APPLIED MICROBIOLOGY, Nov. 1973, p. 699-704

Vol. 26, No. 5
Printed in U.S.A.

Prasinons A and B: Potent Insecticides from *Streptomyces prasinus*

S. J. BOX, M. COLE, AND G. H. YEOMAN

Beecham Research Laboratories, Brockham Park, Betchworth, Surrey, RH3 7AJ, England

Received for publication 22 June 1973

Two metabolites which have high activity against sheep blowfly larvae (*Lucilia sericata* and *L. cuprina*) were found to be produced by *Streptomyces prasinus* NCIB 10719. These substances were isolated from culture filtrate by solvent extraction and chromatography and named prasinons A and B. Fermentation factors affecting the formation of these substances are described together with their physical, chemical, and biological properties.

Several substances isolated from actinomycetes as antifungal or antibacterial compounds have been found subsequently to have various types of insecticidal activity. Examples of such compounds are antimycin A (4), flavensomycin (1), novobiocin (2), and cycloheximide (3), but others such as the piericidins (7), aureothin (5), and macrotetralides (6) have been found by specifically screening culture filtrates for insecticidal activity.

In our screen of culture filtrates from actinomycetes, we specifically looked for activity against the larval stage of the sheep blowfly (*Lucilia sericata*). The present paper describes the fermentation, isolation, and properties of two very active larvicides produced by *Streptomyces prasinus* NCIB 10719, prasinon A and prasinon B (British patent application no. 34138/72). These substances are believed to be novel microbial insecticides.

MATERIALS AND METHODS

Culture and cultural conditions. The strain of *S. prasinus* described in this paper was isolated from a South African soil sample and identified by I. Bousfield of the Torry Research Station, Aberdeen, Scotland. The culture has been deposited in the National Collection for Industrial Bacteria under the number NCIB 10719.

A lyophilized stock spore preparation was used to inoculate yeast extract agar slants having the following composition: yeast extract, 20.0 g; (Yeatex, English Grain Co., Burton-on-Trent, England); glucose monohydrate, 10.0 g; agar, 30.0 g (Oxoid no. 3, Oxoid Ltd., Southwark Bridge Road, London, S.E.1.); deionized water, 1 liter; pH adjusted to 6.8. Agar slants were incubated for 7 to 14 days at 26 C until sporulation had occurred. Two milliliter volumes of a spore suspension, produced by adding 10 ml of sterile deionized water to this slant, were used to inoculate each 500-ml conical flask containing 100 ml of seed stage medium, which was then shaken for 48 h. Two-milliliter volumes of this seed stage were then used to inoculate each 500-ml flask containing 100 ml of fermentation medium which was shaken for 5 days. All flasks were closed with cotton plugs and incubated at 26 C on a rotary shaker (240 rpm, 1-in [2.54 cm] radius). In steel fermenters, the fermentation stage was 4 days at 26 C, the fermentation being aerated at 0.2 to 0.5 vol per vol per min and stirred at 130 rpm with a vaned disk impeller.

The seed and fermentation media used for the production of prasinons A and B were identical and had the following composition: dried distillers solubles, 20.0 g (Scotasol, Thos. Borthwick Ltd., 60 Wellington St., Glasgow, W.C.2. Scotland); sucrose, 10.0 g; deionized water, 1 liter; pH adjusted to 6.5. All media were sterilized by autoclaving at 121 C for 15 min.

Assay for activity against larvae of L. sericata. The test sample at the appropriate concentration in 0.5 ml of acetone was distributed over fine paper tissue (2.5 by 16 cm) contained in a glass tube (7.5 by 27 cm). The acetone was driven off in a stream of air, and when the paper tissue was dry 0.5 ml of sterile calf serum was added to the tissue. Twenty-five first-instar larvae of the British sheep blowfly (*L. sericata*) or the Australian sheep blowfly (*L. cuprina*) were counted onto the tissue with a fine paint brush. The larvae were 12 to 17 h old and were hatched and reared on ox liver at 27 C (75% relative humidity [RH]). The glass tube was closed with terylene (dacron) fabric and placed in a humidity chamber (75% RH) at 27 C for 48 h, at which time the number of dead larvae was recorded using the stereo-microscope if necessary. Cultures of *Lucilia* sp. were maintained by the method of Yeoman and Warren (8).

