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IN VITRO THIOCARBAMATE RESISTANCE OF *TRICHOPHYTON MENTAGROPHYTES*

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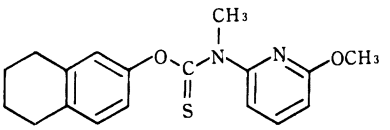
The *in vitro* resistance of *Trichophyton mentagrophytes* to piritetrate (M-732), a new thiocarbamate antidermatophytic compound, was studied in comparison with a reference thiocarbamate, tolnaftate. It was found that this fungus was not readily able to develop resistance to either of these compounds. After a long period of serial subculturing of six strains in Sabouraud dextrose broths and on Sabouraud dextrose agar slants containing increasing concentrations of compound, only one strain of each eventually became highly resistant to tolnaftate in either media. None of the strains acquired resistance to piritetrate. The two tolnaftate-resistant strains thus obtained manifested morphological, biochemical and pathological changes and showed partial cross-resistance to piritetrate.

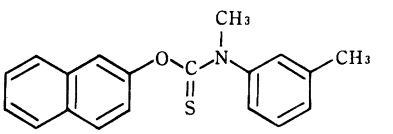
Key words : Piritetrate (M-732), Tolnaftate, Thiocarbamate resistance, *Trichophyton mentagrophytes*

INTRODUCTION

Piritetrate (M-732) is a new thiocarbamate compound [methyl (6-methoxy-2-pyridyl) carbamothioic acid *O*-5, 6, 7, 8-tetrahydro-2-naphthalenyl ester ; $C_{18}H_{20}N_2O_2S$] which had been synthesized by the Chemical Research Laboratory of the Tosoh Corporation (formerly, Toyo Soda Manufacturing Co., Ltd.), SHINNANYO-SHI, YAMAGUCHI-KEN, JAPAN. The structure of piritetrate is shown in Fig. 1, together with that of a reference thiocarbamate, tolnaftate [methyl (3-methylphenyl) carbamothioic acid *O*-2-naphthalenyl ester; $C_{19}H_{17}NOS$][1)]. Piritetrate is a white crystalline powder with a melting point of 98.5-99.5℃. It is insoluble in water, slightly soluble in *n*-hexane, methanol and ethanol, and readily soluble in benzene, ether, acetone, chloroform, dimethylsulfoxide and *N*, *N*-dimethylformamide.



Piritetrate (M-732)



Tolnaftate

Fig. 1. Structures of piritetrate (M-732) and tolnaftate.

The potent *in vitro* and *in vivo* antidermatophytic activity of piritetrate had been initially

identified in our screening program on a number of members of the thiocarbamate series which had been synthesized as agricultural herbicides by the Chemical Research Laboratory. Later[2], we described in detail the results of comparison of the *in vitro* antifungal activity of piritetrate, tolnaftate, tolciclate (a recently developed thiocarbamate compound ; [*O*-(1, 2, 3, 4-tetrahydro-1, 4-methanonaphthalen-6-yl-*m*-*N*-dimethylthiocarbanilate][3] and clotrimazole (a classic azole compound ; [1-{(2-chlorophenyl-) diphenylmethyl} imidazole][4,5], and found that piritetrate possessed the strongest antidermatophytic activity among these antifungals. In the same paper, we also demonstrated the more potent *in vivo* activity of piritetrate than tolnaftate in dermal model infections of guinea pigs with *T. mentagrophytes*. More recently[6], we reported the action mechanism of piritetrate compared with that of tolnaftate; the former was a much more potent inhibitor of fungal squalene epoxidation. This paper deals with the results of comparative *in vitro* resistance studies conducted on piritetrate and tolnaftate against *Trichophyton mentagrophytes*. (A preliminary report of this work was presented at the 24th Interscience Conference on Antimicrobial Agents and Chemotherapy, October 8 ~ 10, 1984, WASHINGTON, D.C.)

MATERIALS AND METHODS

Antifungals. Piritetrate and tolnaftate were supplied by the Tosoh Corporation. These compounds were dissolved in dimethyl sulfoxide (DMSO), and stock solutions of 20 mg/ml were kept at 4℃ in the dark. Twofold dilutions in test media were prepared from these stock solutions.

