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SCREENING OF MARINE INVERTEBRATES FOR THE PRESENCE OF ERGOSTEROL-SENSITIVE ANTIFUNGAL COMPOUNDS

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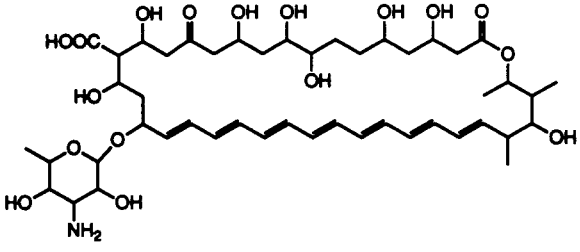
ABSTRACT.—A simple in vitro agar disk diffusion assay has been employed to detect the presence of ergosterol-sensitive antifungal activity in extracts of marine invertebrates. A collection of 116 marine sponges, ascidians, and cnidarians was assayed to reveal 10 samples (8.3%) with significant activity against *Candida albicans*. The antifungal activities of three of these extracts were unaffected in the presence of increasing concentrations of ergosterol, while another three were significantly reduced by ergosterol. The activity of jaspamide [**3**], a potent antifungal from the sponge *Jaspis* sp., was also reduced by ergosterol concentrations as low as 10 ppm. This ergosterol sensitivity is paralleled by the well-known ergosterol dependence of polyene antifungals, such as amphotericin B [**1**], and suggests a common mode of activity. The assay may be useful in mechanism-selective screening for new antifungals and as a dereplication tool.

Many new compounds possessing antibacterial and antifungal properties have been reported from marine sponges, soft corals, algae, bryozoans, tunicates, cnidarians, and other invertebrates (1). In recent years marine invertebrates have been recognized as promising targets in the search for new leads to antimycotic drugs and compounds. Fusetani (2) has tabulated the distribution of antibiotic activity in lipophilic and aqueous extracts of 568 species of marine invertebrates from Japanese waters against three species of yeast, *Mortierella ramannianus, Candida albicans,* and *Penicillium chrysogenum*. Of 282 sponges tested, 7.4% were found to be active, while 7.6% of cnidarian species displayed antifungal activity against one or more test organisms. Earlier screening (3,4) of marine invertebrate extracts from the Caribbean revealed similar levels of activity against *C. albicans*. The chemistry of 62 antifungal natural products from marine invertebrates was reviewed in 1988 (2); however, many more antifungal compounds have been added to this list since then (1).

The question remains whether bioassay-directed screening of sessile marine invertebrates may uncover new leads to antifungal agents effective in the treatment of disseminated mycoses in humans. In the past, in vitro screening for antifungal compounds has been successful in identifying new clinically important antifungals, including amphotericin B [**1**]. In our search for new antifungal agents from marine invertebrates we felt it would be useful to provide early evidence of the mechanism of action of new antifungals. This may increase the likelihood that a novel antifungal compound may represent a new lead with possible therapeutic promise. We sought a selective bioassay which targeted particularly susceptible components of fungal cell structure or metabolism. In this report we describe a screen based on a simple susceptibility assay



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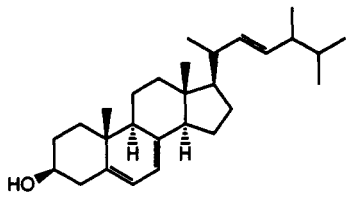

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which selects marine extracts with amphotericin-B-like activity against *C. albicans*, a common human pathogen. In addition, we have elucidated a possible common mechanism of activity, similar to that exhibited by amphotericin B, for a known marine antifungal cyclodepsipeptide.

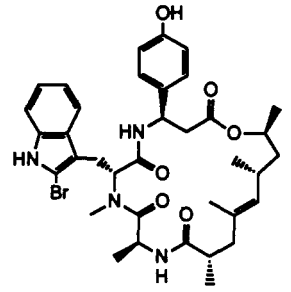
The polyene antibiotics, such as amphotericin B [**1**], are selectively toxic to yeast cells. Amphotericin B binds selectively to ergosterol [**2**], present in yeast cell membranes, leading to spontaneous self-assembly of ordered channels or pores (5). These channels, permeable to potassium and other cytosolic compounds, lead to collapse of membrane potential, lysis, and cell death. Mammalian cells, containing cholesterol instead of ergosterol, are less susceptible to polyenes. This differential sterol affinity, is presumably the basis of polyene selectivity. In order to explore this attractive combination of polyene-like activity with non-polyene chemical structure, we have sought to detect polyene-like activity in marine-derived antifungal extracts. New fungicidal agents, with membrane-ergosterol activity similar to that of amphotericin B but without the toxicity associated with polyenes, may have therapeutic utility in the treatment of AIDS-related systemic mycoses.

