Full Papers

A New 2D-TLC Bioautography Method for the Discovery of Novel Antifungal Agents To Control Plant Pathogens

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A new bioassay has been developed combining the simplicity of direct bioautography with the improved chromatographic resolution of 2D-TLC. Mixtures of structurally diverse antifungal agents were tested to establish the validity and utility of this method in the discovery of new natural products with activity against agriculturally important fungal pathogens.

The past three decades have seen a dramatic increase in pathogen resistance to both agrochemical and pharmaceutical fungicides. In agriculture this translates into the need for repeated applications of chemicals to crops or the insufficient control of plant diseases. In addition, a major public concern is the need for safer and less environmentally harmful agrochemicals. New prototype antifungal agents are needed to address this situation. These agents must function by new modes of action, different from those of existing synthetic agents, to avoid cross-resistance with currently available antifungal products.[1,2] Successful discovery of novel natural product fungicides has necessitated the development of new bioassay techniques and protocols that allow for the detection of small amounts of biologically active chemicals, which should be selective enough to determine optimum target pathogens, and amenable to the analysis of complex mixtures. Two sensitive detection systems have been used by our laboratories to screen for new antifungal agents: a high-throughput 96-well microbiassay[3] and one-dimensional direct bioautography assay[4] to study antifungal agents effective against *Colletotrichum* and *Phomopsis* species. Moreover, bioassays for agricultural antifungal agents usually require specialized equipment and are not well suited for use in natural products chemistry laboratories. Herein, we describe a new 2D-TLC-based directed bioautography method that uses plant pathogenic fungi as antifungal indicators, is easy to run, requires no specialized equipment, and is well suited to chemically complex natural product-rich samples.

The strobilurins, inspired by a group of natural products produced by wood-inhabiting forest mushrooms (*Strobilurus tenacellus* (Pers. ex Fr.) Sing. and *Oudemansiella mucida* (Schrad.) Höhn.), are now available commercially as azoxystrobin (**1**) and kresoxim-methyl.[5] Azoxystrobin (*β*-methoxyacrylate) was selected from 1400 compounds synthesized by one company and based on these naturally occurring antifungal products. It has high levels of fungicidal activity, a broad-spectrum activity, low mammalian toxicity, and a benign environmental profile. Strobilurin trials demonstrated LC_{95} values below 1 mg AI/L (active ingredient/liter) and activity against important diseases caused by ascomycete, basidomycete, deuteromycete, and oomycete plant pathogens. These natural products possess a novel mode of action by inhibiting mitochondrial respiration through prevention of electron transfer.[6−8] Because of this novel mode of action, the strobilurins will offer control of pathogens resistant to other fungicides. The strobilurins have both disease preventative and curative properties and are active against spore germination, mycelial growth, and sporulation. The importance and future of the strobilurins as a new class of fungicides is seen by the fact that 21 companies have filed 255 patent applications primarily for use as agricultural fungicides.[6] Because of the importance of this new class of naturally derived fungicides, we used a mixture of azoxystrobin and other commercial fungicides to evaluate the resolving power of 2D-TLC direct bioautography for application in natural products research.

Bioautographic assays are usually used to screen for antimicrobial activity by absorbing chemicals onto the surface of chromatographic plates and placing them directly in contact with a medium that is then inoculated with bacterial or fungal cultures. These assays detect "clear zones" where microbial growth is inhibited. They are often difficult to read and work best with water-soluble compounds that readily diffuse into water-based microbial media. Unfortunately, antimicrobial compounds that do not dissolve or diffuse in water may, therefore, not be detected. Antifungal bioautographic assay systems have used classically one-dimensional thin-layer chromatography (TLC) to separate the chemical constituents of plant extracts.[9−11] These methods must be repeated using a large number of different solvent systems to further separate both polar and nonpolar chemical constituents often found in crude extracts. Herein, we describe a new 2D-TLC direct bioautography method where the TLC plates are developed once with a polar solvent, turned 90°, and then developed a second time with a nonpolar solvent system. The 2D-TLC plates are then dried and sprayed with a nutrient broth