Trichophyton mentagrophytes strains. Six strains, namely, MTU numbers 19021, 19023, 19024, 19025, 19027 and VUT 74023, were used. These had been maintained in our laboratories by subculturing on Sabouraud dextrose agar (SDA; 2% dextrose, pH 6.5±) slants. None of them were known to have been exposed to drugs, including piritetrate and tolnaftate, *in vitro* or *in vivo*. Strain VUT 74023 had been isolated from a naturally-infected guinea pig and identified as *Arthroderma vanbreuseghemii* by Dr. HASEGAWA A, the Animal House, Faculty of Agriculture, University of Tokyo. Prior to the present study, we reconfirmed that this strain was highly virulent for guinea pigs upon dermal infection and produced the ascomycetous form. Later, it proved to be the only strain which developed resistance to tolnaftate. Strain MTU 19025, which had been isolated by us from a patient with tinea pedis, was representatively selected for a comparative study of primary resistance with strain VUT 74023. Strains MTU 19025 and VUT 74023 were highly susceptible to both piritetrate and tolnaftate, with each showing MICs of 0.01 μg/ml and 0.01 μg/ml in Sabouraud dextrose broth (SDB; 2% dextrose, pH 6.5±) and 0.02 μg/ml and 0.1 μg/ml on SDA, respectively.

Media. SDA and SDB were used for various tests. These will hereafter be simply called solid medium and liquid medium, respectively, unless otherwise specified. 'Ordinary Sabouraud dextrose agar' (hereafter referred to as ordinary SDA; 4% dextrose, pH 6.5±) was employed to obtain a sufficient amount of conidia for inoculation in tests on the morphological and biochemical properties as well as on the virulence of resistant mutants. For demonstration of the perfect stage of strain VUT 74023 and its subcultures, Takashio's medium[7] was used ; its ingredients are neopeptone (Difco) 0.1 g, dextrose 0.2 g, KH_2PO_4 0.1 g, $MgSO_4 \cdot 7\ H_2O$ 0.1 g, and Bactoagar 2.0 g, made up to 1,000 ml with distilled water.

Animals. Male guinea pigs, weighing ca. 300 g, were purchased from the Shizuoka Laboratory Animals Corporation, Ltd., SHIZUOKA, JAPAN and used for determination of the virulence of the subcultures in comparison with their sensitive parent strains.

Preparation of inoculum. An appropriate volume of saline containing 0.1% Tween 80 was poured onto the culture of *T. mentagrophytes* grown on ordinary SDA slants at 27℃ for 4 weeks, and the surface of the culture was scratched with a platinum loop to free the conidia. The conidium suspension mixed with a small amount of the hyphae was transferred into a flask and allowed

to stand for an appropriate period until small pieces precipitated. After filtering the suspension, excluding the precipitates through sterile folded gauze, the supernatant, containing almost pure conidia, was suspended in the same saline and adjusted to ca. 10^7 CFU/ml of conidia on the basis of an optical density of 0.35 at 540 mμ. Immediately prior to inoculation, the conidium suspension was shaken gently to ensure a homogeneous state.

Determination of MICs. The MICs of piritetrate and tolnaftate were determined by the broth and agar dilution methods. Conidia were inoculated into 5 ml of broth in L-form tubes and onto 20 ml agar plates containing twofold dilutions of antifungal. The inoculum sizes were ca. 10^3 CFU/ml and ca. 10^3 CFU, respectively. After incubation at 27℃ for one week in the liquid medium with shaking, and for 3 weeks on the solid medium, the MICs were read.

Testing for development of resistance. For development of resistance in liquid medium, the culture, which had grown fairly well at a subinhibitory concentration closest to the MIC, was gently crushed in a mortar to obtain a homogeneous inoculum suspension and adjusted to ca. 10^7 CFU/ml. The conidium suspension was then inoculated to a series of the same or a little higher twofold dilutions of the antifungal. Subculturing at 27℃ with shaking was performed every week for 27 weeks. At the time of transfer of solid medium cultures, conidia were harvested from colonies grown on plates containing the antifungal at a subinhibitory concentration closest to the MIC. Subculturing was carried out every 3 weeks, 27 times in total. The inoculum sizes in liquid medium and on solid medium were the same as those indicated in the determination of MICs.

Testing for demonstration of primary resistance. The existence and number of cells resistant to both compounds in the population of strains VUT 74023 and MTU 19025 were determined. About 10^9 CFU of conidia harvested from each strain after growth on ordinary SDA at 27℃ for 4 weeks were plated on plates containing 0, 0.01, 0.1 and 1 μg/ml of the compounds. The number of visible colonies formed after incubation for one week at the same temperature was counted.

