CLAVAMYCINS, NEW CLAVAM ANTIBIOTICS FROM TWO VARIANTS OF *STREPTOMYCES HYGROSCOPICUS*

I. TAXONOMY OF THE PRODUCING ORGANISMS, FERMENTATION, AND BIOLOGICAL ACTIVITIES

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In a selective screening for antifungal metabolites, six new clavam antibiotics, clavamycins A, B, C, D, E and F, have been detected from two variants of *Streptomyces hygroscopicus* NRRL 15846 and NRRL 15879.

The goal of our antifungal screening was the discovery of new compounds which are active against *Candida* sp. and which display features different from those of the known antibiotics. Our strategy was based on the selection of active substances inducing morphological changes on growing *Candida* cells, on antagonism tests and on extraction behavior. Among the metabolites discovered by this procedure were six new clavam antibiotics from two strains of *Streptomyces hygroscopicus*. This report presents the taxonomy of the producing strains, the fermentative production and the characteristics of the two main metabolites, clavamycins A (Fig. 1) and D (Fig. 2). The isolation procedures, the physicochemical characterization and the structures of the different clavamycins are described in the following publication[1]. The strain NRRL 15846 also produces hydroxymethylclavam, an extractable clavam with antifungal activities[2].

Materials and Methods

Taxonomy

The two actinomycetes strains were identified according to BERGEY's Manual of Determinative Bacteriology[3].

Fermentation

The fermentative procedure was identical for both strains.

One liter of seed medium (malt extract 10 g, yeast extract 2 g, glucose 10 g per liter, pH adjusted to 7.2) in a 2-liter shake-flask was inoculated with *ca.* 10^9 spores. The seed culture was incubated for 3 days at 27°C on a rotary shaker at 200 rpm. This seed culture was then transferred to 20 liters of the

Fig. 1. Structure of clavamycin A.

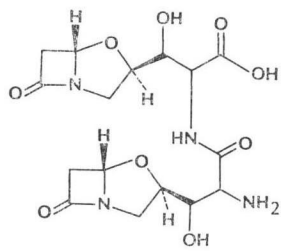
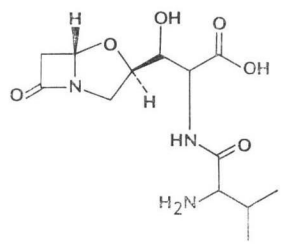


Fig. 2. Structure of clavamycin D.



same medium in a 22-liter fermentation tank. This intermediary culture was incubated at 27°C for 3 days with an air-flow of 0.5 liter/liter/minute and 100 rpm stirring. Ten liters of the intermediary culture were transferred to a 100-liter production medium (glucose 10 g, soluble starch 20 g, yeast extract 5 g, NZ Amine Type A 5 g, $CaCO_3$ 0.1 g per liter, pH adjusted to 7.0) in a 120-liter fermentation tank. The fermentation was carried out at 24°C for 5 days with an air-flow of 0.8 liter/liter/minute and 100 rpm stirring.

The biomass, the pH and the antibiotic content (determination of the broth activities against *Candida albicans*) of the production cultures were monitored.

At the end of the fermentation, the broth was adjusted to pH 7.0 and the antibiotics isolated as described in the subsequent paper[1].

Biological Activities

Test strains used were from the culture collection maintained in our laboratories. Fungi were cultivated on a 2% malt agar medium and bacteria on a 1.5% Bacto Tryptone blood agar base. In addition, *Escherichia coli* was also grown on a minimal medium containing K_2HPO_4 0.7%, KH_2PO_4 0.3%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4$ 0.1%, NaCl 0.01%, glucose 0.4% and agar 1.5%.

The morphological changes of growing cells of *C. albicans* were observed on a 2% malt agar medium osmotically stabilized with 27% sucrose. The minimal inhibitory concentrations (MICs) were evaluated by arithmetic series type of dilution test as described by KAVANAGH[4].

The *in vitro* test with chitin synthase from *Coprinus cinereus* was preformed according to BRILLINGER's method[5].

