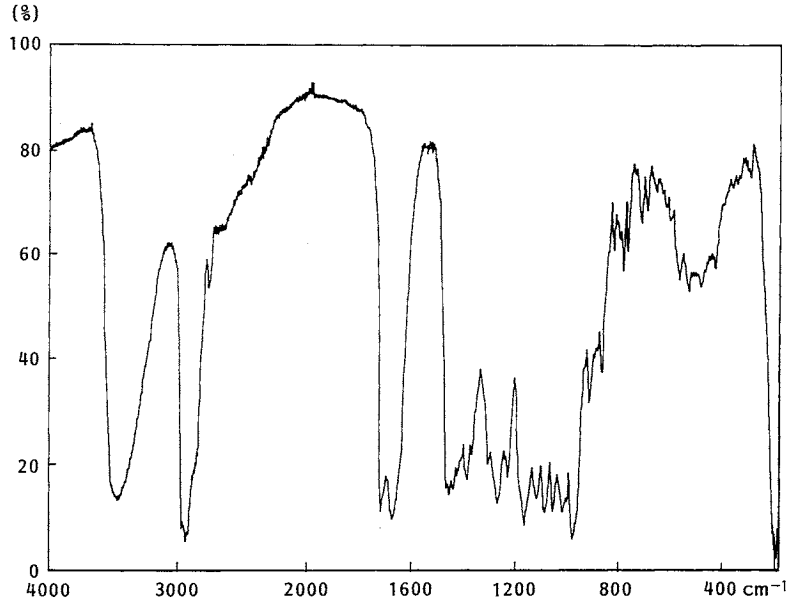
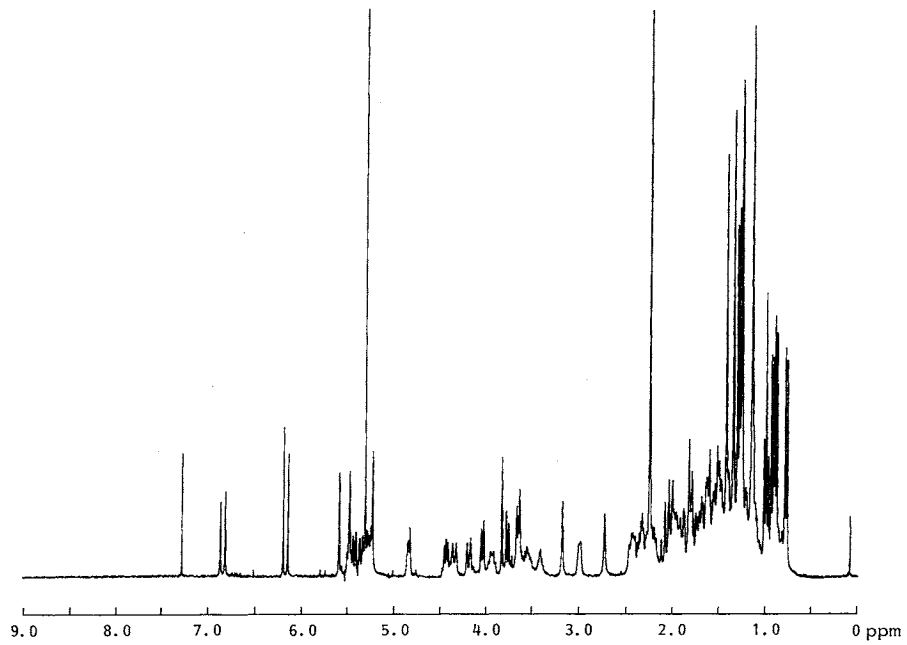


Fig. 2. ^{1}H NMR spectrum of component A (300 MHz, $CDCl_3$).



by diet-cup, topical or injection insecticidal bioassay and the activity was accomplished by three partitions, followed by size exclusion chromatography and preparative TLC.

Component A was obtained as a colorless waxy solid. The IR spectrum of component A showed broad bands at 3450 cm^{-1} (–OH stretchings), and 1716 and 1668 cm^{-1} indicated the presence of an

Fig. 3. ^{13}C NMR spectrum of component A (75 MHz, $CDCl_3$).

