Communications to the Editor

VERLAMELIN, A NEW ANTIFUNGAL AGENT

Sir:

A new antifungal agent, previously coded as MSD-A43F[1~3] has been found to be produced by *Verticillium lamellicola* (F. E. V. Smith) W. Gams. Verlamelin is a lipopeptolide of new structure. In this communication, the production, isolation, biological activity, characterization and structure of verlamelin are reported.

Verlamelin is produced when *V. lamellicola*, Merck strain MF-4683, is cultured in shaken, solid or liquid static conditions. In shaken culture, the fungus does not produce a filamentous growth. Short hyphae are formed from which yeast like cells bud off at the ends. These buds produce short hyphae to complete the cycle. Most of the verlamelin is retained in the cells and productivity is much greater, at least 30×, in static culture over shaken culture. The culture is maintained in vegetative stage by freezing at −70°C. Two ml of thawed vegetative cells are inoculated in 40 ml of medium in 250-ml baffled Erlenmeyer flasks. The medium contains corn steep liquor 0.5%, tomato paste 4.0%, Cerelose 1.0%, $FeSO_4 \cdot 7H_2O$ 0.001%, $MnSO_4 \cdot 4H_2O$ 0.001%, $CuCl_2 \cdot 2H_2O$ 0.000025%, $CaCl_2 \cdot 2H_2O$ 0.0001%, H_3BO_3 0.000056%, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.2% in distilled water at pH 6.8. After 24 hours shaking at 220 rpm at 28°C, 1 ml of growth is inoculated into 40 ml of production medium in 250-ml baffled flasks. The production medium contains sucrose 6.0%, Bacto peptone 1%, yeast auto-lysate 0.2% in distilled water at pH 6. Maximum broth potency of 30 μg/ml occurs after 4 days incubation at 25°C with liquid static conditions. Verlamelin also has been produced and isolated from shaken cultures. Productivity is much lower and requires larger amounts of cells. These can be produced by using inoculum, developed through successively larger stages, to inoculate required volumes of production medium. The inoculum and production media described above are useful.

Verlamelin must be extracted from the cells. The viscous, non-filtrable whole broth from agitated fermentations was stirred with one volume of methanol for 1 hour and filtered through Super-cel. The filtrate was evaporated *in vacuo* to an aqueous phase and extracted with an equal volume of ethyl acetate (2×). The ethyl acetate layers were pooled, concentrated and dried with sodium sulfate. The ethyl acetate concentrate was chromatographed on a silica gel column formed in ethyl acetate. Elution of the column with ethyl acetate and mixtures of ethyl acetate - methanol of increasing methanol concentration segregated the antifungal activity in the fraction eluting with 30% methanol in ethyl acetate. The active fraction was rechromatographed on a silica gel column formed in methanol and subsequently washed with acetone and ethyl acetate. Elution of the column as above gave the antifungal activity in a

Fig. 1. IR spectrum of verlamelin in KBr.

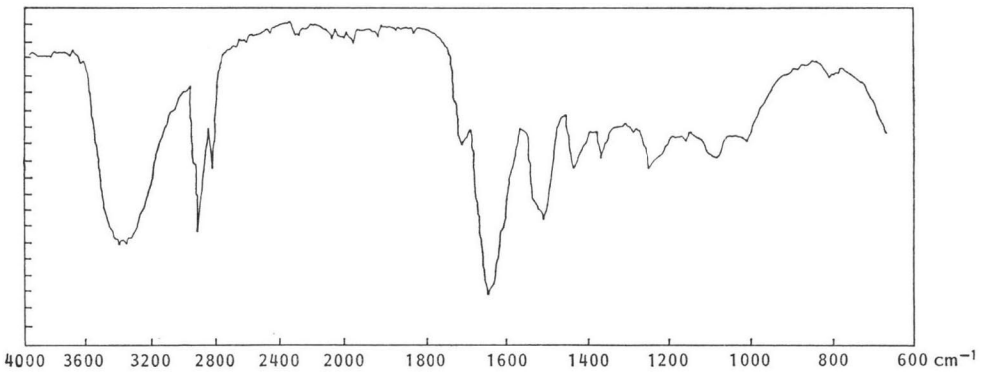


Fig. 2. ^{1}H NMR spectrum of verlamelin in $CDCl_3$.