Demonstration of back mutation. The two tolnaftate-resistant mutants which were eventually obtained were passed 10 times in liquid medium in the absence of antifungal. The level of resistance was checked after 5 and 10 passes to determine if back mutation occurred.

Demonstration of cross-resistance. Two resistant mutants and their parent strain (VUT 74023) were examined for cross-resistance to piritetrate and tolnaftate in terms of the MIC on plates containing appropriate concentrations of antifungal.

Testing for cultural and morphological changes in the resistant mutants. To examine for possible cultural and morphological changes, the mutants were incubated at 27℃ in liquid medium with shaking or on ordinary SDA plates, and their growth rates and colony forms were observed over a period of 4 and 6 weeks, respectively, in comparison with their parent strain. Cells of the mutants grown in ordinary slide culture using SDA at the same temperature were also compared with those of their parent strain by means of light microscopy. Possible loss of ascomycetomatous form was examined in cultures of the resistant mutants grown on Takashio's medium at 27℃ for 2 months.

Testing for changes in virulence of the resistant mutants. The resistant mutants were compared with their parent strain for their virulence in each ten of guinea pigs. An area (ca. 8 by 10 cm) on the dorsal trunk of each animal was shaved, and two circular areas 3 cm in diameter separated by an appropriate distance were gently abraded with sandpaper. About 10^6 CFU of conidia of these strains grown on ordinary SDA at 27℃ for 5 weeks were then inoculated onto these treated sites.

RESULTS

Development of resistance. Five *T. mentagrophytes* strains, i.e., 19021, 19023, 19024, 19025 and 19027, did not develop resistance to either compound in

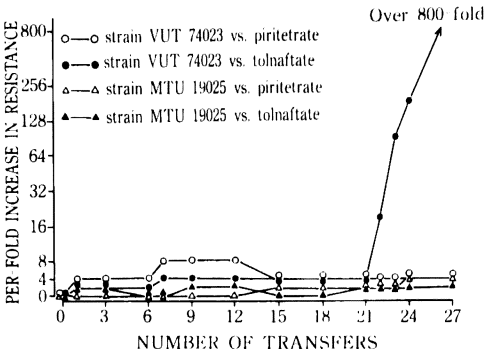


Fig. 2. Development of resistance of strains of *Trichophyton mentagrophytes* to piritetrate and tolnaftate in liquid medium. Initial susceptibility of strain VUT 74023 to piritetrate and tolnaftate was 0.01 and 0.1 μg/ml, while final susceptibility was 0.04 and >80 μg/ml. The respective figures for resistance of strain MTU 19025 were 0.01 and 0.1 μg/ml and 0.08 and 0.2 μg/ml.

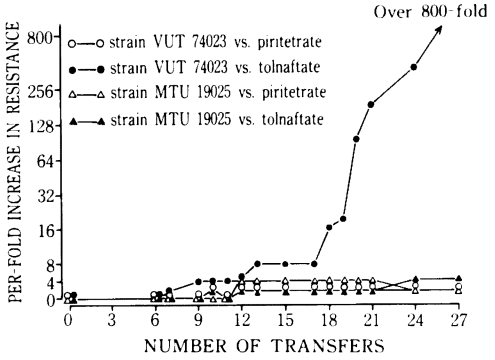


Fig. 3. Development of resistance of strains of *Trichophyton mentagrophytes* to piritetrate and tolnaftate in solid medium. Initial susceptibility of strain VUT 74023 to piritetrate and tolnaftate was 0.02 and 0.1 μg/ml, while final susceptibility was 0.08 and >80 μg/ml. The respective figures for resistance of strain MTU 19025 were 0.02 and 0.1 μg/ml and 0.04 and 0.04 μg/ml.

either medium. Their MIC values increased only 2- or 4-fold. Strains 19023 and 19025 showed a transient 8-fold increase in resistance to piritetrate and tolnaftate, respectively, in liquid medium. In contrast, only strain VUT 74023 developed high-level resistance to tolnaftate in both media. This strain developed gradual resistance to tolnaftate midway through the series of subculturing in liquid medium and progressive