EXPERIMENTAL

MATERIALS.—Cholesterol, miconazole, and amphotericin B [**1**] were obtained from Sigma Chemicals and used as supplied. Ergosterol [**2**] (Sigma) was recrystallized from EtOH. Jaspamide [**3**] and specimens of *Hexabranchus sanguineus* (collected from the Fiji Islands in 1987) were generously provided by Professor Chris Ireland, University of Utah. Kabiramide C [**4**] was isolated from *He. sanguineus* by using standard techniques [unpublished results, T.F. Molinski; cf. Experimental section in Kernan *et al.* (15)]. *C. albicans* was obtained by subculture of a patient isolate (UC Davis Medical Center), and all other microbes were from the ATCC. All microbiological manipulations were carried out using standard sterile procedure (9).

STEROL DEPENDENT DISK DIFFUSION ASSAY.—A simple agar disk diffusion assay for antifungal susceptibility was modified in the following way. Tryptic soy agar (TSA) plates were prepared from bactoagar (4% w/v), tryptic soy digest (3% w/v, sterol free by ^{1}H nmr and tlc), and deionized H_2O, together with different concentrations of ergosterol. Ergosterol was added from stock solutions in EtOH to the warm agar mixture and homogeneously dispersed by vortexing or vigorous stirring prior to autoclaving and pouring into Petri plates. The EtOH concentration was kept constant 1% (v/v) before autoclaving. Final sterol concentrations are reported in ppm (w/v). Control plates were prepared containing 1% EtOH but without sterol. Agar plates prepared both with and without 1% EtOH and tested with various antifungal agents showed no difference in zones of inhibition. All assays were performed in duplicate. The plates were spread evenly with a lawn of *C. albicans* at the beginning of the incubation. Sterile paper disks (6.5 mm diameter, Difco) were saturated with 15 μl of standard solutions of crude extracts or pure antifungal compounds and allowed to dry. Disks saturated with solvent and dried in the same manner were used as control. The plates were inverted and incubated overnight at 37° (16–19 h), and the diameters of the clear zones of inhibiton were measured to the nearest 0.5 mm. Error was estimated from measurements of duplicate plates of *C. albicans* incubations with amphotericin B over a range of dosages.

DOSE RESPONSE.—Dose response curves were determined by the addition of measured doses of antifungal compound (determined volumetrically) to sterile blanks followed by incubation on TSA plates, as



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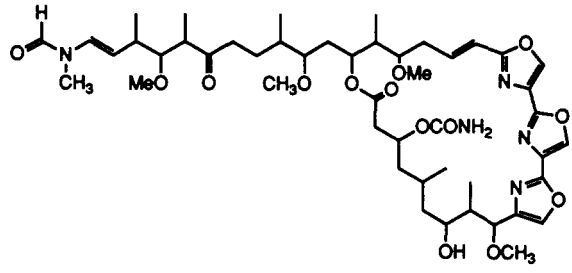

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above, and measurement of the resultant zones of inhibiton. It was found that the zones of inhibition were approximately proportional to $\log_{10}$ of the dose for amphotericin B, jaspamide, and kabiramide C (Figure 1).

STEROL DEPENDENCE OF ANTIFUNGAL COMPOUNDS.—A dose of the test substance was chosen to produce a zone of inhibition at least twice the diameter of the sterile blank. Zones of inhibition were measured at constant dosage of antifungal extracts and pure compounds in the presence of increasing ergosterol or cholesterol in the TSA medium. Activity was calculated as percent of control by dividing the zone of inhibition given by the test substance by the corresponding zone produced by the test substance on a control plate containing no sterol (see Figures 2–4).