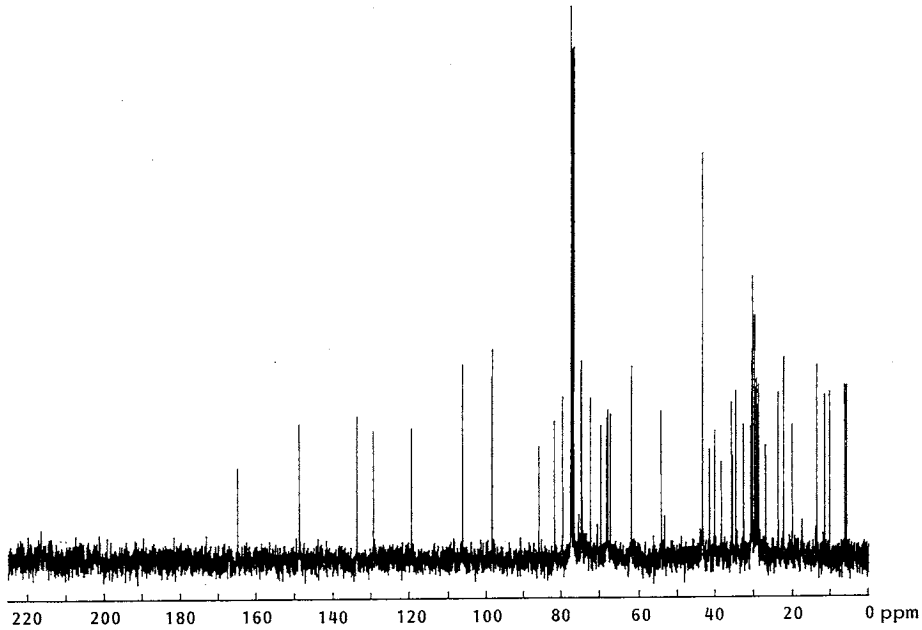


Fig. 4. Mass spectrum of component A (FAB).

(A) Positive ions, (B) negative ions.

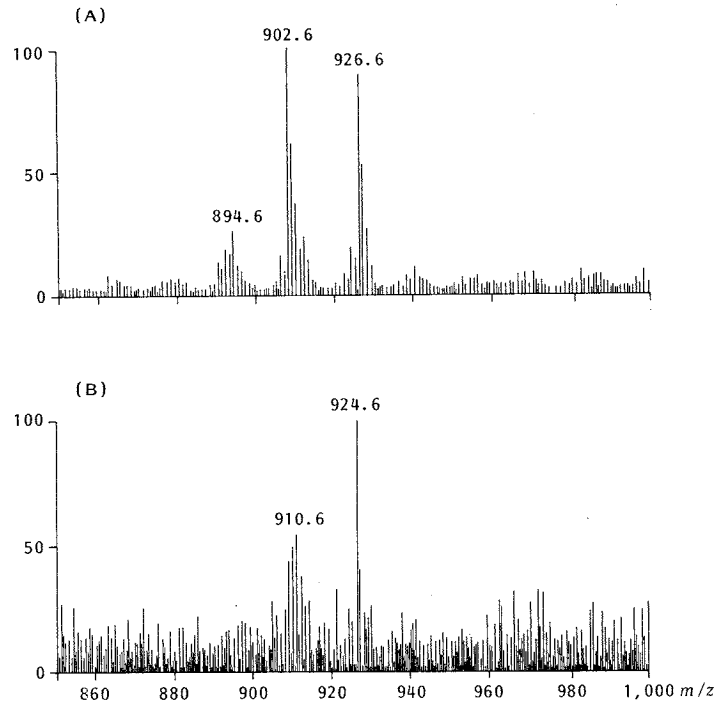


Table 1. Diet, injection, and topical insecticidal activity of W719 crude extract and pure metabolite complex.