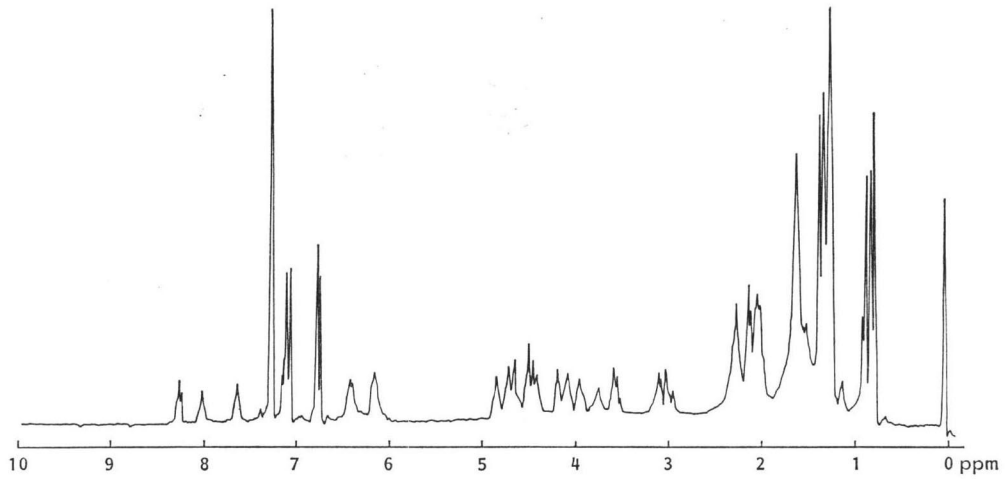
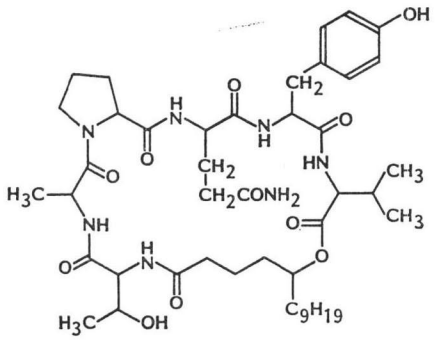


Fig. 3. Structure of verlamelin.



fraction eluted with 20% methanol in ethyl acetate. The antifungal activity was purified to homogeneity on a Sephadex LH-20 column equilibrated with methylene chloride - hexane - methanol (10: 10: 1.2) and run isocratically in the same system. The bioactive fractions were pooled, concentrated and the residue judged to be pure by TLC and HPLC.

Verlamelin is a white amorphous solid of elemental composition $C_{45}H_{71}N_7O_{11}$. The molecular weight 885 was determined by high resolution mass spectrometric measurement at *m/z* 867 ($M-H_2O$) and confirmed by field desorption mass spectrometry. UV absorption of verlamelin was measured in neutral and acid (0.01 N HCl) methanol and in basic (0.01 N NaOH) methanol.

Neutral and acid methanol
λ_{max} 225 nm, $E^{1\%}=230$
λ_{max} 278 nm, $E^{1\%}=38$

Table 1. *In vitro* activity of verlamelin.

