Detection of Xanthomegnin in Epidermal Materials Infected with *Trichophyton rubrum*

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Xanthomegnin, a mutagenic mycotoxin best known as an agent of nephropathy and death in farm animals exposed to food-borne *Penicillium* and *Aspergillus* fungi, was first isolated about 35 y ago as a diffusing pigment from cultures of the dermatophyte, *Trichophyton megninii*. This study investigates the production of xanthomegnin by the most common dermatophytic species, *Trichophyton rubrum*, both in dermatologic nail specimens and in culture. In view of the labile nature of xanthomegnin, a chromatographic procedure was developed to allow high-performance liquid chromatography analysis within 1 h of sample extraction. In cultures, *Trichophyton rubrum* produced xanthomegnin as a major pigment that appears to give the culture its characteristic red colony reverse. Xanthomegnin was also repeatedly extracted from human nail and skin material infected by *Trichophyton rubrum*. The level of xanthomegnin present, however, varied among the clinical samples studied. Xanthomegnin was not detected in uninfected nails. These results show that patients with *Trichophyton rubrum* infections may be exposed to xanthomegnin, although the consequences of such an exposure are not currently known. ***Key words: human dermatophytosis/mutagenic mycotoxin/nephropathy/*Trichophyton rubrum*/xanthomegnin. J Invest Dermatol 115:901–905, 2000***

Trichophyton rubrum is the most common agent of human dermatophytosis, and, in general, is one of the most common and economically significant microbial dermatopathogens (Summerbell and Gupta, 1999). One of the striking features of this fungus in pure culture is its intense pigmentation, usually blood red but occasionally yellow. The pioneering studies of Wirth *et al* (1965) and Blank *et al* (1963, 1966) showed that both colors were ascribable to different pH forms of a previously uncharacterized fungal metabolite, xanthomegnin, found in *T. rubrum* and the closely related pathogens *T. megninii* (from which the compound derives its name) and *T. violaceum*.

In recent decades the production of this compound by dermatophytes has been all but forgotten, and it is seldom mentioned in reviews of dermatophyte physiology. Over the same time period, however, xanthomegnin has become regarded as a significant animal toxin when produced in poorly stored feed materials by fungi in the mold genera *Aspergillus* and *Penicillium* (Carlton *et al*, 1973; Stack and Mislivec, 1978). Certain food-contaminating members of these genera have been shown to produce a high level of this compound both on natural substrates and in laboratory cultures (Hald *et al*, 1983; Scudamore *et al*, 1986; Frisvad, 1995). In animals, oral intake of xanthomegnin leads to kidney and liver abnormalities, often first presenting as jaundice (Carlton *et al*, 1976). Death may result. The compound has been shown to interfere with cellular respiratory processes (Kawai *et al*, 1976) and has given positive results in tests for genotoxic potential (Mori *et al*, 1984). This significant agricultural problem has engendered official protocols for the reduction of animal exposure to xanthomegnin and related fungally produced anthraquinone compounds. Both the US Food and Drug Administration (Stack *et al*, 1977, 1978; Carman *et al*, 1983, 1984) and European agencies (Van Egmond, 1991; Frisvad and Thrane, 1993; Muller and Boley, 1993; Frisvad, 1995) have promulgated such protocols. The aim of this study was to determine if xanthomegnin could be detected in human skin and nail specimens colonized by *T. rubrum*. There were two rationales. One was to conduct a preliminary investigation to determine whether there was a basis for regarding this distinctive compound as a potential diagnostic indicator in patient specimens. (The small number of food-borne mold species coincidentally producing the same compound does not include any verified human pathogens or commensal colonizers of human body surfaces.) The other was to determine if patients might be exposed, at some level, to this well known toxin when infected by *T. rubrum*.

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MATERIALS AND METHODS

Fungal cultures and epidermal specimens To obtain *in vitro* cultures of common dermatophytic species, two freshly isolated *T. rubrum* strains were grown as a hyphal lawn in a 15 ml Petri dish. The cultures were grown at 28°C on Sabouraud's CCG agar medium (10 g Bacto peptone per liter, 40 g glucose per liter, 15 g Difco agar per liter, 50 mg chloramphenicol per liter, 300 mg cycloheximide per liter, and 60 mg gentamicin per liter, where the last three antibiotic additions define CCG). For dermatologic specimens, portions of nail cuttings and skin scrapings received by courier at the Laboratories Branch of the Ontario Ministry of Health in Toronto were stored immediately at –70°C. The remainder of the sample was used for diagnostic culture and microscopic analysis for identifying the fungal species.