The influence of ergosterol, of amino acids and short peptides (50 mM) (Serva Biochemical Ltd.) on the activity of clavamycins A and D was examined by cross-test on agar plates[6].

A mixture of broad spectrum β-lactamases (*Bacillus cereus* 569-H9, Enzyme Biochemical Ltd.) (2 mg/100 ml) was used to analyze the degradation of clavamycins A and D. Enzyme inhibition was checked with TEM β-lactamases.

Acute toxicity was determined by intraperitoneal application, using 5 mice per dose.

Detection with TLC

Ten to 50 μl of the centrifugated broths were directly applied to cellulose polygram plates. The plates were developed in propanol - H_2O (7: 3). The clavamycins were visualized with the van Urk reagent.

Results and Discussion

Taxonomy

Both strains were identified as belonging to the genus *Streptomyces* (Type I cell wall) and to be variants of *S. hygroscopicus*. NRRL 15846, designated *S. hygroscopicus* var. S 81-3615, was isolated from a cobalt-rich soil in Berne, Switzerland. NRRL 15879, designated *S. hygroscopicus* var. *antillensis*, was isolated from a loam from Guadeloupe, French West Indies. Both strains have the cultural characteristics of *S. hygroscopicus*. The sporophores have narrow compact spirals that are borne in dense clusters (Fig. 3). The grey spores are oval to rectangular in shape and the spore surfaces are smooth to warty as determined by the electron microscope (Fig. 4). The distinctive hygroscopic character and blackening on most agar media is typical.

The physiological properties and carbon utilization studies are summarized in Tables 1 and 2.

The diversity of the bioactive metabolites produced by strains of *S. hygroscopicus* is large, *e.g.* macrolides[7], glycopeptides[8], guanidylfungins[9], the clavamycins are now added to this list. The producer strains were isolated from soil samples from different geographical regions, indicating the wide distribution of such producer strains in nature.

Fig. 3. Photomicrograph of *Streptomyces hygroscopicus* (malt extract - yeast extract - agar, ×850).

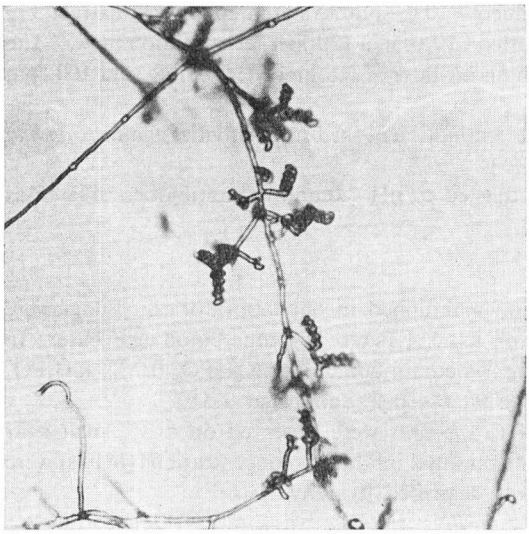


Fig. 4. Electronmicrograph of spores of *Streptomyces hygroscopicus* (malt extract - yeast extract - agar, ×5,350), showing warty surfaces.

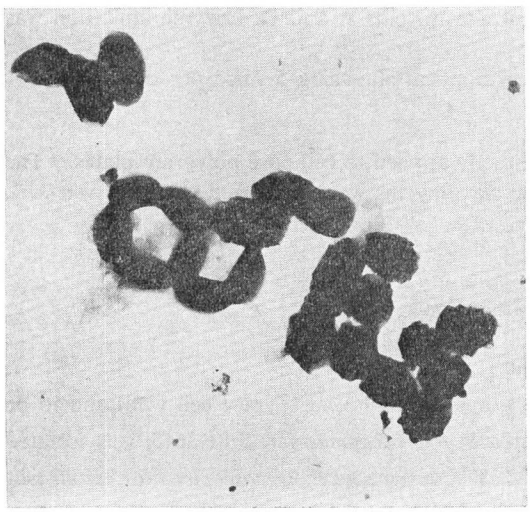


Table 1. Physiological properties.

