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STRUCTURE AND FUNCTIONS OF FUNGAL CELL SURFACES

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(NASA-TM-77439) STRUCTURE AND FUNCTIONS OF  
FUNGAL CELL SURFACES (National Aeronautics  
and Space Administration) 23 p  
HC A02/MF A01

N85-15347

CSCI 06C

Unclass

G3/51 11471

Translation of "Shinkinsaibohyoso no Kozo to Kino", Shinkin to  
Shinkinsho, Vol. 18, No. 3, 1977, pp. 147-155



# STRUCTURE AND FUNCTIONS OF FUNGAL CELL SURFACES

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## I. Introduction

Information on the structure of the surface of fungal cell /147\* walls can be said to be very scarce compared to that for bacterial cells. It is primarily for this reason that it is difficult to isolate the structural substances of the surface layers of fungal cells. That is to say, with respect to bacteria, the separation of the plasma membrane and cell wall of bacteria has already been accomplished for some time now, and based on the detailed analysis of the chemical constituents, even the structure of the molecular construction is being elucidated. Moreover, recently, even the in vitro reconstitution of the structure of the superficial layers has been attempted, and a large amount of important information has been obtained as a result. On the other hand, differing from this kind of situation with bacterial cells, in approaching fungi, the research has been centering around the yeast-type fungi, *Saccharomyces* and *Candida*, and this research also has focused on the chemical analysis of the structure of the surface layers. It can be thought that understanding of the construction of the structure at the molecular level is a problem almost of the past. With respect to pathological filamentous fungi, research has just entered only the early stages, and now it is necessary to study this field diligently. In this study, the discussion of the structure related to the surface layers will be limited to pathological filamentous fungi (Dermatophytes).

## II. Chemistry of the Cell Wall and the Construction of the Structure

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\*Numbers in the margin indicate pagination in the foreign text.

## 1) Separation of the Cell Wall and Ultramicrostructure

The attempts at separating the cell walls from dermatophytes began late compared to those for yeast type fungi. By 1968, Shah and Knight [1] and Itoh et al. [2] had separated and purified the cell walls from Trichophyton mentagrophytes, but both groups had the same difficulty with the cell wall fraction, that of the purity of the fraction, and it was necessary to remove foreign substances using other membrane systems. Nozawa et al. [3] achieved the separation of an almost completely clean and purified fraction of cell walls by means of using Epidermophyton floccosum, which is thought by others to easily undergo massive destruction of the cell. In other words, in that method, after creating a homogenous suspension of fungi in a Waring blender, the suspension is put into a Ribi cell fractionator (Sorvall Co., U.S.A.) and destruction of the cells is performed under high pressure (30,000 psi) three times. The resultant is rinsed with water, centrifuged in a sucrose gradient, and treated with SDS (sodium dodecyl sulfate). In this manner a purified fraction of cell walls was obtained. The results of observing the cell wall fraction obtained in this way, by means of scanning electron microscope, are shown in Figure 1. It shows a hollow cylindrical structure, and it is clear that there is very little foreign substance originating from the inner membrane system, including the plasma membrane. Even in the ultra-thin layer image there are no other substances which are from non-membrane system areas attached to the cell wall. From the standpoint of these electron microscopic findings, it is certain that the separation of the cell walls of E. floccosum as done using this method results in a very high degree of purity.

Furthermore, the results of the observation of the structure of the outer surface of this fraction of cell walls by means of shadowing electron microscopy showed that the bundled microfibriles were aligned mainly in the direction of the axis of the fungal filaments, and with the microfibriles which crossed these, a net



FIGURE 1. Scanning Electron  
Microscopic Image  
of Separated *E. floccosum*  
Cell Walls  
(X 10,000)

mesh like structure was exhibited (Figure 2a). On the other hand, /148 with respect to the inner surface, a net mesh like structure of narrow microfibriles which was more pronounced than that seen in the outer layer, and a condition in which amorphous substances filled the spaces between the microfibriles, were observed (Figure 2b). This morphological difference between the inner and outer surfaces was clarified, and it suggests that there are differences in the chemical components which formed these structures.

