INCIDENCE OF ANTIPROTOZOAL AND ANTIVERMAL ANTIBIOTICS IN FUNGI. I

CLASS FUNGI IMPERFECTI

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The antiprotozoal and antivermal activities of 22 species of Fungi imperfecti as well as a description of the method used for screening are presented. In the microorganisms studied a very high incidence of antiprotozoal and antivermal activities was found. The results indicate that this class of fungi is a good source of antiprotozoal and antivermal antibiotics.

A method of specific screening for antiprotozoal antibiotics using *Trypanosoma cruzi* as a test organism was described by us several years ago[1]. The technique of this method was recently modified, and more protozoa and one nematode were included into the test system. By testing crude extracts of the culture broths the method enabled us to identify the eventual production of antiprotozoal or antivermal substances and to work out procedures for their isolation[2,3,4,5].

We are interested in the isolation of antibiotics specifically active against protozoa (and antivermaloly active substances), *i. e.* in the isolation of substances which are active only against protozoa (or nematodes), and are not active against bacteria or fungi. These antibiotics, therefore, cannot be detected by the usual plate screening methods using only bacteria and fungi as test organisms.

The incidence of antiprotozoal antibiotics in various groups of microorganisms is surprisingly high: we have found that 27.8 % of the cultures of Aspergillaceae studied produce substances having antiprotozoal activity[6]; in *Penicillia* 60 % of the cultures tested had antiprotozoal activity[7], whereas in aquatic Phycomycetes a 92 % incidence of antiprotozoal substances was found[8].

In this paper the modified method of screening and the antiprotozoal, antivermal as well as antibacterial and antifungal activities of 23 species of Fungi imperfecti, obtained from the culture collection of the Centralbureau voor Schimmelcultures in Baarn, Holland, are described.

Nearly all of the strains of Fungi imperfecti studied are known to be "predacious fungi" which are characteristic in their ability to trap and kill protozoa and microscopic worms present in water. This peculiar killing ability as well as various forms of trapping organs of the "predacious fungi" were already described a long time ago by several authors[9,10].

It is very interesting to note that the death of the trapped organisms takes place

in a relatively short period of time so that it is very unprobable that the death is caused only by the "effect of strangulation". For this reason the production of antiprotozoal and of antivermal substances by Fungi imperfecti could well be expected. According to our present knowledge nobody has systematically screened for antiprotozoal antibiotics or for antivermal substances in Fungi imperfecti.

Materials and Methods

Butanolic extracts of 100 ml of the fermentation broth and mycelium of the microorganisms cultivated on a rotary shaker are evaporated to dryness *in vacuo*. The residue is again dissolved in a small volume of a suitable organic solvent, and into this solution a strip of chromatographic paper is immersed for a few seconds. After drying at laboratory temperature segments of the paper, which serve only as carriers of the extracted materials, are cut into very thin strips and added to small test tubes containing 0.5 ml of suspensions of the test protozoa. These are observed microscopically, 10 fields per sample, after 24 and 48 hours. Active extracts cause the death (immobilisation), or lysis of the protozoa. The results are evaluated according to the following key :

0	extract inactive, protozoa as in control
+	about 25 % of the protozoa dead
++	about 50 % of the protozoa dead
+++	about 75 % of the protozoa dead
(+++)	only single protozoa living
D	all protozoa dead
L	all protozoa dead ; a pronounced lysis of the organisms is observed

The following protozoa are used as test organisms (the approximate number of organisms per ml and the composition of the medium are in brackets): *Trypanosoma cruzi* (100,000 ; NNN agar+physiological saline), *Leishmania brasiliensis* (200,000 ; SALE-SCHMIDT's medium), *Strigomonas culicidarum* (200,000; NNN agar+physiological saline), *Euglena gracilis* and *Euglena gracilis* depigmented (500,000 ; bactopepton Difco 5.0 g, sodium acetate 2.0 g, water 1,000 ml), *Astasia chattoni* (500,000 ; sodium acetate 1.0 g, beef extract Difco 1.9 g, tryptone 2.0 g, yeast extract Difco 2.0 g, calcium chloride 0.01 g, distilled water 1,000 ml), *Tetrahymena piriformis* (500,000 ; LWOFF's medium).