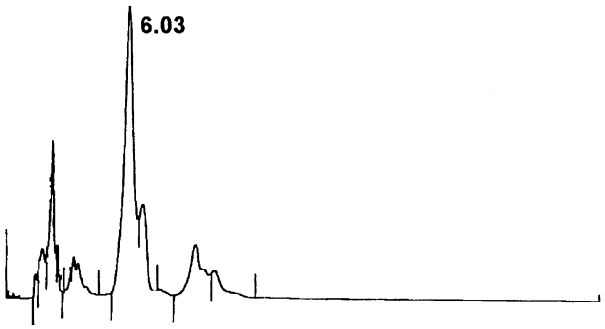

Figure 1. HPLC chromatogram of a xanthomegnin standard. A dried fraction of authentic xanthomegnin was dissolved in acetonitrile about 30 min prior to HPLC analysis.

Sample preparation and extraction For *in vitro T. rubrum* cultures, the entire fungal lawn grown for 4 wk on a 56.7 cm^2 agar plate was harvested for pigment extraction. The culture was ground to a paste with a pestle and mortar and extracted for 10 min with 100 ml of methanol–chloroform–acetic acid (50:50:1 vol/vol/vol). The extract was clarified by passing through a loosely packed layer of glass wool and the solvent removed at 40°C by vacuum evaporation. The oily residue was extracted with 50 ml of acetonitrile for 5 min, filtered through a layer of glass wool and the solvent removed by vacuum evaporation. The dried residue was mixed with 5 ml acetonitrile on a vortex mixer, allowed to settle in the rotary flask and the top layer transferred to a glass vial wrapped with aluminum foil. The total preparation time of *in vitro* culture extracts was less than 90 min.

The xanthomegnin analysis of the clinical skin and nail specimen was carried out within 7 d of its collection at the physician's clinic. After weighing, the sample was immersed in liquid nitrogen, powdered with a pestle and mortar and extracted in 25 ml of methanol–chloroform–acetic acid (50:50:1 vol/vol/vol). The extract was filtered through a layer of glass wool and dried at 40°C by vacuum evaporation. The dried residue was mixed with 0.5 ml acetonitrile on a vortex mixer, allowed to settle in the rotary flask for 2–3 min, and the clear top layer was gently transferred with a Pasteur pipette to the sample vial. The entire period of sample preparation was less than 45 min. Caution was taken to reduce the exposure to light during the entire period of sample preparation.

For reference, an authentic dried sample of xanthomegnin was obtained from Dr M. E. Stack, Food and Drug Administration, Washington DC, U.S.A.

High-performance liquid chromatography (HPLC) HPLC was performed on a Waters M-45 solvent delivery system with a Rhyeodyne injector, a 20 µl loop, and a Brownlee 4.6 mm × 22 cm C_8 (10 µm silica) reverse phase column. Detection was carried out with a Spectra Chrom 100 variable wavelength detector attached to a Waters 745 B integrator. The sample was monitored at 405 nm with an amplitude of 0.2 absorbance units full scale (a.u.f.s.) The column was equilibrated at 1 ml per min with a mobile phase of acetonitrile–water–KH_2PO_4 (55:45:0.14 vol/vol/wt) adjusted to pH 3.0 with phosphoric acid. All separations were carried out isocratically. Standard xanthomegnin solutions were prepared by dissolving a dried film of an authentic sample in acetonitrile about 30 min prior to HPLC analysis. The identification of xanthomegnin in nail samples was confirmed by agreement with the retention time of xanthomegnin in the most recent standard chromatogram.

RESULTS

The HPLC procedure used in this study eluted an authentic sample of xanthomegnin in about 6 min (**Fig 1**). The retention time and peak area of the xanthomegnin standard was monitored routinely after every 10 samples and at the start of each fresh batch of mobile phase. An examination of these standard xanthomegnin HPLC profiles revealed a shift of less than 0.5 min in the retention time and a variation of less than 10% in the peak area during the entire course of this study. There was no apparent change in the overall recovery of xanthomegnin from the column during this period.