Thin-layer chromatography. All thin-layer chromatography was carried out at 21 C on glass-backed silica gel plates (Merck F254, 0.25-mm layer thickness) and stored in a desiccator before use. Prasinons A and B were visualized by their ability to quench the fluorescent indicator incorporated into the plate and

also by spraying with van Urk's reagent. This reagent was prepared by dissolving 1 g of 4,5-dimethylaminobenzaldehyde in 50 ml of concentrated HCl and adding 50 ml of ethanol. Prasinon A gave a yellow zone and prasinon B gave a pink zone immediately after staining with this reagent. After the plates were heated for 2 to 3 min at 120 C, both zones turned brown.

RESULTS AND DISCUSSION

Fermentation studies. The larvicidal metabolites of *S. prasinus* are readily produced in certain complex media (Table 1). The mixture of larvicidal metabolites contained in a neutral ethyl acetate extract of culture filtrates is described below. The corn-steep-based media, although giving high yields of ethyl acetate-soluble material, did not give prasinon. Low final pH values are given by media 3, 4, 6, and 7 (Table 1) and are associated with low yield. A final pH value of 7.1 to 7.8 seems to be most favorable as judged by media 1, 2, and 8. The first medium in Table 1, based on distillers solubles, gives the best yield of ethyl acetate-soluble material coupled with high larvicidal activity. High-activity extracts were also obtained for media 2 and 8, but the yields were much lower.

Prasinon was produced in certain chemically defined media, the most active extract being obtained when glucose or starch was used as carbohydrate (Table 2). The yields of active extract were not as great as for the control medium, but the purity was higher.

With sucrose as carbohydrate, the effect of varying the nitrogen source is shown in Table 3. It is clear that a wide variety of amino acids may be used but that phenylalanine is particularly good, giving extracts with high activity and dry weight. The adverse effect of very high pH is shown by the medium containing glutamic acid. Prasinon is also produced by using inorganic nitrogen in the form of NH_4Cl or $NaNO_3$, but the weight yield is poorer than in the phenylalanine medium.

With the dried distillers solubles, sucrose medium, the yield of prasinon was decreased by high aeration and high starting pH (Table 4).

Isolation of prasinons A and B. Prasinons A and B were isolated by the procedure outlined in Fig. 1 and described below. *S. prasinus* NCIB 10719 was grown for 4 days in the dried distillers solubles, sucrose medium (Table 1) contained in a stainless-steel fermenter. The culture fluid obtained after centrifugation was adjusted to pH 7 with HCl and extracted with 0.25 volume iso-butyl acetate. After separation the iso-butyl acetate extract was evaporated under reduced pressure to yield a dark-brown oil. This oil was washed with about 0.2 volume of petroleum ether (100 to 120 C), and the washings were discarded.

The petroleum ether-insoluble material was dissolved in a minimum of benzene-methanol

TABLE 1. *Complex media for the production of prasinon*

Composition of culture medium[a]	pH at harvest	Weight of ethyl acetate-soluble material; (μg/ml of culture filtrate)	Larvicidal activity of ethyl acetate-soluble material from 5-day cultures[b]		
			50 μg/ml	10 μg/ml	2.5 μg/ml
1. Dried distillers solubles (Scotasol), 2.0%; sucrose, 1.0%; pH adjusted to 6.5	7.8	102	100	98	0
2. Soyabean flour (Arkasoy), 3.0%; glucose, 6.0%; $CaCO_3$, 0.5%	7.1	22	100	100	0
3. Soyabean meal, 6.0%; glucose, 5.0%; $CaCO_3$, 1.0%; Na_2HPO_4, 0.1%	6.0	82	100	0	0
4. Corn-step liquor, 7.0% (vol/vol); brown sugar, 2.0%; $CaCO_3$, 1.0%	6.2	138	0	0	0
5. Corn-steep liquor, 2.0% (vol/vol); lactose, 1.0%; $CaCO_3$, 1.0%; pH adjusted to 7.0	8.4	198	0	0	0
6. Malt extract, 2.5%; mycological peptone (Oxoid), 1.0%; maltose, 4.0%	5.0	76	0	0	0
7. Peanut meal, 2.0%; cotton seed meal, 1.0%; glucose, 4.0%; $CaCO_3$, 0.5%	6.4	22	88	0	0
8. Peanut meal, 1.0%; cotton seed embryo (Pharmamedia), 1.0%; glucose, 4.0%; $CaCO_3$, 0.5%	7.4	20	100	100	29

[a] Made up in deionized water (weight/volume).
[b] Percent kill of *L. sericata* larvae for various concentrations in larval culture serum.