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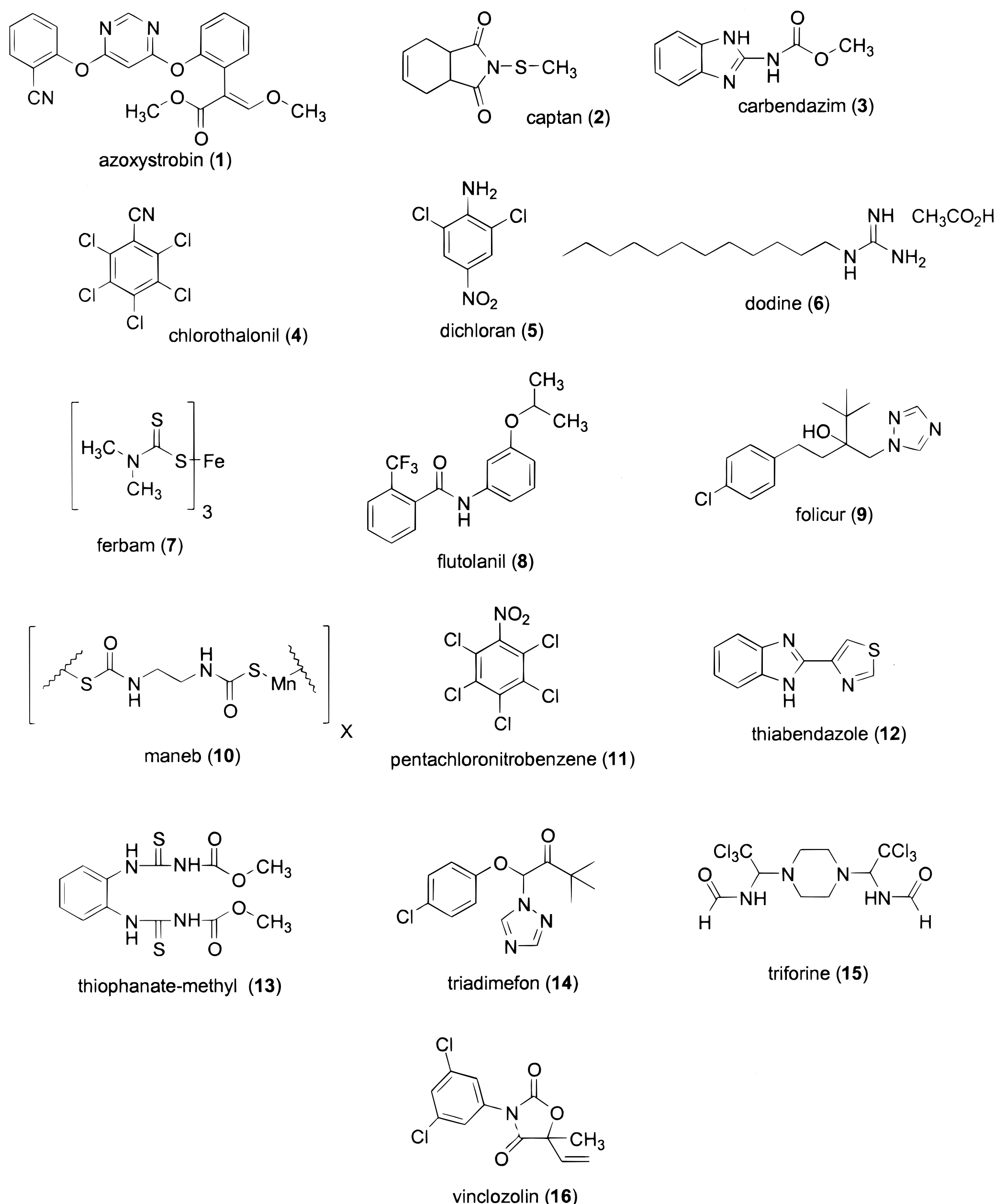
Chart 1



spore suspension, and the fungal culture is grown directly on the silica gel surface of the TLC plate. This new method takes advantage of the resolving power of 2D-TLC to separate chemically diverse mixtures found in crude plant and algal extracts. Two-dimensional TLC bioautography is well suited to resolving extracts containing lipophilic natural products that are difficult to separate by single elution TLC. Antifungal metabolites can be readily located on the plates by visually observing clear zones where active compounds inhibit fungal growth. This method eliminates the need for the development of large numbers of plates in multiple solvent systems, reduces the amount of waste solvents for disposal, and substantially reduces the time needed to identify active compounds.