The HPLC profiles of extracts from two *in vitro* cultures of *T. rubrum* showed the elution of several distinct compounds, including a major peak coeluting with xanthomegnin (**Fig 2**). The concentration of the secondary products was higher in culture

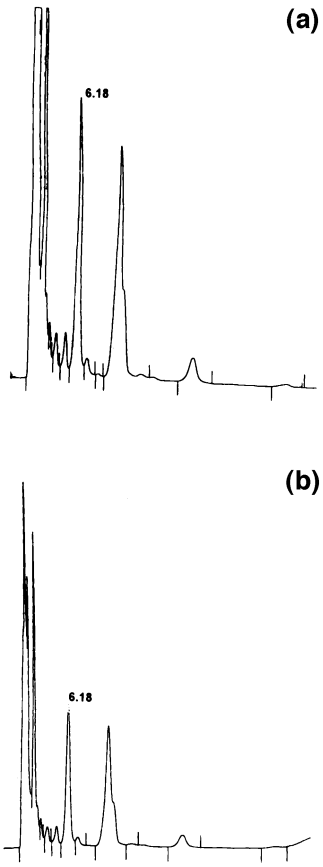

Figure 2. Separation of *T. rubrum* pigments by HPLC. Fungal lawns of clinical strains F-06317 (*a*) and F-06038 (*b*) were analyzed within 90 min of extraction.

F-06317 than in F-06038. The colony reverse of the former culture also appeared in visual examination to be more intensely colored than that of the latter. A comparison of HPLC profiles from the two cultures shows that they produced similar compounds, but at different levels.

Table I shows the variation in amounts of xanthomegnin seen in uninfected and infected nails, as well as infected skin, based on 20 µl fractions of 0.5 ml extracts loaded on to the column. It includes extracts from normal toenail specimens (normal A–J) obtained from 10 different healthy volunteers with no history of mycoses. In addition, extracts are included from 10 clinical toenail samples as well as two skin samples (from left buttock and an unspecified skin site), all from different individuals with conventionally laboratory-proven *T. rubrum* infection. No attempt was made in the preliminary investigation of skin specimens to analyze analogous normal controls, as completely undamaged skin does not yield the quantities of scale needed to perform a rigorously parallel study. As **Table I** shows, there was no detectable xanthomegnin (i.e., xanthomegnin below the HPLC lower detection limit of 2.5 mV per s) in normal nail material, whereas xanthomegnin quantities obtained from infected nails showed a more than 10-fold variation. Although part of this variation was attributable to different amounts of extractable material fortuitously becoming available from different infected nails, there was also strong variation in quantity per unit nail. For example, sample F-05903 with 44 mg extractable nail yielded a peak of 25.8 mV per s, whereas a 42 mg sample, F-06337, yielded a 350.2 mV per s peak.

Four of the 10 essentially identical HPLC profiles for normal nails are shown in **Fig 3**. **Figure 4** shows profiles representing the diversity among extracts of infected samples. Each profile prominently features the characteristic xanthomegnin peak at 6.00–6.5 min range, as seen in all infected samples. In addition to this peak, infected specimens showed a number of smaller peaks,

Table I. Specimens examined[a]

Specimen	Specimen weight (mg)	Fungus grown	Peak area response at 6.0–6.5 min elution time (mV per s)
Nail specimens			
Normal A	335	None	< 2.5
Normal B	252	None	< 2.5
Normal C	167	None	< 2.5
Normal D	87	None	< 2.5
Normal E	78	None	< 2.5
Normal F	65	None	< 2.5
Normal G	35	None	< 2.5
Normal H	44	None	< 2.5
Normal I	32	None	< 2.5
Normal J	25	None	< 2.5
F-00150	151	*T. rubrum*	225.2
F-00634	61	*T. rubrum*	169.4
F-04132	70	*T. rubrum*	491.9
F-04307	55	*T. rubrum*	405.3
F-05609	71	*T. rubrum*	104.9
F-05903	44	*T. rubrum*	25.8
F-06337	42	*T. rubrum*	350.2
F-07170	43	*T. rubrum*	10.5
F-07192	73	*T. rubrum*	23.1
F-08111	30	*T. rubrum*	31.1
Skin specimens			
F-05538	3	*T. rubrum*	7.6
F-06499	5	*T. rubrum*	17.4

[a]Normal toenail specimens were from volunteers with no symptoms of mycoses. Specimens designated with F-numbers were clinical toenail and skin samples sent to MOH laboratories for fungal identification. The threshold for peak area response was set at 2.5 mV per s.