TABLE 2. *Chemically defined media for the production of prasinon: effect of variation of carbohydrate*

Composition of culture medium[a]	pH at harvest	Weight of ethyl acetate-soluble material; (μg/ml of culture filtrate)	Larvicidal activity of ethyl acetate-soluble material from 5-day cultures[b]			
			50 μg/ml	10 μg/ml	5 μg/ml	2.5 μg/ml
Basal + 3.0% glucose	7.2	20	100	100	100	97
Basal + 3.0% fructose	5.5	6	100	100	82	
Basal + 3.0% sucrose	8.5	12	100	30		
Basal + 3.0% mannitol	7.4	55	100	100	98	0
Basal + 3.0% starch	7.7	22	100	100	99	47
Basal + 3.0% glycerol	7.4	26	100	93	0	
Dried distillers solubles, 2.0%; sucrose, 1.0% (complex medium control)	7.3	81	100	94	81	

[a] Basal medium: glycine, tyrosine, cystine, proline, glutamic acid, and lysine each at 0.1%; Na_2HPO_4, 0.1%; KNO_3, 0.2%; KCl, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4$, 0.001%; pH adjusted to 7.0; deionized water.
[b] See footnote *b*, Table 1.

TABLE 3. *Chemically defined media for the production of prasinon: effect of variation of nitrogen source*

Composition of culture medium[a]	pH at harvest	Weight of ethyl acetate-soluble material; (μg/ml of culture filtrate)	Larvicidal activity of ethyl acetate-soluble material from 5-day cultures[b]		
			50 μg/ml	10 μg/ml	2.5 μg/ml
Basal + 0.5% glycine	8.2	24	100	96	26
Basal + 0.5% phenylalanine	6.9	26	100	100	99
Basal + 0.5% cysteine	6.5	10	100	94	0
Basal + 0.5% proline	8.3	20	100	96	0
Basal + 0.5% glutamic acid	8.9	10	100	0	
Basal + 0.5% lysine	7.1	22	100	86	0
Basal + 0.2% NH_4Cl	6.3	6	100	100	69
Basal + 0.2% $NaNO_3$	7.1	12	100	100	0
Dried distillers solubles, 2.0%; sucrose, 1.0% (complex medium control)	7.9	75	100	88	0

[a] Basal medium: sucrose, 3.0%; Na_2HPO_4, 0.1%; KCl, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.05%; Fe SO_4, 0.001%; pH 7; deionized water.
[b] See footnote *b*, Table 1.

TABLE 4. *Effect of cultural conditions on the production of prasinon*

Cultural conditions[a]			pH at harvest	Weight of ethyl acetate-soluble material (μg/ml of culture filtrate)	Larvicidal activity of ethyl-acetate soluble material[b]		
Type of flask	Starting pH	Cultivation period (days			100 μg/ml	10 μg/ml	5 μg/ml
Plain conical	6.5	3	7.9	74	100	90	45
		4	7.8	58	100	96	94
	8.0	3	8.0	74	100	86	0
		4	7.9	104	100	42	0
Baffled conical (glass spikes)	6.5	3	8.0	50	100	84	0
		4	7.9	56	100	84	0
	8.0	3	8.1	122	100	0	
		4	7.7	66	100	0	

[a] Medium: dried distillers solubles, 2.0%; sucrose, 1.0%; 100-ml volumes in 500-ml flasks.
[b] See footnote *b*, Table 1.