Results and Discussion

Less than 1 mg of crude extract applied to each plate was shown to be suitable for high-resolution 2D-TLC development and seldom produced chromatographic overloading. The quantity applied could be adjusted to reflect differences in chemical composition related to the specific chemical nature of the organism extracted and the extraction method used to prepare the extract. Considerable variation was encountered between extracts of diverse organisms (plants, algae, microbes, and invertebrates) due to pronounced differences in lipid, alkaloid, and polysaccharide levels. Glass-backed TLC plates were found to work well and avoided chromatographic aberrations often associated with cracking of the Si gel surface due to bending experienced when cutting aluminum-backed plates. The added weight of the glass also stabilizes the plates when sprayed with conidial suspension. Our studies indicate that antifungal activity of many commercial fungicides can be detected on these TLC plates with less than 1×10^{-5} μmol applied per spot.

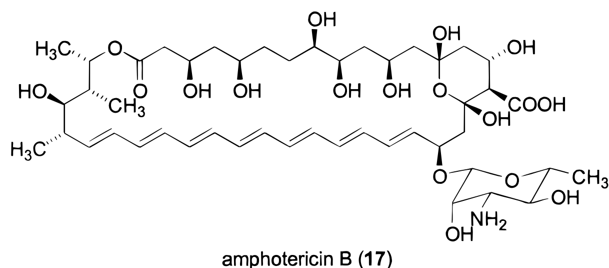
Plant pathogenic fungi were evaluated for their sensitivity to commercial agricultural fungicides (compounds **1**–**16**, Chart 1) by direct 1D-TLC bioautography. The relative detectability of each compound (1×10^{-5} μmol of each

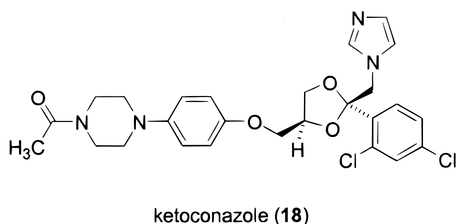
Table 1. Sensitivity of *C. fragariae* to Selected Commercial Fungicide Standards by Direct Bioautography

compd	compd name[a]	detectability[b] (±)	zone dimens[c] (mm)
1	azoxystrobin	+	8 × 10
2	captan	+	12 × 15
3	carbendazim	+	4 × 6
4	chlorothalonil	+	14 × 20
5	dichloran	+	8 × 10
6	dodine	+	4 × 5
7	ferbam	+	30 × 50
8	flutolanil	−	no zone
9	folicur	+	26 × 35
10	maneb	+	5 × 9
11	pentachloronitrobenzene	±	1 × 5
12	thiabendazole	−	no zone
13	thiophanate-methyl	+	4 × 6
14	triadimefon	+	11 × 11
15	triforine	−	no zone
16	vinclozolin	+	6 × 8

[a] Concentration 1.0×10^{-5} μmol/spot, [b] Detectability measured by visual observation of clear zone of fungal inhibition. [c] Approximate dimensions of zones of fungal inhibition produced by fungicides.

Chart 2





standard per spot) and size of fungal inhibition zone produced by each agent are shown in Table 1.

Mixtures composed of agricultural fungicides and antifungal drugs were used to examine the resolving power of the 2D-TLC system on structurally diverse chemical standards. Combinations of agricultural fungicides (**1**−**5**, **10**, **12**, **14**, **15**) and antifungal drugs (**17** and **18**, Chart 2) were readily separated and produced clear zones of fungal inhibition (Figure 1).

Lipid extracts of aquatic plants, algae, and marine cyanobacteria were evaluated by this 2D direct bioautography method (Figure 2). Chemically complex mixtures of extract constituents were easily resolved by 2D-TLC, and active metabolites were located by characteristic zones of fungal growth inhibition. Polar compounds were resolved upon initial elution, while lipids and compounds of intermediate polarity were readily separated when developed in the second dimension. Secondary metabolites were resolved from complex mixtures in crude extracts even in the presence of pigments and membrane lipids.