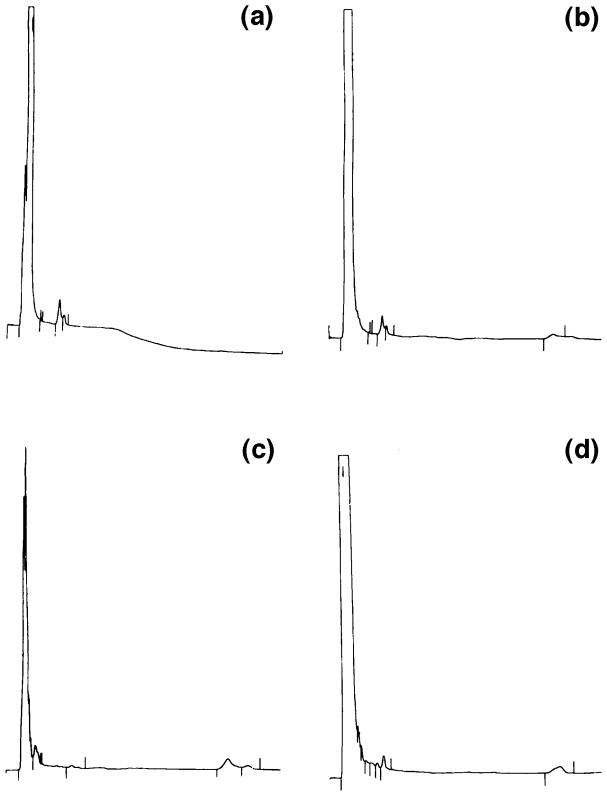

Figure 3. Separation of pigments from four uninfected normal nail samples. Pigments from powdered nail specimens were analyzed within 45 min of extraction.

particularly in samples where the amount of xanthomegnin was relatively high. These compounds, which were similar to those seen in profiles made from pure cultures of *T. rubrum*, were not characterized.

DISCUSSION

"Secondary metabolites" is a standard physiologic term for often unusual biochemicals produced abundantly by filamentous fungi (Griffin, 1993). With the exception of the earlier work of Smith and Marples (1964) demonstrating the presence of penicillin-resistant *Staphylococcus aureus* on the skin of hedgehogs infected with *T. mentagrophytes* as evidence for the excretion of penicillin by the fungal pathogen, there have been few studies on the possible excretion of secondary metabolites in fungal dermal infections. This study was directed towards the necessary initial step of optimizing the purification techniques for extracting one particular such compound, the mycotoxin xanthomegnin, from human dermal samples, and at the same time simply demonstrating that this material was produced by *T. rubrum* in at least some infected hosts *in vivo*. This study demonstrates the presence of xanthomegnin in nail and skin samples of patients diagnosed with *T. rubrum* infection.

With regard to technical aspects of purification, the detection and measurement of xanthomegnin have been problematic in the past, hindering the acquisition of information about this metabolite. It becomes unstable after extraction (Carman *et al*, 1983) and its reliable elution from chromatographic columns has also been difficult (Wall and Lillehoj, 1983). Over the past 20 y, a number of chromatographic procedures have been developed for the detection of xanthomegnin in grain-infesting mold species in the genera *Aspergillus* and *Penicillium* (Stack *et al*, 1978; Carman *et al*, 1983; Wall and Lillehoj, 1983; Muller and Bolley, 1993). These procedures are based on reverse phase HPLC with an acidified mobile phase (Frisvad and Thrane, 1993). Verification of the