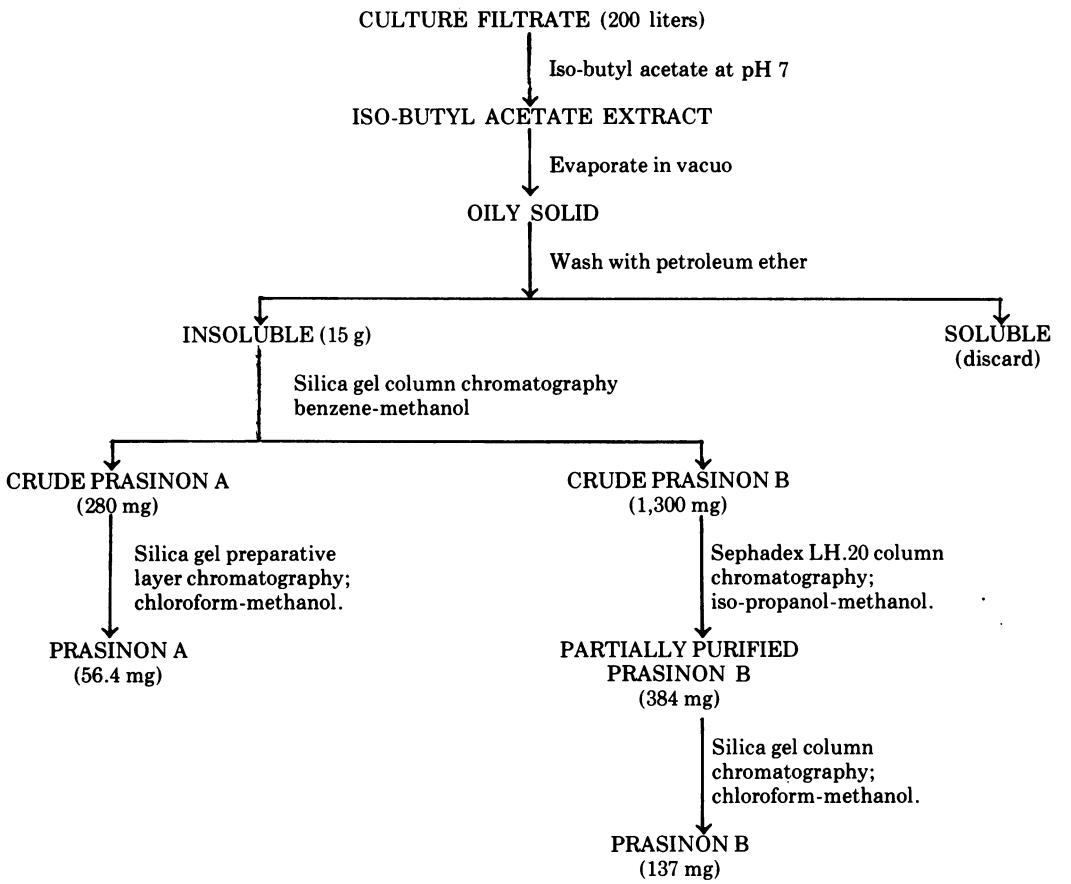

FIG. 1. *Isolation of prasinon A and prasinon B.*

(9:1, vol/vol) and chromatographed on a column of silica gel (Merck Keiselgel H, TLC grade, 5 by 45 cm) equilibrated with the same solvent mixture. The column was eluted with benzene-methanol (9:1, vol/vol), and the fractions (15 ml) were monitored for their larvicidal activity and reaction with van Urk's reagent after thin-layer chromatography. Two zones of larvicidal activity were eluted from the column. The first zone gave a crude preparation of prasinon B and the second gave a crude preparation of prasinon A.

Column fractions containing prasinon A were bulked and evaporated to dryness to yield an oily solid which was further purified by preparative layer chromatography on silica gel (Merck F 254, 3-mm layer) with chloroform-methanol (95:5, vol/vol) as eluant. The bright-yellow band running between R_f values of 0.15 and 0.26 was scraped off the plate and eluted from the silica gel with ethyl acetate. The solvent was filtered free of silica gel and evaporated to dryness to give a yellow amorphous solid which was chromatographically pure.

The fractions containing crude prasinon B obtained from the benzene-methanol column were evaporated to an oily solid which was dissolved in iso-propanol-methanol (70:30, vol/vol) and chromatographed on a column of Sephadex LH20 (2.5 by 35 cm) with iso-propanol-methanol (70:30, vol/vol) as the eluant. Those fractions showing larvicidal activity were bulked and evaporated to dryness to yield a yellow amorphous solid. This preparation was still not chromatographically pure and was further purified by chromatography on a column of silica gel (1.25 by 25 cm) with chloroform-methanol (95:5, vol/vol) as eluant. Fractions containing prasinon B were evaporated to dryness to give a yellow amorphous solid which was chromatographically pure. The R_f values for prasinons A and B are given in Table 5.