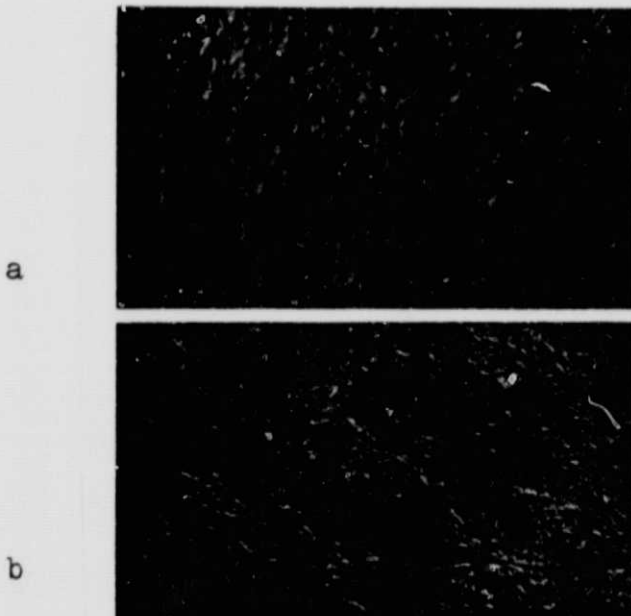


FIGURE 2. Structure of the  
Surfaces of Identical  
Cell Walls  
(X 36,000); a: Outer  
Surface; b: Inner  
Surface

TABLE 1. Chemical Constituents of Individual Fractions of Cell Walls from *Epidermophyton floccosum* Extracted with Acids and Bases

Structural Components	Untreated Cell Wall	1N NaOH	1N NaOH + 1N NaCl	4N Acetic Acid	4N Acetic Acid	Exo-layer + 1N NaOH
Neutral Sugars	46.3	31.4	3.1	43.1	26.2	11
Glucose	35.2	26.5	2.9	37.2	22.3	trace
Mannose	5.3	3.0	0	5.5	2.0	10
Galactose	trace	0	0	0	0	trace
N-acetylglucosamine	30.9	66.7	95.3	47.2	68.4	17
Peptides	10.3	0.6	0.3	1.7	0.3	63
Lipids	4.6	0	0	0	0	0

## 2) Chemical Constituents of Isolated Cell Walls

The chemical constituents of the purified fraction of cell walls separated by means of the above-mentioned method, as shown in Tabel 1, included about 80% sugar components, this being the primary constituent as is the case with the cell walls from other types of cells. Other than this, there were a variety of peptides and lipids. Moreover, it was shown that the chemical constituents of the cell walls of *T. mentagrophytes* exhibited almost the same trend [4]. With respect to the sugar components, glucose and glucosamine were the primary constituents, composing close to 70% of the sugars. On the other hand, with respect to the amino acid make-up, along with asparaginic acid, glutamic acid, proline, and glycine, there were the important ligands in which a sugar is added to alanine, namely serine and threonine. With respect to the lipid components, there were the phospholipids phosphatidaethanolamine and phosphatidalcholine and a number of neutral lipid ergosterols.

Next, having clarified the chemical constituents of the whole cell wall, in order to clarify the correlations between the structure of the constituents of the cell wall and the chemical constituents, study of the dissolved fractions, obtained by treating the purified cell walls with acid or bases, and the remaining fractions was carried out [5]. And in particular in the latter case, morphological observations were performed with the aid of electron microscopy. Changes in the chemical constituents of the cell walls which was obtained after the extraction with acids and bases are shown in Table I. The peptides and lipids were dissolved and removed by treating with the 1 N NaOH, and the proportion showing the stable acetylglucosamine increased. When examined with the aid of electron microscopy, mainly the changes in the inner /149 surface of the wall were obvious. The contours of the microfibriles became more distinct and the width of the fibers reached 15-20 nm. Furthermore, when this fraction was treated with 1 N NaCl, a fraction formed almost exclusively of acetylglucosamine was obtained. Morphologically, it appeared to be linkages of thick rod-like fibers. Because, it was identified as chitin by means of x-ray diffraction, this acid and base resistant fraction was judged to be chitin fibers. On the other hand, when the cell wall was initially treated with 4 N acetic acid, the fibrous structure became blurred, and a fraction was obtained in which microfibriles filled in within the amorphous space-filling substance. Furthermore, when this fraction was extracted with a base, a fraction with a small quantity of glucose (the successor to glucan) in it was formed. Morphologically, the microfibriles reappeared distinctly. These fibers differed from the ones initially formed with the treatment with the base and were generally narrow.