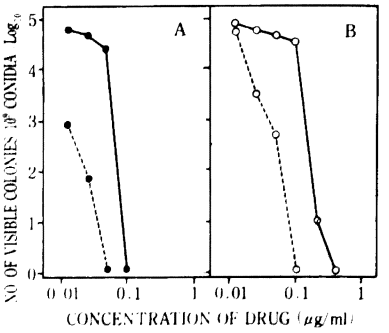


Fig. 4. Comparison of the distribution of piritetrate- or tolnaftate-resistant mutant cells existing in the sensitive strains of *Trichophyton mentagrophytes* VUT 74023 and MTU 19025. Ca. 10^9 CFU of the conidia of strains grown on SDA slants by incubating at 27℃ for 4 weeks were plated on SDA plates containing drug at 0.01 to 80 μg/ml and the visible colonies formed after incubating for one week at the same temperature were counted.
Symbols : A, piritetrate ; B, tolnaftate ; ——, VUT 74023 strain, ; ------ , MTU 19025 strain.

resistance to the drug in the late stage of serial subculturing on solid medium. These resistant progenies were designated L-1 mutant and S-1 mutant, respectively. We consider that both progenies reached the highest-level resistance, over an 800-fold increase, because they grew at 80 μg/ml, the highest tested concentration of tolnaftate and its maximum solubility in the media used (piritetrate also). For simplicity, Figs. 2 and 3 show the results for strain MTU 19025, selected as representative of the five strains which did not develop resistance, and strain VUT 74023.

Distribution of resistant mutant cells existing in the sensitive strains. Using about 10^9 CFU of conidium of strains VUT 74023 and MTU 19025, all the cells of strain MTU 19025 were sensitive to 0.1 μg/ml or less of both compounds, whereas relatively many cells of strain VUT 74023 were a little less sensitive to tolnaftate, with MICs between 0.1 and 1 μg/ml (Fig. 4). This difference in susceptibility to piritetrate and tolnaftate, although not very conspicuous, may evidence the fact that strain VUT 74023 eventually developed a high level of resistance to tolnaftate during the repeated subculturing in

Table 1. Degree of back mutation of L-1 and S-1 tolnaftate-resistant mutants of *Trichophyton mentagrophytes* VUT 74023

Organism	MIC of tolnaftate (μg/ml) after transfers (number) of :		
	0	5	10
L-1[a]	>80	>80	>80
VUT 74023 parent	0.08	0.08	0.08
S-1[b]	>80	2.5	1.25
VUT 74023 parent	0.08	0.08	0.08

[a, b] Resistant mutants obtained by serial transfers of the parent strain sensitive to piritetrate and tolnaftate in liquid and on solid medium containing increasing concentrations of drug (see Fig. 1.).

[a] L-1 : Tolnaftate-resistant mutant obtained in SDB ; tested for back mutation by serial transfers in drug-free SDB.

[b] S-1 : Tolnaftate-resistant mutant obtained on SDA ; tested for back mutation by 10 serial transfers on drug-free SDA.

Table 2. Piritetrate sensitivity of the tolnaftate-resistant mutants of *Trichophyton mentagrophytes* VUT 74023

Organism	MIC (μg/ml) of :	
	Tolnaftate	Piritetrate
L-1[a]	>80	5
VUT 74023 parent	0.08	0.02
S-1[b]	>80	10
VUT 74023 parent	0.08	0.01

[a, b] See the footnote to Table 1.

liquid medium and on solid medium, in contrast to the five other strains, including MTU 19025, which failed to develop resistance to either compound.

Stability of resistance. As shown in Table 1, L-1 mutant retained the highest level of resistance and was able to grow at a concentration of 80 μg/ml of tolnaftate even after 10 transfers, whereas S-1 mutant lost much of its resistance after 5 transfers. S-1 mutant, however, did not revert completely to its original susceptibility even after 10 transfers. Their parent strain had not changed from its initial susceptibility, the MIC 0.08 μg/ml.

Cross-resistance. The results of the test for cross-resistance on solid medium are presented in Table 2. There was slight but definite cross-resistance between piritetrate and tolnaftate in both L-1

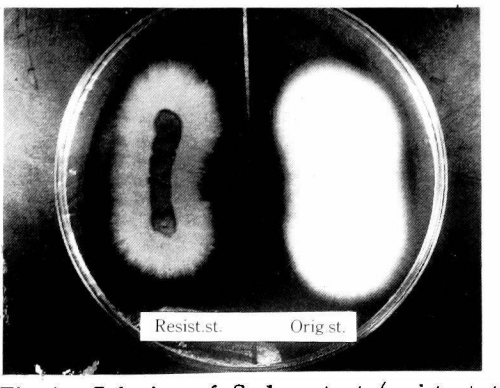


Fig. 5. Colonies of S-1 mutant (resistant to tolnaftate; left) and its parent strain (VUT 74023; right).
Note the pigment formation by the mutant.