Physical and chemical properties. Prasinons A and B are pale-yellow solids having high

solubility in methanol, ethanol, *n*-butanol, benzene, toluene, chloroform, ethyl acetate, butyl acetate, and acetone. They have poor solubility in diethyl ether and petroleum spirit (100 to 120 C) and are insoluble in water. Other physical and analytical data are given in Table 6. The molecular weight derived from the analytical data and empirical formula is much higher than the highest mass ion. Further work is in progress to find the reason and elucidate the structures of prasinons A and B, which are clearly closely related.

When dissolved in methanol, prasinon B degrades to a compound which has much lower larvicidal activity, i.e., 80% kill of *L. sericata* larvae at 25 μg/ml. Breakdown of prasinon B in methanol may be followed by thin-layer chromatography, the product having a slightly higher R_f value than prasinon B (Table 5). More than 50% breakdown occurred within 24 h at room temperature. There is no apparent breakdown of prasinon A under the same conditions.

TABLE 5. *Silica gel thin-layer chromatography of prasinons A and B and the methanol decomposition product*

Solvent	R_f value (mean of two values)		Methanol decomposition product of prasinon B
	Prasinon A	Prasinon B	
n-Butyl acetate	0.10	0.20	0.27
Benzene-acetone (50:50, vol/vol)	0.49	0.59	0.68
Benzene-ethyl acetate (50:50, vol/vol)	0.10	0.22	0.33
Benzene-methanol (90:10, vol/vol)	0.13	0.20	0.30
Chloroform:methanol (95:5, vol/vol)	0.50	0.58	0.74

The infrared spectrum of prasinon A (Fig. 2) is very similar to that for prasinon B and the methanol degradation product of prasinon B, except in the region 1,600 to 1,750 cm^{-1}. All three compounds have maxima in this region at 1,615 and 1,670 cm^{-1}, but these maxima are of different relative intensity. Prasinon B and its methanol degradation product, unlike prasinon A, have a clear peak at 1,720 cm^{-1} but not at 1,700 cm^{-1}.

Insecticidal properties. Table 7 shows the activity of prasinons A and B against the larval stage (first instar) of a susceptible strain of *L. sericata* and both susceptible and insecticide-resistant strains of *L. cuprina*. These substances are somewhat more active than butacarb but less active than diazinon. When tested against organophosphorus and carbamate- or organochlorine-resistant strains, the activity of prasinon is somewhat reduced but not as much as would be expected if cross-resistance were present. These resistant strains were field strains in origin, not obtained from the sensitive strains

TABLE 6. *Physical properties of prasinons A and B.*

Prasinon	Optical rotation $[\alpha]_D^{20}$	Ultraviolet spectrum		Mass spectrometry (highest mass ion—*m/e*)	Analytical data			
		λ max; (nm)	$E_{1\,cm}^{1\%}$		C	H	N	O
A	+ 16.3 (3.19 g/liter of $CHCl_3$)	248	570	538	64.42	8.21	2.00	26.34
B	+ 29.9 (2.88 g/liter of $CHCl_3$)	249	540	538	65.47	8.21	2.16	24.16[a]

[a] By difference.

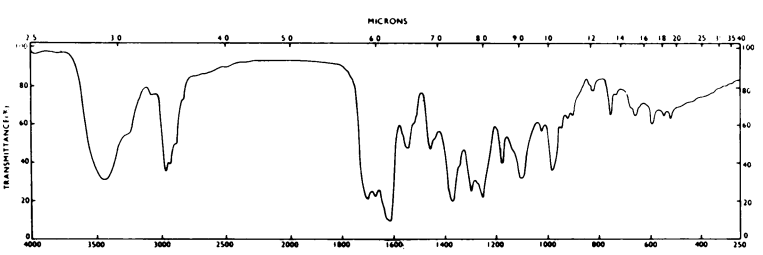

FIG. 2. *Infrared spectrum of prasinon A (KBr disk).*

TABLE 7. *LC_{50} values (μg/ml) for prasinons A and B against Lucilia species*

Compound	Susceptible strains		Insecticide resistant strains of *Lucilia cuprina*	
	L. sericata (British strain)	*L. cuprina* (Australian strain)	Organophosphorous and carbamate resistant[a]	Organochlorine resistant
Prasinon A	0.5–1.0	0.5–1.3	3.3–3.5	1.8–2.3
Prasinon B	0.7–0.8	0.5–0.9	2.0–3.0	<1.0
Butacarb	1.6–1.8	1.4–1.7	27	
Diazinon	0.1–0.2	0.1–0.2	1.2–1.7	