By the way, this type of fibrous structure appeared to be wholly without any import. It has been ascertained even morphologically that there are substances presenting a membranous structure on the outermost layer of the cell wall, and the fraction with this structure generated a lot of interest. Nozawa et al.



FIGURE 3. Electron Microscopic Image of "Exo-layer" (X 60,000)

compared this structure to the structure of other cell walls. It showed a peculiar resistant to *Helix pomatia* enzyme, and even after treating with this enzyme the membranous structure remained intact. This fraction was obtained by centrifugation and was named the "exo-layer" [6]. The electron microscopic presentation of this structure, as shown in Figure 3., shows a membranous structure, but the structure is not like that commonly found in biological membranes, the typical two-layer structure of a simple membrane. Even from the standpoint of the chemical analysis of the fraction, because no lipids were detected, this was not judged to be a so-called membrane structure. The characteristics of this structure were that of a type of sugar protein with the sugar portion composed only of mannose. According to disc electrophoresis, the molecular content of the primary peptide fractions (I-V) consisted of various molecules with molecular weights ranging from 42,000 to 154,000. Among these, fraction I (molecular weight, 154,000) was judged to be a sugar protein containing mannose which stained positively as a sugar. Also, with respect to the amino acid content, as a item of deep interest, it was found that the primary constituents were the amino acids which are seen often in bacterial cell walls, namely, asparaginic acid, glutamic acid, glycine, and alanine. However, while the biochemical significance of this unique outer layer structure is currently completely unknown, it is possible that it



may have some important function involving immunological activity. At any rate, both of these may be answered by the results of current research.

### 3) Structure of the Cell Walls of Dermatophytes

Based on the above-described changes in the structure of the cell walls treated with acids and bases and in the chemical constituents, the basic components of the structure of the basic cell wall was inferred. Accordingly, it is indicated that the cell wall of *Epidermophyton floccosum* is formed by the primary component, 2 layers, an inner and an outer, each approximately 0.1-0.2 microns thick, and of an outermost layer, a thin "exo-layer." Moreover, the outermost layer exhibits a coarse net mesh like structure composed mainly of sugar proteins; the outer layer present under this is formed of finely integrated microfibriles. These microfibriles (200 Angstroms) are suggested to be composed of a core region of chitin fibers surrounded by a layer of glucan, and all of this is covered with a base resistant, acid-dissolvable polysaccharide. The inner layer of the cell wall is thought to be formed from almost identical microfibriles and amorphous substances which are base dissolvable polysaccharides which fill in the spaces between the microfibriles.

However, this model of the components of this structure is no more than a tentative proposal, and a more detailed molecular structure should be shown by the current elucidation on the form of bonding of the polysaccharides. Recently, the results of the systematic chemical analysis of *T. mentagrophytes* cell walls by Poulain et al.[7] led them to report that the cell wall structure determined from the results matched the model proposed by Nozawa et al rather well. This suggests that there are similarities in the structure of the cell walls of both types of fungi. On the other hand, Nozawa et al have already discussed the similarities and differences with the model of the cell wall of *E. floccosum* from the standpoint of the analysis of the chemical components and the

morphological observation of the cell wall of *T. metagrophytes*, and the resemblances have been pointed out [8,9].

Next the most recent general remarks by Kitashima et al. [10] on details of such things as the cell walls of other types of fungi is presented.