Our results suggest that chemically different populations of cyanobacteria, for example, can easily be distinguished by their characteristic "2D-TLC fingerprint" and antifungal zone patterns. Thus, this system represents a powerful technique for the identification and selection of chemically unique chemotypes from microbial isolates and plant collections.[12] Chromatographic properties (relative polarity, UV absorbance, chemical reactivity, etc.) associated with

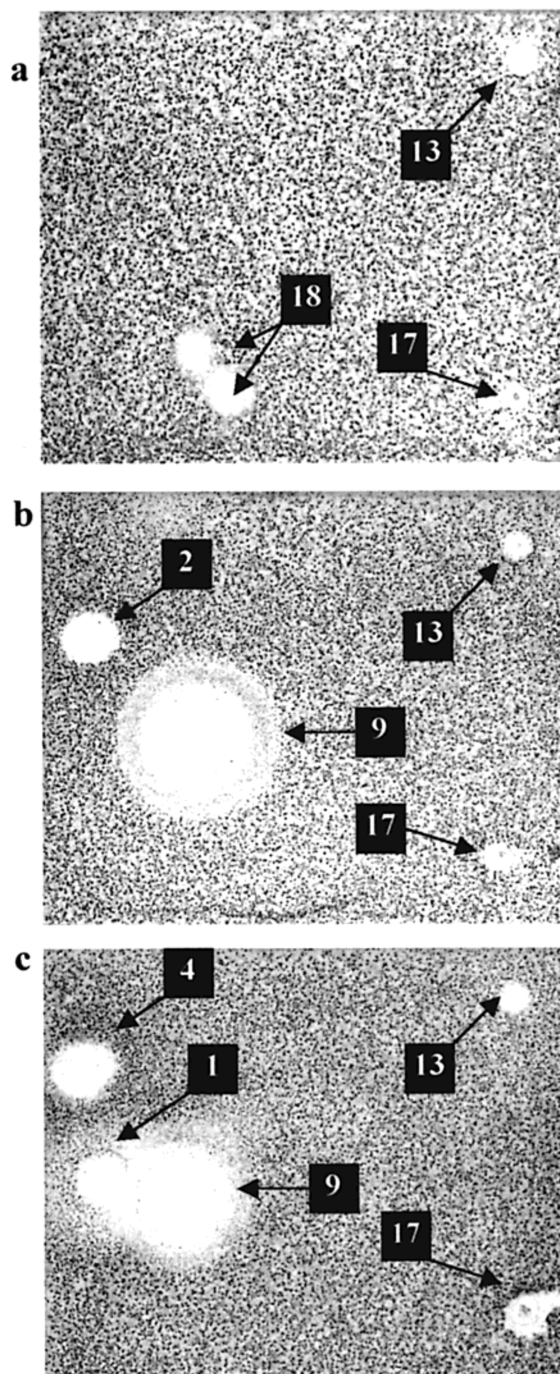


Figure 1. 2D-TLC direct bioautography of commercial agricultural fungicides and antifungal drug standards. Antifungal standards were each evaluated at 1×10^{-5} μmol per spot. TLC plates were developed in the first dimension (right to left) in 10% MeOH in CH_2Cl_2 (v/v) and developed in the second dimension (bottom to top) with 50% EtOAc in hexanes (v/v). Antifungal activity was clearly observed by "zones" of fungal growth inhibition using *Colletotrichum fragariae* as the indicator species. Antifungal standard combinations evaluated for each bioautography plate as follows: (a) ketoconazole (**18**, two spots), amphotericin B (**17**, origin); (b) captan (**2**), folicur (**9**), amphotericin B (**17**); (c) chlorothalonil (**4**), azoxystrobin (**1**), folicur (**9**), amphotericin B (**17**). Plates were spotted in the upper right-hand corner with thiophanate-methyl (**13**), as an internal control, prior to conidial inoculation.