COLLECTION OF MARINE INVERTEBRATES AND PRE-TESTING FOR ANTIBIOTIC ACTIVITY.—Marine invertebrate samples ($N = 116$), coded and cataloged, were collected using scuba on the Great Barrier Reef (16°S, 146°E) and in Port Phillip Bay (37°50′S, 145°E), Australia, in February 1990. Individual samples were immediately frozen on dry ice, transported to Davis, California, and stored at −30°. Voucher specimens are archived in the chemistry department, University of California, Davis. A portion of each sample (approximately 1–5 g) was cut and soaked in EtOH for 48 h. The solvent was removed in a centrifugal vacuum evaporator and the weighed residues made up to 20 mg/ml in MeOH. The MeOH solutions were tested at 300 μg per disk as described on TSA plates containing no sterol against *C. albicans*, *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* DH1 (ATCC 33849).

RESULTS AND DISCUSSION

The binding of sterols to polyene antifungals has been the subject of extensive study (6,7). Zygmunt and Tavormina (8) demonstrated that exogenous ergosterol added to broth cultures of *C. albicans* completitively binds to amphotericin B, reducing the available concentration of polyene in the medium, resulting in apparent diminished activity. We have employed a variation of this competitive binding experiment using a simple antifungal susceptibility assay which uses the disk diffusion method on agar plates treated with known concentrations of ergosterol. A disk diffusion assay was chosen as a compromise over the more rigorous but time-consuming broth dilution method (9) in order to expedite the rapid screening of hundreds of samples while still obtaining biologically relevant results and to keep consumption of limited samples to a minimum

(the present method can be run with as little as 1 mg of extract). Using this method we have screened crude extracts of marine invertebrates and pure antifungal compounds to detect polyene-like activity and found ergosterol-dependent activity in a large number of our extracts. We have also discovered ergosterol-dependent activity in jaspamide [**3**], a potent antifungal cyclodepsipeptide first isolated from the pacific sponge *Jaspis* sp. in 1986 (10,11). Amphotericin B [**1**], jaspamide [**3**], and kabiramide C [**4**] all exhibited activity against *C. albicans* with roughly $\log_{10}$ dependence of activity (zone of inhibition in mm) versus dose in μg (Figure 1).

Figure 2 shows the results of ergosterol-dependent antifungal activity of two marine natural products displaying potent activity against *C. albicans*: jaspamide [**3**], the first member of a series of modified cyclodepsipeptides incorporating a unique polypropionate hydroxyacid moiety (10–13) from the sponge *Jaspis* sp., and kabiramide C [**4**], isolated from the egg ribbons of the spongivorous dorid nudibranch *He. sanguineus* (14) and also from the sponge *Halichondria* sp. (15). Both jaspamide and kabiramide C are inactive against Gram-positive and Gram-negative bacteria (T.F. Molinski, D.J. Faulkner, and M.K. Harper, unpublished results), but both inhibit *C. albicans* in the disk diffusion assay at 1 μg or less. In the agar disk diffusion assay, compound **4** is more potent than **2** or **3**.

Compounds **1**, **3**, and **4** were tested for the effect of ergosterol. In the presence of 20 ppm of ergosterol, amphotericin B [**1**] activity is reduced to 50% (Figure 2). This is interpreted as competitive association of **1** with added ergosterol, leading to reduction of available **1** and attenuation of antifungal activity. In the presence of increasing concentrations of ergosterol, the activity of kabiramide C [**4**] was undiminished, suggesting a mechanism of action for **4** unrelated to membrane ergosterol binding. Since it has been shown that **4** has potent activity against murine L1210 leukemia cells (14) and acute toxicity in mice (15), these results suggest that another mechanism of cytotoxicity toward eukaryote cells is operative. This is a subject of study in our laboratories. In parallel assays, the activity of miconazole was shown to be undiminished by ergosterol at concentrations of up to 100 ppm, the highest used in this study (data not shown).