	NRRL 15846	NRRL 15879
Nitrate reduction	+	+
Starch hydrolysis	+	+
Cellulolytic activity	−	−
Milk coagulation	+	+
Milk peptonization	+	+
Gelatin liquefaction	−	+
Hydrogen sulfide production	−	+
Melanin formation	−	+
NaCl inhibition	≥5% but <7%	≥5% but <7%
Temperature for growth	18~35°C	18~33°C

+ Positive, − negative.

Table 2. Carbon utilization.

	NRRL 15846	NRRL 15879
D-Glucose	+	+
D-Xylose	+	+
D-Galactose	+	+
Raffinose	+	+
L-Arabinose	+	+
D-Fructose	+	+
L-Rhamnose	+	+
D-Mannitol	+	+
i-Inositol	+	+
Salicin	+	−
Sucrose	+	+

+ Utilized, − not utilized.

Fermentation

The production of clavams by both *S. hygroscopicus* strains is optimal at 24°C, although for good growth and sporulation, 28 to 35°C is necessary. The seed culture needs a low aeration rate, less than 20 mM O_2/hour, while the production culture has an oxygen transfer rate of 20~40 mM O_2/hour. The best fermentation media contain glucose and starch as carbon sources and NZ Amine Type A, corn steep liquor or ammonium succinate as nitrogen sources. Both strains can also grow and produce clavams in a minimal synthetic medium consisting of glucose, starch, ammonium succinate and mineral salts.

A typical time-course production of the strain NRRL 15846 is shown in Fig. 5. The antibiotic production starts 2 days after inoculation to reach a maximum titer of 200 mg/liter clavamycins and hydroxymethylclavam after 5 days and decreases rapidly afterwards. It is after the period of intensive growth that the biosynthesis of the antifungal compounds begins. The maximum rate is reached at the beginning of the stationary phase. The strain NRRL 15879 has a similar fermentation course. With the addition of amino acids to the production media, it is possible to direct the biosynthesis of the

clavamycins. For example, by adding 5×10^{-3} M L-valine, clavamycin D can be produced exclusively.

Biological Activities

The clavamycins are potent anti-*Candida* compounds as demonstrated by their MICs against 3 *Candida* species (Table 3). In an osmotically stabilized medium, the severe morphological changes

Fig. 5. Time course of clavamycin fermentation with *Streptomyces hygroscopicus* NRRL 15846.

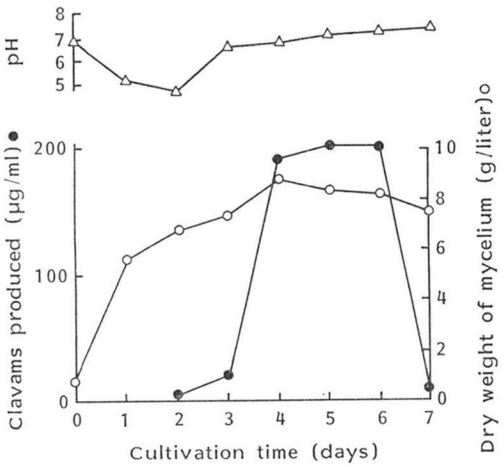


Table 3. Minimal inhibitory concentrations (μg/ml).

Organism	Clavamycin A	Clavamycin D
Candida krusei	6.0	0.5
C. tropicalis	0.2	0.2
C. albicans	0.2	0.1

Fig. 6. Morphological changes induced by clavamycin D to *Candida albicans* (×250).

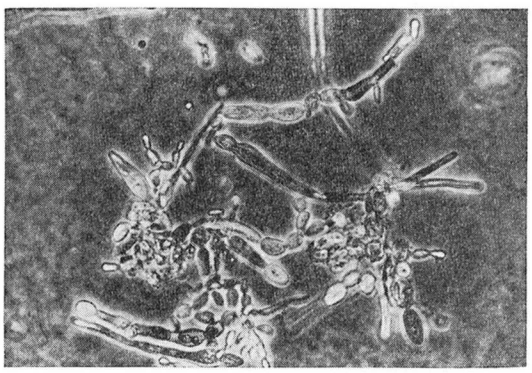


Table 4. Antimicrobial spectrum of clavamycins A and D.