### III. The Plasma Membrane as the Site of the Effects of Polyene Antibiotics

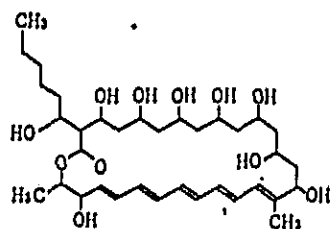
A method to separate the plasma membrane from dermatophytes with a high rate of recovery and a high degree of purity has not yet been established. Consequently, in order to understand the cytoplasmic membrane which incorporates many functions even in the layers on the surface of fungal cells, a very significant approach is to obtain information by introducing polyene antibiotics which have a particular localization of effect on this membrane. The effects of the polyene antibiotics on this membrane are described below.

#### 1) Chemical Structure of Polyene Antibiotics

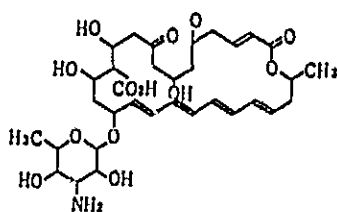
Since the discovery of the antifungal substances (the current Naistatin) produced by *Streptomyces noursei* which was separated from the soil of cattle ranches in Virginia by Hazen et al. in 1951, many polyene antibiotics have been reported. Actually, there are not a large number of varieties which can be put to practical service, and those are limited to only a few. Here, if one is to show a representative chemical structure, it would be like Figure 4. As the names indicate, there are many trans-type conjugated double bonds in the macrolide rings. Among the larger ringed molecules are naistatin and amphotericin B, and among smaller ones is filipin. Also, they are classified according to the number of double bonds. Included in the tetraenes are naistatin, pimaricin, and etholscomicin, in the pentaenes is filipin, and in the heptaenes is amphoteriin B.

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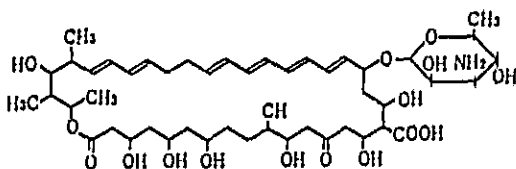
図4 代表的なポリエン抗生物質の化学構造



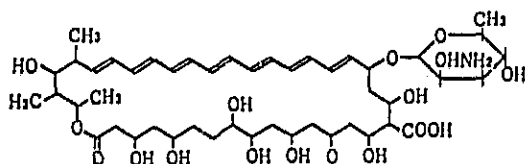
Filipin



Pimaricin



Naistatin



Amphotericin B

FIGURE 4. Chemical Structure of Representative Polyene Antibiotics

In all of these substances, as clarified by the structure, there are hydroxide groups on one side of the macrolide ring, and on the other side there are a series of double bonds; it is a so-called substance that alternates between both parents.

## 2) Morphological Changes in the Cytoplasmic Membranes of Fungi Due to Polyene Antibiotics

The creation of a "pit" structure when polyene substances are applied to a membrane containing sterols as membrane components has been clarified already by Kinsky et al. (1967) [11]. However, the question of whether or not the resulting "pit" structure observed by means of negative staining electron microscopy is the "gap" commonly found in membranes has been argued since that time. Since the introduction of freeze fracture electron microscopy, it has become possible to observe the structure of the channel region within the double-layers of living membranes. Thus, Nozawa et al applied various polyene antibiotics to *Epidermophyton floccosum* and observed the morphological changes. As a result, it was shown that changes in the distribution of the membrane protein particles (80-105 Angstroms) were induced, and this trend was particularly evident on the inner fracture face (PF) [12,13]. It is thought that the movement of membrane proteins and the change in the distribution pattern was brought about by the rearrangement of the membrane lipids. In other words, by means of the interaction of the polyene antibiotics and the ergosterols within the membrane, rigid and fluid areas were created in the lipids, and the membrane protein particles moved like icebergs from solid areas to fluid areas. Consequently, it may be permissible to think of this also as a type of phase separation. As shown in Figure 5, the morphological changes in the cytoplasmic membrane can vary according to the type of polyene antibiotic. These have been divided into 3 major types [14]. Type I is the "pit" form type like in the case of filipin; a consistent structure with 250-300 Angstrom diameter elevated areas can be seen on the PF surface, and 70 Angstrom diameter depressions can be observed in the center of the elevated areas. Because of this information, it has been suggested that the large gaps previously thought to be common in membranes do not exist. Actually, the correlation of the depression in the central area of the pits with the unusual