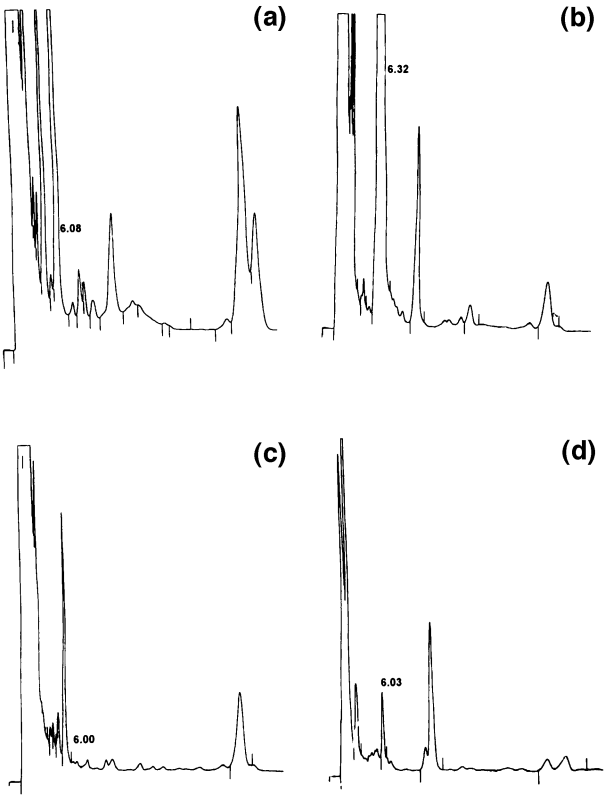

Figure 4. Separation of pigments from clinical nail samples infected with *T. rubrum* strains F-0150, F-04132, F-05609, and F-05903. (*a*) F-0150, (*b*) F-04132, (*c*) F-05609, (*d*) F-05903. Pigments from powdered nail specimens were analyzed within 45 min of extraction.

identity of xanthomegnin is currently assisted by the availability of reference material from the US Food and Drug Administration (Stack *et al*, 1978). Our study has employed these up-to-date procedures and quality control checks combined with an extraction technique optimized for obtaining xanthomegnin from human skin and nails.

Considerable precaution was exercised to ensure the presence of xanthomegnin in infected materials was not detected as an artifact. Although analysis could not be done directly in the dermatologist's office, sample material was sent by rapid courier to the laboratory and frozen at –70°C upon arrival. The total time, 24–48 h, was considerably less than that needed to induce the *de novo* production of secondary metabolites in fungal colonies *in vitro*. Therefore, even if, hypothetically, removal of substrate skin material from the patient induced a switch from pathogenic to saprobic growth in the dermatophyte, there was insufficient time for the fungus to enter the secondary metabolic phase of that growth prior to being frozen. Therefore, any xanthomegnin in the samples was produced during growth on the host. In reality, the most likely metabolic status of fungal elements in sampled skin and nail scrapings is long-term dormancy, mostly related to the very dry condition of these materials (Sinski *et al*, 1980). This further increases the likelihood that any secondary metabolites present derived from on-host growth.

There are various ways in which the production of such a mycotoxin in the host may be significant. The toxin may act strictly within the lesion as a local virulence factor, or it may have more far-reaching deleterious effects on the host, either through direct toxicity or mutagenesis. Alternatively, it may have no effect on host biology at all, but may aid the fungus in defending itself against bacterial or fungal competitors in dermal materials. Finally, it may exert no adverse effect against either the host or other microbes, but might fortuitously be a convenient ''signature'' indicator of the presence of the organism, and thus may be of use in determining the etiology of problematic cases. Each of these possibilities is briefly discussed.

The study of fungal secondary metabolites as virulence factors is in its infancy. The opportunistic pathogen *A. fumigatus* has been found to produce a metabolite, gliotoxin, which genetically damages macrophages by inhibiting transcription factor NFκB and causing DNA fragmentation similar to that seen with toxins known to induce apoptosis (Waring, 1990; Pahl *et al*, 1996). This metabolite has been found in naturally occurring and controlled laboratory animal infections (Sutton *et al*, 1996; Richard, 1997). Its damage to macrophages may be one of the factors making this species a particularly virulent agent of opportunistic pulmonary infection in the immunocompromised host (Sutton *et al*, 1996). *A. flavus*, another opportunist mainly invading the immunocompromised human host via the respiratory tract, produces secondary metabolites called aflatoxins, which impair alveolar macrophage phagocytosis (Jakab *et al*, 1994). Excretion of these compounds in human pulmonary infection has recently been documented (Mori *et al*, 1998). The potential pulmonary effects of aflatoxins have only recently been investigated, but these compounds have long been known as potent chemical carcinogens with particular affinity for hepatic DNA (Olsen *et al*, 1988). Xanthomegnin is derived from the same polyketide chemical pathway giving rise to aflatoxins. In animal feeding studies it has mainly been found to affect the kidney and liver (Carlton *et al*, 1976) but, as with the more extensively investigated aflatoxins, may prove to have other effects when investigated in the context of other tissues. In structure, it is a naphthaquinone, and fungal compounds in this group have been found to generate harmful activated oxygen species using electrons from the respiratory metabolism of bacterial competitors (Haraguchi *et al*, 1997). Whether elements of the cellular immune system might be similarly affected has not been investigated. Study of the interactions of xanthomegnin with the dermal immune components would be of interest.