Test format	Sample	Test level	% Mortality
Tobacco budworm diet incorporated:			
One 2nd instar larva/vial	Crude extract	30 ml broth/vial	100
3 ml laboratory diet/vial	(broth equiv)	10 ml broth/vial	100
		3 ml broth/vial	0 s
	Pure complex	20 μg/vial	45 ss
		6.6 μg/vial	11 s
		2.2 μg/vial	0
Injection:			
50 mg larvae	Crude extract	250 μl/larva	100
Sample in 0.5 μl acetone	(broth equiv)	50 μl/larva	100
		10 μl/larva	0 s
	Pure complex	10.0 μg/larva	100
		3.3 μg/larva	100
		1.1 μg/larva	100
		0.37 μg/larva	75
		1.0 μg/larva	80
		0.33 μg/larva	20
		0.11 μg/larva	0
Tobacco budworm topical:			
50 mg larvae	Crude extract	500 μl broth equiv	0
Sample in 1 μl acetone	Pure complex	20 μg/larva	40 s
		6.6 μg/larva	50
		2.2 μg/larva	40
		0.75 μg/larva	0

s: Stunted, ss: severely stunted.

Table 2. Insecticidal activity of W719 samples in foliar greenhouse assays.

Sample	% Mortality			
	Tobacco budworm	Beet armyworm	2-Spotted spider mite	Cotton aphid
Crude extract:				
50 × broth conc	29 s	100	25	35
10 × broth conc	0	30	25	15
2 × broth conc	0	0	11	0
Pure complex:				
500 μg/ml	90 ss	100	12	90
167 μg/ml	40 ss	45 s	0	12
56 μg/ml	0	0	0	0
Standard:				
100 μg/ml	71 a	80 b	81 c	100 b
25 μg/ml	29	0	45	100
6.25 μg/ml	0 s	0	0	92

a: Pydrin, b: chlorpyrofos, c: cyhexatin, s: stunted, ss: severely stunted.

α,β-unsaturated amide functionality (Fig. 1). The high field ^{1}H NMR spectrum of component A was quite well resolved in the down field region between 5 and 7 ppm. However, the bulk of protons absorbed in the region 0.9～1.9, indicated that component A was highly saturated (Fig. 2). The ^{13}C NMR indicated the presence of 5 sp^2 carbons, 9 methyl groups, 13 CH_2-groups, and 20 CH-groups (Fig. 3). The HRFAB mass spectrum (Fig. 4) of component A showed a positive ion at m/z 926.6205 $(M+H)^+$ which requires

Table 3. Residual contact activity of W719 pure complex in foliar greenhouse tests with 4 hours direct sunlight exposure per day.

Day after treatment	% Mortality						
	Tobacco budworm				Beet armyworm		
	0	1	2	4	0	1	2
W719 pure complex (μg/ml):							
300	80	50	50	100 LK	nt	96	100
100	10	30	0	55 LK	nt	76	72
33	10	0	0	35 LK	nt	16	16
11	0	0	0	0	nt	8	0
Pydrin (μg/ml):							
100	100	90	60	50	nt	100	76
25	20	20	0	50 LK	nt	64	44
6.25	0	0	0	0	nt	4	0

nt: Not tested, LK: late kill with significant leaf damage.

the molecular formula $C_{50}H_{88}NO_{14}$. The mass spectrum also showed a prominent peak at m/z 909 which is interpreted as the loss of one molecule of water. Based on the initial physico-chemical and spectral data, component A seemed to be a polyhydroxylated macrolide similar to cytovaricin[3], but containing an amide moiety.

The insecticidal activity of the W719 dichloromethane extract and pure complex (mixture of components A and B) in diet, injection, and topical assays is summarized in Table 1. The activity in the extract compared to the pure complex is consistent with a broth production level of about 1 ppm. The stunting of growth observed at sublethal doses in the diet assay suggests an antifeedant effect. The relatively potent injection activity compared with the flat dose-response in the topical assay suggests that the compound either does not penetrate the cuticle well or is metabolized rapidly. Alternatively, the low activity seen in the topical assay could be due to ingestion of the cuticle after molting.

The activity in foliar greenhouse assays is presented in Table 2. The metabolites are moderately active against beet armyworm and tobacco budworm, with weak activity against cotton aphids.

The residual contact activity data presented in Table 3 show that the metabolites retain approximately half their activity against tobacco budworm after 2 days with 4 hours sunlight per day, and show slightly better residual activity against beet armyworm.

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