[a] Results refer to a field organo-phosphorus-resistant strain subjected to laboratory carbamate pressure (RD strain).

listed, and it was noted that they appeared to have some low-level resistance to insecticides which do not inhibit cholinesterase.

At a final concentration of 8 μg/ml, neither prasinon A nor B had any inhibitory effect on the acetylcholinesterase of *L. sericata* fly heads or human red blood cells. At 80 μg/ml, there was only about 10% inhibition of both enzymes, whereas in the test used the known inhibitor eserine gave 50% inhibition of the insect enzyme at about 0.03 μg/ml.

The prasinons were not active against the adult stage of *L. sericata* or *L. cuprina* by topical application (10 μg/fly) or surface contact test (5 μg/cm²). Against *Pieris brassicae* larvae (Cabbage White Butterfly), they showed weak activity giving an LC_{50} at 500 to 700 μg/ml on dipped leaves, and against adult *Sitophilus granarius* 100% kill was obtained with grain treated at 1 mg/ml. These results suggest that the compound is acting via the oral route. No activity was seen against *Aphis fabae* nymphs (bean aphis), *Tetranychus urticae* (red spider mite), or *Boophilus decoloratus* larvae (African blue cattle tick).

Other biological properties. In tests for antibacterial activity, the prasinons were inactive at 100 μg/ml against gram-negative and gram-positive bacteria, except in the case of prasinon A which showed a minimum inhibitory concentration of 100 μg/ml against *Bacillus subtilis* and *Staphylococcus aureus*. The compounds did not show any activity at 100 μg/ml against a range of animal and plant fungal pathogens, but prasinons A and B did show pronounced activity against *Trichomonas foetus*, with minimum inhibitory concentrations of 1.25 and 0.08 μg/ml, respectively.

The acute oral toxicity tests in mice gave LD_{50} values of about 90 mg/kg for prasinon B and 500 mg/kg for prasinon A.

ACKNOWLEDGMENTS

We are most grateful to E. la Croix, R. A. Edmondson, G. Hanscomb, and M. S. Verrall for their help and to T. A. Bell, G. Hughes, and B. C. Warren for technical assistance.

LITERATURE CITED

1. Craveri, R., and G. Giolitti. 1957. An antibiotic with fungicidal and insecticidal activity produced by *Streptomyces*. Nature (London) **179**:1307.
2. Harries, F. H. 1967. Fecundity and mortality of female codling moth treated with novobiocin and other antibiotics. J. Econ. Ent. **60**:7-13.
3. Harries, F. H., and V. J. Mattson. 1963. Effects of some antibiotics on three aphid species. J. Econ. Ent. **56**:412-414.
4. Kido, G. S., and E. Spyhalski. 1950. Antimycin A, an antibiotic with insecticidal and miticidal properties. Science **112**:172-173.
5. Oishi, H., T. Hosokawa, T. Okutomi, K. Suzuki, and K. Ando. 1969. Pesticidal activity of aureothin. Agr. Biol. Chem. (Tokyo) **33**:1790-91.
6. Oishi, H., T. Sugawa, T. Okutomi, K. Suzuki, T. Hayashi, M. Sawada, and K. Ando. 1970. Insecticidal activity of macrotetrolide antibiotics. J. Antibiot. **23**:105-106.
7. Takahaski, N., A. Suzuki, Y. Kimura, S. Miyamoto, S. Tamura, T. Mitsui, and J. Fukami. 1968. Isolation, structure and physiological activities of piericidin B, natural insecticide produced by a *Streptomyces*. Agr. Biol. Chem. (Tokyo) **32**:1115-1122.
8. Yeoman, G. H., and B. C. Warren. 1965. Vet. Rec. **77**:922-928.