Test organism	Inhibition zones (mm)*	
	Clavamycin A	Clavamycin D
Staphylococcus aureus	0	0
Micrococcus lysodeikticus	0	0
M. luteus	0	0
Enterococcus faecalis	0	0
Proteus vulgaris	0	0
Escherichia coli K12	0	0
E. coli K12 (on minimal media)	0	0
Pseudomonas fluorescens	0	0
P. aeruginosa	0	0
Comamonas terrigena	0	0
Candida albicans	23	41
C. tropicalis	17	38
C. parapsilosis	24	46
Kloeckera apiculata	24	32
Hansenula anomala	20	29
Trichophyton quinckeanum	40	65
Curvularia lunata	14	39
Pythium paroecandrum	0	0
Neurospora crassa	22	39
Aspergillus fumigatus	0	16

* 20 μl of a 10^{-3} M solution on filter disc.

Table 5. Effects of amino acids and peptides on the activity of clavamycins A and D against *Candida albicans*.

Addition of	Reversion of activity			
	Clavamycin A	Clavamycin D	Compound **7**	Hydroxy-methylclavam
Alanine	−	−	−	−
Alanyl-alanine	+	+	−	−
Alanyl-alanyl-alanine	+	+	−	−
Methionine	−	−	−	−
Methionyl-methionine	+	+	−	−
Methionyl-methionyl-methionine	+	+	−	−

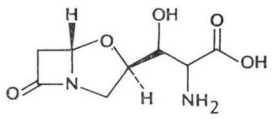
+ Positive, − negative.

Table 6. Comparison of the properties of the clavamycins to other antifungal compounds.

	Clavamycins	Hydroxy-methylclavam	Polyoxins	Nikkomycins	Polyens
Butanol extraction	−	+	−	−	+
van Urk reaction	+	+	−	−	−
Morphological changes of *Candida albicans*	+	+	+	+	+
Chitin synthase inhibition	−	−	+	+	−
Reversion of antifungal activity by ergosterol	−	−	−	−	+
Reversion of antifungal activity by di- and tri-peptides	+	−	+	+	−
Antibacterial activity	−	(±)	+	+	+

+ Positive, (±) doubtful, − negative.

Fig. 7. Structure of derivative **7**, the common part of all clavamycins.



induced by clavamycin A or D to the growing cells of *Candida* (Fig. 6) indicate an activity against cell wall synthesis. However, the chitin synthase is not inhibited *in vitro* by the clavamycins. Addition of ergosterol does not reverse the antifungal activity indicating that there is no interference with sterol biosynthesis.

The clavamycins are also active against fungi belonging to different classes (Table 4), but are not active against bacteria.

The antagonism tests (Table 5) show that the activity of clavamycins A and D against *C. albicans* can be reversed by di- and tri-peptides, but not by amino acids. This indicates that clavamycins are recognized as peptides and therefore transported in the yeast cell by the peptide transport system. By enzymatic degradation with aminopeptidase M, the derivative **7** can be obtained[1]. This derivative is common to all clavamycins (Fig. 7). The antifungal activity of derivative **7** as well as that of hydroxymethylclavam are not reversed by small peptides. Their antibacterial activities are antagonized by methionine and peptides containing methionine.

The clavamycins A and D are not β-lactamase inhibitors, nor are they affected by β-lactamases. The non-affinity to β-lactamases could be explained by the stereochemistry of the clavamycins[1]. Clavamycins A and D are toxic to mice, LD_{50} values are around 10 mg/kg.

In Table 6, the main features of the clavamycins are compared to other antifungal compounds from actinomycetes. They present different features from the known antifungal compounds, even from other clavams[2,10] and as such they form a new class of antifungal substances.

Acknowledgments

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