The above-mentioned interference with bacterial metabolism may be significant to the fungus in human infections, especially in the microbially diverse nails and toewebs. In these sites, dermatophytic lesions are likely to suffer secondary invasion by aggressive, fungus-parasitizing *Pseudomonas* species such as *P. aeruginosa* (Molina *et al*, 1991). Although *T. rubrum* also secretes the lactam antibiotic penicillin *in situ* as a secondary metabolite (Youssef *et al*, 1979), most pseudomonads are resistant to lactams, and xanthomegnin may be an important part of the dermatophyte's ability to avoid destruction by these bacteria.

The ability of xanthomegnin to adversely effect animal organ systems, and its potential genotoxicity (Mori *et al*, 1984) raise the question of whether long-term exposure in humans with dermatophytosis may have adverse effects. Although the xanthomegnin produced in a nail may be more or less isolated from living tissue and the circulatory system, the extent to which this material may be absorbed from extensive skin dermatophytoses such as ''moccasin foot'' tinea pedis, where the whole surface of the soles of both feet may be involved, is an open question. The HPLC system used in our study allows the determination of xanthomegnin at a μg range (Wall and Lillehoj, 1983). In our study, the amount of xanthomegnin measured in a 20 μl injection was only a small fraction of the 500 μl extract prepared from each skin sample. As the total amounts of skin scraping extracted from specimens F-06446 and F-06499 were 4 and 7 mg, respectively, detection of μg quantities of xanthomegnin in these small specimens indicate a significant presence of this mycotoxin in the infected skin area. Although these amounts are not likely to cause serious kidney or liver disorders reported in farm animals (Carman *et al*, 1983; Wall and Lillehoj, 1983), the potential for considerable exposure over time in extensive skin infections is nevertheless not negligible. Many fundamental questions, however, such as the mobility of xanthomegnin in epidermal tissues, need to be answered before the significance of the compound can be estimated. It is interesting to note that dermatophyte infections are often associated with so-called ''id'' reactions giving rise to inflamed tissues at body sites far removed from the dermatophyte lesions (Svejgaard *et al*, 1976; Veien *et al*, 1994; Gianni *et al*, 1996); such reactions suggest that some dermatophyte components, including materials with antigenic activity, are mobilized into the circulation. Whether xanthomegnin is among them is unknown.

In animal ingestion studies, xanthomegnin has been listed as a class C toxin causing jaundice at 448 mg per kg of feed (Abramson, 1997). The relatively high quantity needed to have a discernible adverse effect in these studies may suggest that humans with dermatophytosis are not exposed to a significant quantity of this toxin, particularly as there is no known association between dermatophytosis and jaundice or kidney damage. It is not known, however, what proportion of xanthomegnin in feed is absorbed; nor is it known whether any of the compound's effects, such as its genotoxicity, may be cumulative.

Finally, the distinctive chemical nature of xanthomegnin, and the ability to detect it in few milligrams of skin scrapings or nail clippings using HPLC technology, raises the question of whether it could be used for rapid detection or identification of dermatophytosis. Certainly, no chemical known to be produced in humans resembles it as we completely lack the polyketide pathway by which it forms (Griffin, 1993). In our study normal nails yielded no coincidental HPLC peaks in extractions. The other fungi producing it, mainly the *Aspergillus ochraceus* and *Penicillium aurantiogriseum* group, have never been reliably reported from human skin disease (Summerbell, 1997), and the latter group lacks the ability to grow at body temperature (Pitt, 1979). Overall, the probability of xanthomegnin being found in skin or nail in any way other than through *in situ* production by infecting dermatophytes is not high. On the other hand, whether this compound will be found in detectable quantities in every *T. rubrum* lesion is not known, either for untreated lesions or for lesions where biosynthesis may have been disrupted by antifungal drugs. It should be noted, however, that on two occasions when frustrated physicians referred us specimens from nails where fungal filaments were seen in direct microscopy but a dermatophyte repeatedly failed to grow in

culture, high quantities of xanthomegnin were detected in the patient materials (data not shown). The possibility that this compound may assist in the diagnosis of dermatophytosis, at least in complex cases where precise information is needed, is worthy of further exploration, and follow-up studies are in progress.

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