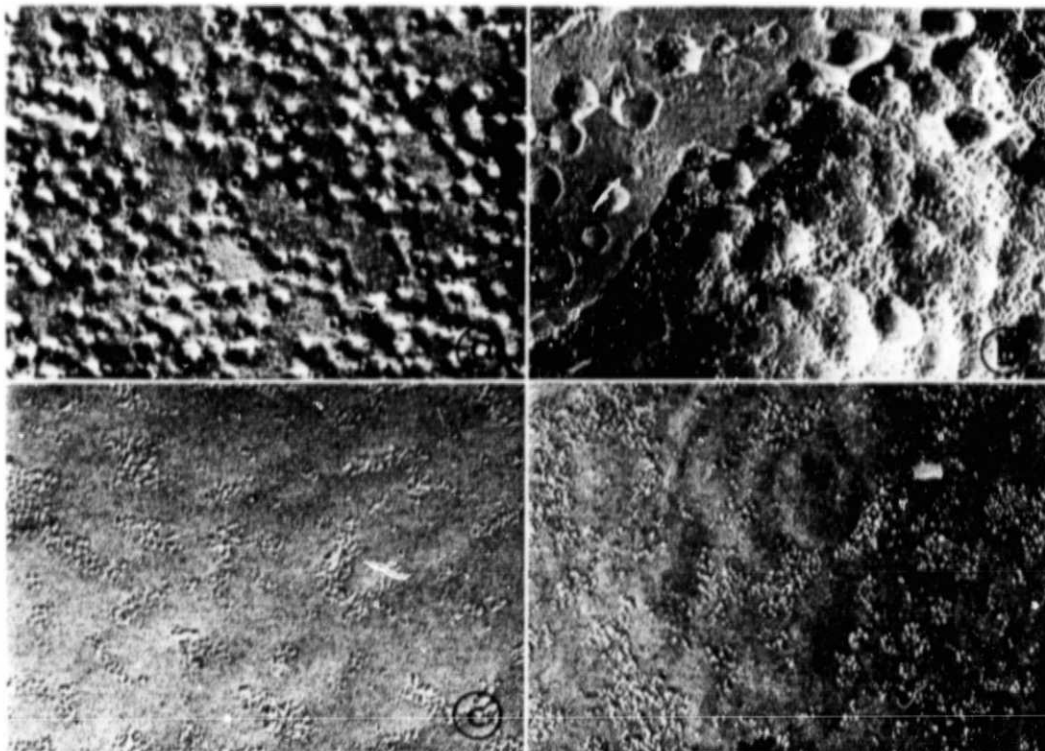


FIGURE 5. Morphological Changes in the Plasma Membrane of *E. floccosum* Due to the Polyene Antibiotics Shown in Figure 4. (X 80,000) a: Filipan b: Pimaricin c: Amphotericin B d: Naistatin

permeability of the inside and outside of the membrane can be adequately explained. Type II is the net mesh type of structure as in the case with pimaricin. On the PF surface there are many protein particles forming a net mesh shape on the periphery of a circular elevated structure that is deficient of membrane protein particles and has a diameter of 1,000 Angstroms. On the EF surface one can observe depressed areas which correspond to these protruding parts. And finally, Type III, is an irregular type, and is seen in the case of antibiotics as amphotericin B and naistatin; there is no pattern to the arrangement of the membrane proteins and one can only see agglutination of various large membrane proteins. Thus, the

creation of these characteristic changes in the morphology of the membrane due to various polyene antibiotics is a true reflection of the differences in the structures of the polyene sterol complexes within the cell membrane.