The antivermal activity is tested on *Anguillula aceti*, a free living nematode using the method described by BAĆIKOVÁ *et al.*[11] but with a modified and less tedious way of evaluation. The paper strips containing the extracts are added to test tubes with the suspension of nematodes (about 1,000 organisms/ml). These are observed microscopically after 72 hours, and the results are evaluated according to the following key :

0	extract inactive, nematodes as in control
+	about 1/4 of nematodes dead, slightly decreased motility
++	about 1/2 of nematodes dead, decreased motility
+++	about 3/4 of nematodes dead, pronounced decrease of motility
D	all nematodes dead, eventually partial lysis of organisms

The evaluation of both antiprotozoal and antivermal activities requires some practice, but reliable, well-overlapping and reproducible results can be obtained even by different observers.

The antibacterial and antifungal activities are determined by the usual agar plate method using paper discs cut from the paper strips containing the dried extract. As test organisms *Bacillus subtilis* (or *Staphylococcus aureus*), *Escherichia coli*, *Candida pseudotropicalis* (or *Candida albicans*) and *Aspergillus fumigatus* are used.

The diameters of the zones of inhibition around the discs were divided into five categories :

0	no zone of inhibition
±	diffuse zones of inhibition not reaching further than 2 mm from the edge of the paper disc
+	inhibition zones of a diameter up to 15 mm
++	inhibition zones of a diameter between 15 and 20 mm
+++	inhibition zones of a diameter of more than 20 mm

The method as described up to this point only gives data on the whole crude extract, *i. e.* on the sum of all activities detected. It says nothing about the number and respective spectra of the individual antibiotics responsible for the effect observed. According to general experience several antibiotics are often produced side by side. In such cases the antiprotozoal activity is mostly due to the production of one specifically active substance which is not active against bacteria or fungi. Another antibiotic is usually responsible for the antibacterial or antifungal activity.

If the isolation of a specifically active antiprotozoal or antivermal antibiotic is attempted, paper chromatography of the crude extract is employed. The chromatograms are cut into short segments enabling the detection of the respective active spots with a variety of test organisms. This gives the answer as to whether or not we are dealing with one substance having a broad spectrum or whether the extract contains several narrow-spectrum antibiotics.

The chromatographic results simultaneously also serve as a guide in working out the isolation procedure.

Results

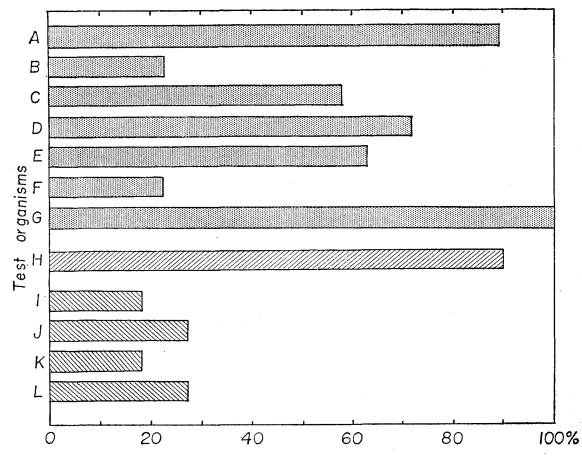
The list of Fungi imperfecti tested and their activities are presented in Table 1.

Fig. 1 gives the percentual incidence of various antagonisms in the cultures studied.

All cultures of Fungi imperfecti tested were active against protozoa, 82 % were active against the nematodal test organism, and 42 % against bacteria and/or fungi.