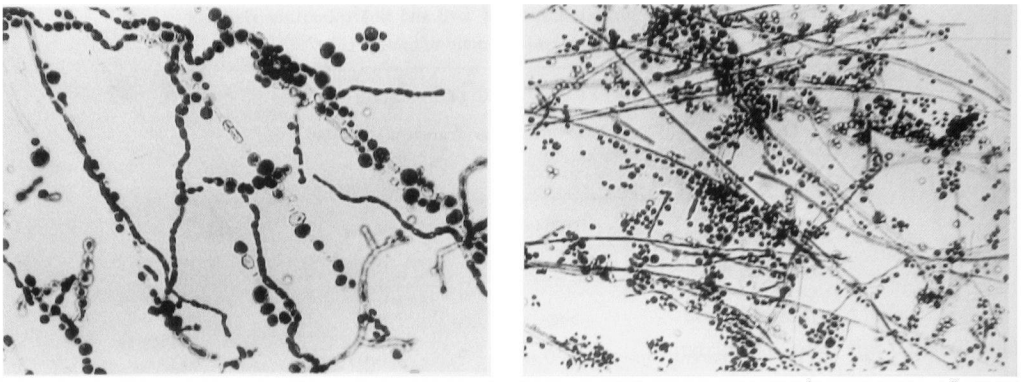


Fig. 6. Light microscopy of cells of S-1 mutant (resistant to tolnaftate ; left) (×400) and its parent strain (VUT 74023 ; right)(×400). Note the differences between the two strains in size and shape.

and S-1 mutants.

Cultural and cell morphologies of the resistant mutants. The growth rates and colony forms of the mutants, when incubated at 27℃ in liquid medium with shaking for one week or on solid medium for six weeks, were not significantly different, and did not differ from their parent strain. The principal change seen in the colonies of S-1 mutant was the formation of a reddish-brown insoluble pigment (s) ; the longer the incubation period, the deeper the colonies became in color tone. As seen in Fig. 5, the reverse of the area where the conidial suspension was inoculated by streaking onto a plate was much deeper in color than the obverse.

Microscopically, the mutants were characterized by the greater size of the conidia, i.e., ranging from 2.5 to 5 μm in diameter, compared with 1.5 to 2 μm in diameter for the parent strain, as shown in Fig. 6 for S-1 mutant. The shape of the conidia of the mutants was globose or ovoid, whereas the conidia of the parent strain were entirely globose. The width of the hyphae was 1 to 2 μm in the mutants and 0.3 to 0.7 μm in the parent strain. The hyphae of the mutants were often distorted and caused conspicuous segmentation that easily produced a number of aleuriospores of various size and shape. The formation of spiral hyphae was also more conspicuous than with the parent strain. Few or no macroconidia were formed by resistant mutants or by the parent strain.

Genetic property of the resistant mutants. Both resistant mutants incubated on Takashio's medium were found to have completely lost the ability to produce a perfect stage during the 3-month observation period.

Virulence of the resistant mutants. Ten guinea pigs given a dermal inoculation of ca. 10^6 CFU of conidia of the two mutants at shaved sites on the back showed no recognizable lesions at any of the 20 infected foci each in total of both groups, while the group of the parent strain gave clearly positive tissue and mycological response at 19 of the 20 infected foci under the same test conditions.

DISCUSSION

Most reports on resistance to antifungal agents in human pathogenic fungi indicate that naturally-occurring resistance is very rare, with the notable exception of resistance to flucytosine[8]. Moreover, the induction of resistant mutants or variants is much more difficult than with bacteria. Fungal resistance has thus received little attention, in contrast to the critical importance of bacterial resistance. Dermatophytes versus antifungals are no exception to this generalization; there have been only a few reports on their resistance, such as to griseofulvin[9-16] and to ambruticin (cyclopropylpyran acid)[17,18]. However, in view of the widespread and frequent use of antifungal agents in recent years, resistance studies have become important, particularly with newly exploited agents.

In this study we found that only one of the six test strains of *T. mentagrophytes* became resistant to tolnaftate after relatively many transfers, while none acquired resistance to piritetrate. These results suggest that this fungal species, and probably other dermatophytes as well, do not readily develop resistance to these thiocarbamate compounds. However, strain VUT 74023 might not necessarily be such an exception in its acquisition of resistance to tolnaftate in view of reports on primary and/or secondary resistance to other antifungals such as azoles in pathogenic fungi, including dermatophytes[4,5,19~21]. The possible existence of a few strains which are able to acquire resistance to such thiocarbamates during long-term therapy might be responsible for the occasional cases of unsuccessful treatment of dermatophytoses.