### 5) Changes in Plasma Membrane Function Due to Polyene Antibiotics

As stated above, obvious morphological changes in the structure of the membrane are the result of application of polyene antibiotics to the plasma membrane, and consequently there are corresponding changes in the function of the membrane [15]. That is to say, the major change is the promotion of the permeability of the membrane. Without looking at the low molecular weight polymers, leakage of /151 such things as high molecular weight polymer proteins and nucleic acid-related substances occurs; this eventually leads to cell death, and this demonstrates capability as an antifungal agent. As a mechanism of this leakage one must assume the presence of some form of a "pore." In actual experiments, by using calculations based on the many molecular-sized substances that leaked out when amphotericin B was applied to artificial lipid membranes, the size of the pores was determined to be in the range of 7-10.5 Angstroms. Many ideas have been proposed regarding the formation of the structure of the pores, and a comprehensive conclusion has not been seen yet. However, in the case of amphotericin B and naistatin, complexes of one molecule of amphotericin B and one molecule of sterol form, group together in rings of 8, and a half pore of one side of a membrane is formed. It is thought that a complete pore is formed when an identical structure on the other layer is joined with that on the first layer by means of hydrogen bonding. On the other hand, in the case of filipin, the process is completely different than this type of ring-shaped structure. The filipin and the sterol form a complex on the top and bottom in 1:1 proportions. This can be said to be a particular structure formed by two-step layering in sheets. However, compared to the above-mentioned pore structure, there is a poor foundation for backing this. At any rate, this

model of the complex of sterols and polyenes stops at the limits of conjecture, and it is necessary to have the backing of present methods of physical chemistry.

By the way, the particular interactions of polyene antibiotics and this type of membrane sterol are not the only basis for the direct affect on the variations in the membrane permeability. Recently, attention has begun to be paid to modifications in the function of various types of plasma membranes due to the loss or weakening of the function of membrane sterols based on these interactions. For example, changes in the activity of adenylic acid cyclase, acceleration of cell fusion, and other things have been reported. However, the details of such things will not be touched upon here.

#### IV. Chemical Structure of Formed Pigments and Their Function

It has been well known for a long time that various types of fungal cell produce pigmented substances. Even with respect to dermatophytes, since about 1964, a positive start in research, beginning with England and moving to the United States and Canada, has commenced. At the same time, Nozawa et al also began researching various problems such as the location of the production of pigments in dermatophytes, the chemical structure of the pigments, the function of the pigments, and the biosynthesis of the pigments. /152 Hereinafter, two or three of these topics will be elaborated upon.

##### 1) Site of the Production of Pigments

The separation of the intracellular organelles from *Trichophyton violaceum*, a strain which has a very marked production of pigments, was attempted, but due to the effort required to destroy the tough cell wall, the damage to the plasma membrane was marked, and eventually separation was deemed impossible. Initially it was determined that close to 60% of the whole pigments were

localized in a fraction obtained by centrifugation of a fraction at 20,000 X gravity for 30 minutes. Because the primary observation was a membrane structure thought morphologically to have originated in the mitochondria [16], it also suggested that it was also a quinone type pigment. It was considered that there also might be a potential correlation with the electron transfer system. Next, based on the electron microscopic observations of pigment producing strains and non-pigment producing strains of *T. violaceum* [17] and *Microsporum cookei* [17], it became clear that the pigmented substances were widely distributed on the cell surface. This led to thinking that the major portion of the pigments were excreted outside of the cell as the products of metabolism and then accumulated on the surface of the cell wall. In other words, the layer which used to be called the "dark outer coating" is thought of as the site where the pigmentous substances are present.

## 2) Chemical Structure of Individual Pigments

With respect to the pigments extracted from the cell walls of individual dermatophytes, a large number of methods were tried, and Nozawa et al finally carried out the extraction and purification using thin layer chromatography and column chromatography [19,20]. By 1970, the extraction of large quantities of pigments was achieved after making substitutions ranging from *Trichophyton* to *Microsporum cookei*, and since then the determination of the chemical structure has become easy [21]. When the pigments that were seen in the cell walls and the culture medium were each extracted by means of chloroform and acetic acid acidified chloroform and separated out by means of thin layer chromatography, five fractions (I-V) were obtained in the former situation and two were obtained in the latter situation. Then, the chemical structures of the individual fractions named aurosporin xanthomegnin, violosporin, citrosporin, rubrosporin, luteosparin, and iridosporin were submitted to investigation. Here, the chemical structures were determined, those of aurosporin, xanthomegnin, and luteosporin, are shown in Figure 6.