Fig. 1. Percentual incidence of various antagonisms in Fungi imperfecti. (Percentages calculated without regard to degree of activity)

A : *Trypanosoma cruzi*
B : *Strigomonas culicidarum*
C : *Euglena gracilis*
D : *Euglena gracilis* depigmented
E : *Astasia chattoni*
F : *Tetrahymena piriformis*
G : *Leishmania brasiliensis*
H : *Anguillula aceti*
I : *Bacillus subtilis*
J : *Escherichia coli*
K : *Candida pseudotropicalis*
L : *Aspergillus fumigatus*



Discussion

The method of screening for antiprotozoally and antivermally active substances in microorganisms enables us to identify cultures producing antibiotics of this type. Substances having a specific antiprotozoal or antifungal activity cannot be detected by the usual agar plate methods and certainly passed unobserved through many screening programs.

The very high incidence of cultures with antiprotozoal activity in Fungi imperfecti (100 %) is remarkable, and this fact is evidently connected with the peculiar ecology of the cultures studied. The high

Table 1. Antibiotic spectrum of 22 Fungi imperfecti.
(For explanation of abbreviations see text)

Number of culture	Order, family, genus, species (variety)	Hour of testing	Activity against: Protozoa after 24 and 48 hours: *Trypanosoma cruzi*	*Strigomonas culicidarum*	*Euglena gracilis*	*Euglena gracilis* (depigmented)	*Astasia chattoni*	*Tetrahymena piriformis*	*Leishmania brasiliensis*	Bacteria and fungi after 24 hours: *Bacillus subtilis*	*Escherichia coli*	*Candida pseudotropicalis*	*Aspergillus fumigatus*	Nematode after 72 hours: *Anguillula aceti*
	Order : Moniliales Family : Moniliaceae													
1	*Arthrobotrys arthrobotryoides* (BERL.) LINDAU	24	++	+	D	D	D	D	D	0	0	0	0	0
		48	D	+	D	D	D	D	D					
2	*Arthrobotrys cladodes* DRECHSLER var. *macroides* DRECHSLER	24	+++	0	0	++	0	0	D	0	0	0	0	0
		48	+++	0	0	++	0	0	D					
3	*Arthrobotrys conoides* DRECHSLER	24	+	0	+++	+++	D	0	D	0	0	0	±	++
		48	D	0	+++	D	D	0	D					
4	*Arthrobotrys dactyloides* DRECHSLER	24	0	0	++	+++	0	0	D	0	0	0	0	++
		48	D	0	++	+++	0	0	D					
5	*Arthrobotrys musiformis* DRECHSLER	24	D	0	+++	D	D	++	D	±	++	++	0	++
		48	D	+	+++	D	D	++	D					
6	*Arthrobotrys oligospora* FRES.	24	+++	0	0	0	0	0	D	+++	0	0	0	0
		48	D	0	0	0	0	0	L					
7	*Arthrobotrys robusta* DUDDINGTON	24	0	0	++	+++	D	0	D	0	0	0	0	++
		48	+++	0	++	+++	D	0	D					
8	*Arthrobotrys superba* CDA. var. *oligospora* COEMANS	24	0	0	0	++	++	+	D	0	++	±	0	+
		48	D	0	0	+++	++	+	D					
9	*Arthrobotrys superba* CDA.	24	+++	0	0	+++	D	0	D	±	++	+	+	++
		48	D	0	0	+++	D	0	D					
10	*Dactylaria candida* (NEES) SACC.	24	0	0	++	++	++	0	D	0	0	0	0	+
		48	D	0	++	++	++	0	D					
11	*Dactylaria lutea* ROUTIEN	24	D	0	++	+++	0	0	D	0	0	0	0	++
		48	D	0	+++	+++	0	0	D					
12	*Dactylaria mycophila* TUBAKI	24	D	++	0	++	D	0	D	±	±	0	±	++
		48	D	D	0	++	D	0	D					
13	*Dactylaria polycephala* DRECHSLER	24	0	0	0	0	0	0	D	0	0	0	0	++
		48	+++	0	0	0	0	0	D					
14	*Dactylaria piriformis* JUNIPER	24	0	0	0	0	+	0	D	0	0	0	+	0
		48	0	0	0	0	+	0	D					
15	*Dactylaria thaumasia* DRECHSLER	24	0	0	++	+++	D	0	D	0	++	+	±	+++
		48	D	0	++	D	D	0	D					
16	*Dactylella bembicodes* DRECHSLER	24	0	0	0	++	0	0	D	0	0	0	+	+
		48	D	0	0	++	0	0	D					
17	*Dactylella cionopaga* DRECHSLER	24	0	0	+++	++	++	0	D	0	++	0	0	+
		48	++	0	+++	++	++	0	D					
18	*Dactylella doedycoides* DRECHSLER	24	0	+	+	++	0	0	D	0	0	0	0	+
		48	D	+	++	D	0	++	D					
19	*Dactylella ellipsospora* (PREUSS) GROVE var. *prolifera* DIXON	24	0	0	0	0	0	0	D	0	0	0	0	+
		48	0	++	0	0	+	++	D					
20	*Dactylella gephyrophaga* DRECHSLER	24	0	0	0	0	++	0	D	0	0	0	0	+
		48	D	0	+	0	++	0	D					
21	*Articulospora tetracladia* INGOLD	24	0	0	++	+++	0	0	(+++)	0	0	0	0	++
		48	0	0	++	+++	0	0	D					
	Order : Sphaeropsidales Family : Zythiaceae													
22	*Aschersonia aleurodis* WEBER	24	0	0	++	+++	++	0	+++	0	0	0	0	+++
		48	0	0	++	+++	++	0	D					