It is noteworthy that the acquired resistance of the tolnaftate-resistant mutants was three orders of magnitude greater than the parent strain. The test strains, including strains MTU 19021 and VUT 74023, transiently developed 8-fold or less resistance in liquid medium and on solid medium. However, such a transient rise in MIC values cannot be regarded as acquisition of resistance in the true sense, since evaluated MIC values do not exceed the upper limit of the MIC range. It seems likely that these multi-step mutants obtained by means of serial subculturing in the presence of increasing concentrations of tolnaftate obviously differ from one-step mutants resistant to flucytosine in their mode of acquiring resistance[8,22]. That the acquisition of resistance was more progressive on solid than in liquid medium might be due to various factors, for instance, the physiological conditions of both media for growth, the preparation of cell suspensions for inoculation, etc.

Overall, VUT 74023 and MTU 19025 showed similar cell population structural patterns in terms of susceptibility to piritetrate and tolnaftate before transfer. However, a culture of VUT 74023 included more cells less sensitive to tolnaftate than to piritetrate. This difference was considered small but not negligible since VUT 74023 eventually became resistant to tolnaftate but not to piritetrate by repeated subculturing in either medium.

The reasons why S-1 mutant became resistant earlier than L-1 mutant, yet L-1 mutant was more stable in the retention of resistance than S-1 mutant, are unclear. Possible factors are slight differences in the techniques for subculturing for development of resistance and for back mutation, or strain variation.

The finding that the cross-resistance of the tolnaftate-resistant mutants to piritetrate was partial may derive from differences in the chemical structures of these thiocarbamates and hence in their *in vitro* and *in vivo* antidermatophytic activity: piritetrate has a pyridine nucleus with a methoxy group and tetrahydronaphthalene moiety, while tolnaftate has a benzene nucleus with a methyl group and naphthalene moiety.

It is of particular interest that the acquisition of resistance of strain VUT 74023 to tolnaftate was accompanied by a characteristic pigment production in the matured colonies of its S-1 mutant. The pigment might be a carotenoid (s) in consideration of its color and insolubility in the solid medium. Changes in cellular morphology of the two resistant mutants were also characteristic, for instance, enlargement of conidia, striking segmentation of hyphae that produced a great many aleuriospores, accelerated formation of spiral hyphae, etc. These features might be due to qualitative or quantitative alterations in important cellular constituents such as nucleic acids and protein, as reported for fungal cells treated with flucytosine[8].

The loss of the perfect stage in the resistant mutants and their concomitant loss or reduction of virulence for guinea pigs upon dermal infection with conidia might be due to a lowering of their general metabolic activity. This 'loss mutation' was found to be irreversible in both mutants. Thus it is clear that these changes in morphological, physiological and pathological properties in the tolnaftate-resistant mutants were closely related to each other.

In summary, a new thiocarbamate compound,

piritetrate, and a reference thiocarbamate, tolnaftate, did not readily induce resistance in *T. mentagrophytes* strains *in vitro* by serial transfer both in liquid and on solid medium containing increasing drug concentrations. However, one of the six test strains became resistant to tolnaftate after many transfers. These results suggest the possible failure of long-term therapy with such thiocarbamates if resistant cells develop. The characteristic morphological changes, concomitant loss of the perfect stage and virulence, cross-resistance and stability of the acquired resistance of the two resultant tolnaftate-resistant mutants are described.

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Trichophyton mentagrophytes のチオカーバメート系抗皮膚糸状菌剤に対する試験管内耐性

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Trichophyton mentagrophytes のチオカーバメート系新抗皮膚糸状菌剤 piritetrate (M-732) に対する試験管内耐性について同族体 tolnaftate と比較研究し，両薬剤ともに本菌種に対し耐性を著しく与えにくいことを認めた。すなわち，被験菌株6株は，薬剤含有サブロー・ブドウ糖液体ならびに寒天培地の継代培養により両薬剤に対し耐性の上昇をみなかった。ただし，1株のみは両培地ともに比較的長期継代培養において tolnaftate に対してのみ高度の耐性を獲得した。これら tolnaftate 両耐性株は形態学的ならびに生化学的に著しい変化を起こしたのみでなく，病原性の消失をきたした。また，両株ともに piritetrate に対し部分的交差耐性を示した。

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