This is consistent with the different mechanism of action displayed by miconazole,

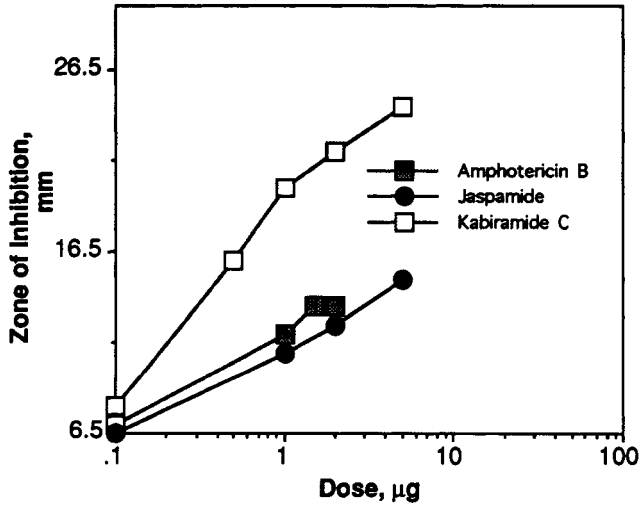

FIGURE 1. Dose response of disk diffusion anti-*Candida albicans* activity. Zone of inhibition versus dose for amphotericin B [**1**], jaspamide [**3**], and kabiramide C [**4**].

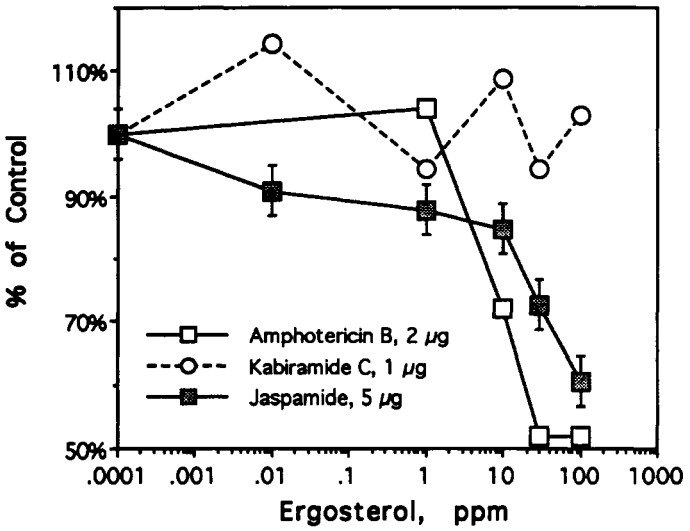

FIGURE 2. Ergosterol dependence of anti-*Candida albicans* activity: Amphotericin B [**1**], jaspamide [**3**] and kabiramide C [**4**].

which blocks the synthesis of ergosterol by inhibiting cytochrome-P450-dependent C-14α demethylase (16).

The antifungal activity of kabiramide [**4**] was undiminished with increasing ergosterol concentration. Jaspamide [**3**] activity, on the other hand, was progressively reduced to 50% in the presence of 120 ppm of ergosterol (extrapolated). Qualitatively, this is the same effect seen for amphotericin B [**1**], although the sensitivity of **3** to ergosterol is lower. Jaspamide activity would appear to be correlated with its binding to ergosterol and, like **1**, it may also interfere with sterol-membrane integrity. This explanation is consistent with the findings of Matthews and co-workers (17) who showed that **3** is growth inhibitory and fungicidal towards *C. albicans* and several other fungi at the same concentration, a property shared by **1** but not by miconazole.

We considered the possibility that exogenous sterol used in the agar may alter yeast cell membrane physical properties or permeability and therefore influence the assay results. Membrane sterol concentrations in several strains of *C. albicans* typically amount to about 5% (50,000 ppm) of the dry cell weight (7). At this concentration it is improbable that the low concentrations of exogenous ergosterol employed in the agar would be sufficient to alter susceptibility of the yeast cells themselves to antifungals.