incidence of cultures with antiprotozoal activity in the species studied certainly does not indicate a too high degree of sensitivity of our method of screening, because in a similar series of experiments a substantially lower incidence of cultures wih antiprotozoal activity was found (27.8 % in *Aspergillaceae*[6], 60 % in *Penicilia*[7]).

The results presented indicate that Fungi imperfecti and especially the so-called "predacious fungi" are rich sources of antiprotozoal antibiotics and of substances with antivermal activity.

In our method of testing the main criterion for ascertaining the activity of the extracts is the loss of motility of the protozoa (or of the nematode). We are well aware of the fact that the loss of motility need not be a sign of the actual death of the tested organisms. In working out our method we have checked on this possibility and found that under the conditions of our screening procedure immobile protozoa inoculated into fresh medium never regained their motility, and a lysis of the organisms was observed after a few days. Similarily a lysis of the protozoa is also always observed after a few days if the organisms are left in the test tubes to which active extracts were added. In the controls, on the other hand, the protozoa are motile and live for a long period of time.

In evaluating our results we did not put great emphasis on the relative activities of the extracts tested. In this stage of screening the potential activity of a given antibiotic cannot be realistically determined because the activity-response obtained in the screening is strongly influenced by the amount of substance produced. A seemingly high activity, *e. g.* an effect symbolized by "D", can be caused by a very high production of a compound which is only slightly active, whereas a seemingly low activity (symbolized by "+") can be due to the production of only very minute amounts of a highly active substance. These problems are further complicated by the fact that in this stage of testing practically nothing is known about the optimal conditions of cultivation (medium, duration of fermentation, *etc.*) and even the effectiveness of the method used for extraction varies with the solubility of the antibiotic in the given solvent.

Chromatographic studies of several extracts having a broad spectrum of activity (*e. g.* antiprotozoal and antibacterial) have shown that the antibacterial or antifungal activity was usually due to the production of one narrow spectrum antibiotic, while the antiprotozoal and/or antivermal activity was due to the production of another specifically active antiprotozoal and/or antivermal substance.

We are now working on the characterisation of several specifically active antiprotozoal antibiotics from the cultures mentioned. The description of these antibiotics will be published later.

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