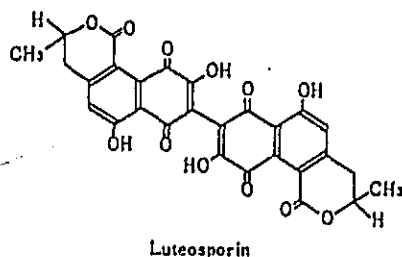
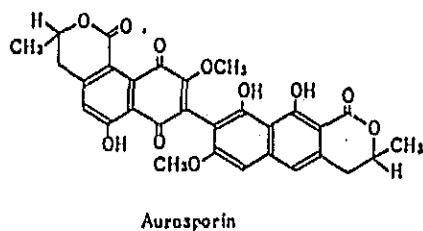
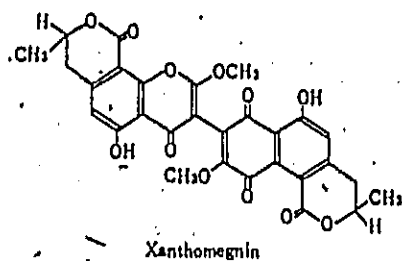


FIGURE 6. Chemical Structures of the Main Pigments

The thing that must be noted is that luteosporin was formed by means of the demethylization caused by treatment of xanthomegnin with sulfuric acid, suggesting an end point of the biosynthetic route. Moreover, when the correlation between the breeding of fungi and the serial pattern of the production of pigment was investigated, aurosporin and xanthomegnin were produced in the logarithmic proliferation stage, and furthermore it became clear that the quantity of violosporin, citrosporin, and rubrosporin increased in that order. With respect to the total quantity of pigment, the pigments which originated in the fungal cell body increased in parallel with the breeding of the fungi, and the pigments that

originated in the culture medium showed a trend of increasing during the quiescent stage. In order to understand the route of biosynthesis, it became necessary to do detailed investigation with the aid of substances labelled with isotopes. However, it is possible to obtain important information from the correlations based on the structures among the pigments by the determination of the chemical structures of the individual pigments, and currently other researchers are focusing attention on determining the structures.

### 3) The Biophysical Significance of Pigments - Centering on the Effects on the Function of Mitochondria

Very few explanations have been made concerning the biophysical significance of these pigments. According to one explanation, the covering of the cell surface with pigment is a response to protect the fungus from ultraviolet radiation, however the strength of this explanation is poor. Actually, the strains of fungi in which /153 the production of pigments is marked are said to be pathogenically strong. In fact, the color of the fungi becomes stronger directly after it is separated from patients, but when it is cultured serially, the capacity to produce pigments has been observed to show a decreasing trend. If one generalizes this to actual situations, there seems to be important meaning in the apparent correlation between the pathogenicity or the infectivity and the pigment, however, no resolute basis for this has been produced by researchers. Nonetheless, it is clear that quinone type pigments that are extracted from fungi have shown strongly harmful effects on the function of rat hepatic mitochondria. Initially, it was thought that the primary pigment xanthomegnin had a strong uncoupling effect on rat hepatic mitochondria at low concentrations ( $10^{-7}$  -  $10^{-8}$ M) [22,23], and it was shown that the same form of uncoupling effect occurred to differing degrees even with other pigments such as violosporin and luteosporin. Figure 7 shows the examples of xanthomegnin and luteosporin [24].