Having demonstrated selectivity in our disk diffusion assay for known antifungal compounds, we next examined crude extracts of marine sponges. Screening of a recent collection of 116 specimens of marine sponges and tunicates collected from the Great Barrier Reef and temperate waters of southern Australia revealed antifungal activity in 10 extracts (8.3%). Of these extracts, here denoted by their accession numbers, nine demonstrated selective activity against *C. albicans* but not *S. aureus, P. aeruginosa,* or *E. coli*. These were tested for ergosterol sensitivity (Figure 3), and extracts of the sponges 90-026 (*Jaspis* sp.) and 90-080 both showed activity with a strong ergosterol dependence, comparable to that of amphotericin B. We have now isolated the active compounds present in 90-026 and have shown that they are, indeed, responsible for the sterol-dependent anti-*Candida* activity of the crude extract. Description of the isolation and structure elucidation of these compounds will be reported in due course. Both were progressively suppressed by ergosterol up to 100 ppm (extrapolated). Extracts of the

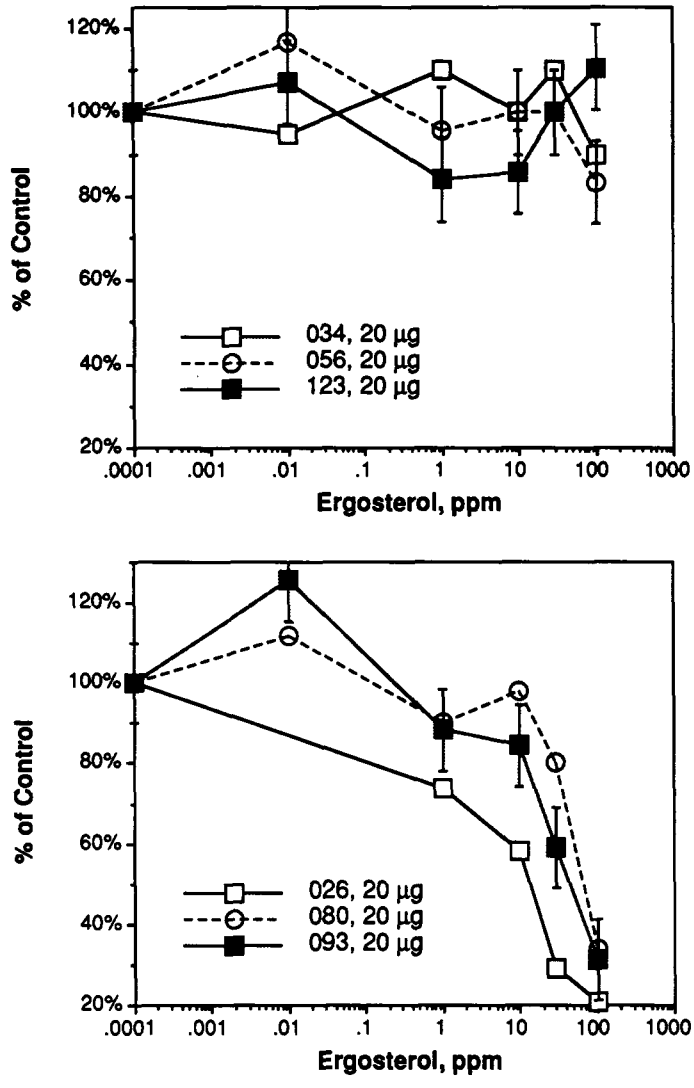

FIGURE 3. Ergosterol dependence of anti-*Candida albicans* activity; antifungal crude extracts from marine invertebrates.

sponges 90-123 (*Latrunculia* sp.), 90-034 and 90-056 showed no ergosterol dependence of anti-*Candida* activity. It is of interest that antifungal norsesterterpene peroxides have been reported from *Latrunculia* sp. from Australia (18,19). Jaspamide is the only antifungal agent reported to date from *Jaspis* sp. (10,11). However, preliminary nmr spectroscopic examination of extract 90-026 did not show the presence of this compound.

On the other hand, sterol selectivity is not observed in the anti-*Candida* activity of crude extract of 90-080; both cholesterol and ergosterol were equally efficient at suppressing antifungal activity. We next examined the effect of sterol structure on the relative sterol dependence of anti-*Candida* activity. When the activity of 90-026 was measured with substitution of ergosterol for cholesterol a dramatic difference was observed (Figure 4). Cholesterol showed a decreased ability to suppress the antifungal activity of 90-026. At a sterol concentration of 1 ppm cholesterol, the activity of 90-026 had di-