Test fungus	MIC (μg/ml)
Ustilago zeae MF-1996	6
Alternaria solani MF-3550	6
Ceratocystis ulmi MF-4042	50
Cochliobolus miyabeanus MF-4626	50
Fusarium oxysporum MF-4344	100
Aspergillus niger MF-442	$\geq$200
Trichophyton MF-4832	$\geq$200
Mucor rouxii MF-4884	$\geq$200
Phytophthora palmivora MF-4653	$\geq$200
Candida albicans MY-992	$\geq$200

Verlamelin was added to potato dextrose agar plates to obtain desired concentrations. Approximately 10^4 spores of each fungus were spotted onto the surface of the agar. Results were scored after incubation for 34 hours at 28°C.

λ_{max} 284 nm, $E^{1\%}=33$
Basic methanol
λ_{max} 245 nm, $E^{1\%}=200$
λ_{max} 294 nm, $E^{1\%}=50$

The structure of verlamelin, as determined by high resolution MS, IR (Fig. 1), ^{13}C NMR and ^{1}H NMR (Fig. 2) spectroscopy and amino acid analysis of the antibiotic and its tyrosine *O*-methyl ether derivatives is shown in Fig. 3. It is a lipopeptolide containing L-valine, L-glutamine, L-proline, D-alanine, D-tyrosine, D-allothreonine and 5-hydroxymyristic acid as an integral part of the ring structure. This structure differs from other lipopeptolides in which a fatty acid is merely attached to the peptide ring such as octapeptins, mycosubtilin and iturin A

Table 2. *In vivo* activity of verlamelin.

Treatment	Concentration (μg/ml)	Wheat leaf rust (pustules/leaf)		Late blight (tomato) rice blast (lesions/5th leaf)		Early blight (tomato) *Phytophthora* (% leaf necrosis)		*Alternaria* (lesions/leaf)		Bean powdery mildew (mildew centers/16 cm^2)	
		Protectant	Systemic	Protectant	Systemic	Protectant	Systemic	Protectant	Systemic	Protectant	Systemic
Verlamelin	1,000	0	5*	≤1	1	8	32	14	58	≤1	12
	500	≤1	15*	0	9	8	60	29	45	≤1	14
	100	6	31*	≤1	26	33	88	38	46	7	17
Standard		Maneb	Carboxin	Hinosan	Hinosan	Maneb	—	Maneb	—	Benomyl	Benomyl
	1,000	—	4	—	31	—	—	4	—	—	0
	100	0	—	≤1	—	11	—	25	—	0	7
	10	12	—	8	—	63	—	58	—	10	—
	1	20	—	16	—	77	—	—	—	26	—
Untreated	—	27	42	52	69	84	88	63	70	25	25

* Phytotoxicity observed.

In the protectant test, spores of the test organisms were sprayed on the lower leaf surfaces two days after solutions of verlamelin were sprayed to run-off on the leaves. In the systemic test, the plants were similarly inoculated with the test organisms two days after verlamelin solutions were added as soil drenchs. Plants were held 24 hours at 21°C in high humidity after inoculation. Activity was determined 3~5 days later.

Table 3. *In vivo* activity of verlamelin against rice blast.

Compound	Rice blast		
	Concentration (μg/ml)*	Lesions per 5th leaf	Remarks
Verlamelin	10	2	No injury
	1	4	No injury
	0.1	8	No injury
	0.01	7	No injury
Benomyl	100	0.1	No injury
	10	4	No injury
	1	4	No injury
Untreated	—	22	No injury

* Compounds dissolved in methanol and diluted with water containing Tween 20.

products by *Bacillus*[4].

Verlamelin shows weak *in vitro* activity and has a narrow antifungal spectrum, Table 1. We have detected no antibacterial activity. The compound induces morphological changes such as swelling and bulging of cells. The fungi most sensitive to the compound may show some lysis. These changes, except for lysis, also occur at concentrations below the MIC but, the effects are not lethal and normal growth resumes with further incubation.

Verlamelin was tested for *in vivo* activity against a series of plant diseases using standard greenhouse tests. The data, Table 2, show clear activity at high concentrations. The compound is particularly active against rice blast, Table 3.

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