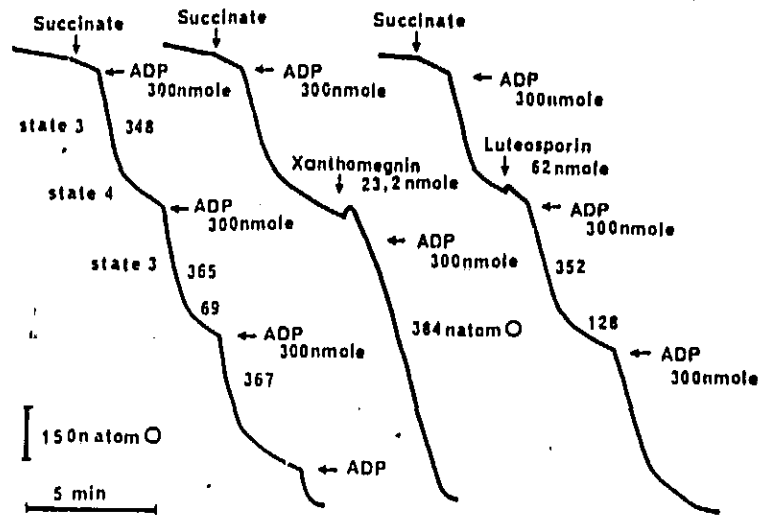


FIGURE 7. Effects of Xanthomegnin and Luteosporin on the Acidified Phosphoric Acidification of Rat Hepatic Mitochondria

On the other hand, the behavior of both pigments with regard to mitochondrial swelling differs. Xanthomegnin gives rise to marked swelling of mitochondria whereas that response does not occur much at all with the luteosporin. These things suggest some sort of correlation between the strength or weakness of the uncoupling response and the affect of mitochondrial swelling, and understanding of the mechanism of uncoupling offers some kind of information.

At any rate, the experimental facts that were obtained in vitro using pigment extracts do not clarify the function of these pigments, but perhaps there is some important significance which can be thought of from the existence of quinone type structures or which cannot be predicted even with respect to the inside of fungal cells. It is hoped that further research results will deal with even these questions.

## V. Conclusion

As mentioned even in the beginning of this study, it is not an exaggeration to say that research concerning the chemical and molecular level structure of the structure of the surfaces of fungal cells, particularly dermatophytes, has been relatively late in being established compared to that of bacterial cells. Moreover, the reason for this has been clarified by the fact that the isolation of the surface structure has been difficult and by other types of information. Moreover, while one can not say that great strides have been made recently, separation of the cell wall structure and chemical analysis have been performed and significant information has been obtained. In particular, the research on the structural constituents of the cell wall has been an important topic of research with respect to its relationship with the mechanism of fungal immunity and infection and with antifugicides. Active research on this topic is necessary in the future.

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## STANDARD TITLE PAGE

1. Report No. NASA-TM 77439	2. Government Accession No.	3. Recipient's Catalog No.
4. Title and Subtitle STRUCTURE AND FUNCTIONS OF FUNGAL CELL SURFACES		5. Report Date APRIL 1984
		6. Performing Organization Code
7. Author(s) Nozawa, Y. Dept. Biochemistry, Gifu University, Gifu, Japan		8. Performing Organization Report No.
		10. Work Unit No.
9. Performing Organization Name and Address Leo Kanner Associates, Redwood City, California 94063		11. Contract or Grant No. NASW-3541
		13. Type of Report and Period Covered Translation
12. Sponsoring Agency Name and Address National Aeronautics and Space Administration, Washington, D.C. 20546		14. Sponsoring Agency Code
15. Supplementary Notes Translation of "Shinkinsaibohyso no Kozo to Kino", Shinkin to Shinkinsho, Vol. 18, No. 3, 1977, pp. 147-155		
16. Abstract A review with 24 references on the biochemistry, molecular structure, and function of cell surfaces of fungi, especially dermatophytes: the chemistry and structure of the cell wall, the effect of polyene antibiotics on the morphology and function of cytoplasmic membranes, and the chemical structure and function of pigments produced by various fungi are discussed.		
17. Key Words (Selected by Author(s))		18. Distribution Statement Unclassified-Unlimited
19. Security Classif. (of this report) Unclassified	20. Security Classif. (of this page) Unclassified	21. No. of Pages 23
		22.