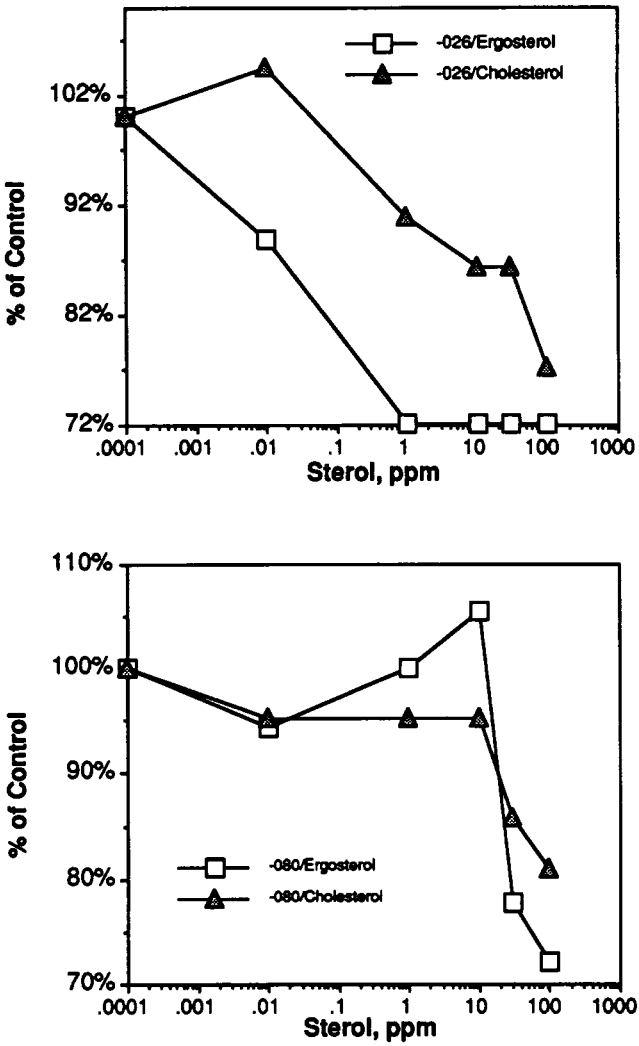

FIGURE 4. Anti-*Candida albicans* activity of selected crude extracts 90-026 and 90-080; differential sterol dependence.

minished to only 90%, but in the presence of ergosterol, activity had dropped to approximately 72%, suggesting that the active component in 90-026 has a higher binding affinity for ergosterol than cholesterol. Ergosterol suppressed the activity of 90-026 at concentrations one order of magnitude lower than that obtained for cholesterol.

The preferential binding of extract 90-026 for ergosterol versus cholesterol is analogous to the properties of amphotericin B. Clejan and Bittman (20) have shown, using uv spectroscopy and cd, that amphotericin B in solution has a similar differential binding affinity for ergosterol versus cholesterol. A comparison of sterol-modulated antifungal activity of crude extracts as a function of sterol structure may serve as a powerful tool for dereplication of known antifungal compounds. For example, the detection of high ergosterol-sensitive activity in a crude extract may automatically eliminate previously identified compounds from marine sources with sterol-dependent antifungal activity which is indifferent to sterol structure. In order to test this as a method of dereplication we intend to examine a larger sample base and investigate the influence of native lipid and sterol content in the extract on in vitro antifungal activity. It is noteworthy

that $\Delta^{5,7}$ marine sterols are moderately common in marine extracts. These sterols, like ergosterol, may suppress antifungal activity and mask the presence of antifungal activity in in vitro assays.

A simple disk diffusion assay has been employed to screen for compounds with sterol-dependent anti-*C. albicans* activity. These results demonstrate the utility of this assay in screening of crude extracts and has identified target extracts in our program for the discovery of sterol-dependent antifungal compounds. The activity of some antifungal marine extracts was confirmed to be sensitive to the structure of the added sterol, comparable to amphotericin B and other polyenes.

This simple assay not only provides a method to identify polyene activity in antifungal extracts, but also identifies extracts whose antifungal action may be due to independent and possibly new modes of activity. Ongoing work includes the isolation and structure elucidation of the active components of the antifungal extracts with priority given to those with sterol-dependent activity. This sterol-dependent antifungal assay may find useful application as a dereplication tool in the search for new antifungals.

ACKNOWLEDGMENTS

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