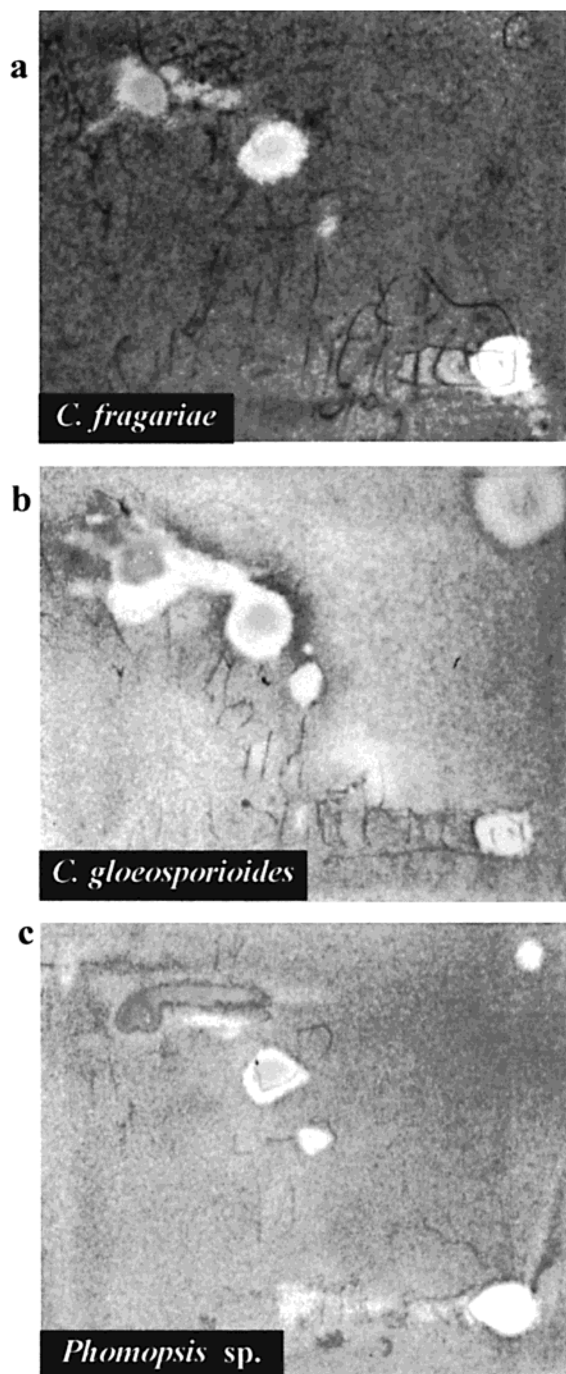


Figure 2. "Zones" of fungal growth inhibition, produced by multiple distinct antifungal metabolites found in a single extract of a marine alga that was prepared in triplicate and inoculated with three different strains of pathogenic fungi: (a) plate inoculated with *Colletotrichum fragariae*, (b) plate inoculated with *C. gloeosporioides*, and (c) plate inoculated with *Phomopsis* sp. Plates were spotted in upper right-hand corner with thiophanate-methyl (**13**), as an internal control, prior to inoculation.

each active metabolite provide valuable information that allows for rapid dereplication of known or nuisance compounds.

When strains of different phytopathogenic fungi with dissimilar fungicide resistance profiles were inoculated onto replicate bioautography plates prepared from any given extract that contains active metabolites, it is possible to visually observe distinct differences in sensitivity of each fungal pathogen to single metabolites (Figure 2). These differences in pathogen sensitivity (fungicide resistance) were observed by direct comparison of inhibition zone dimensions produced by active metabolites (and control standards) against each pathogenic strain tested. Chemical profiles can provide a valuable method for rapidly selecting specific antifungal metabolites with unique activity against fungicide-resistant pathogens and identify new lead compounds with novel mechanisms of action.

Experimental Section

Two-Dimensional-TLC Analysis (2D-TLC). Glass Si gel plates with a fluorescent indicator (10 × 10 cm, 250 μm, Silica Gel GF Uniplate, Analtech, Inc., Newark, DE) were activated for 1 h at 100 °C and stored in a desiccator to increase reproducibility. Crude extract material (less than 1 mg) was dissolved in either Et_2O, CH_2Cl_2, or MeOH and applied to form a single small spot (2−4 mm diameter) in a corner 1 cm from either of two sides of a Si gel plate.

TLC plates were placed in a presaturated solvent chamber (MeOH in CH_2Cl_2, 1:9, v/v), and the extract spot was allowed to develop along the left-hand side of the plate. TLC plates were then removed from the solvent chamber immediately before the solvent front reached the top of the plates. These plates were allowed to air-dry and subsequently placed in another presaturated solvent chamber (EtOAc in hexanes, 1:1, v/v) so that the developed lane of extract was placed horizontally just above the solvent. Each plate was allowed to develop until the solvent front reached 1 cm from the top of the plate and then removed from the chamber and air-dried. Pigmented compounds were marked on the developed TLC plate with a pencil, photographed, and digitized. Each plate was placed in an UV viewing cabinet (254 nm), and the locations of nonpigmented UV_{254}-fluorescing compounds were recorded directly on the plate by penciled-in circles or photographed and digitized. A reference plate was developed for each extract and sprayed with a light even coating of H_2SO_4 in EtOH (1:19, v/v) and gently heated. Locations and any notable colors associated with acid-charring compounds were marked directly on the reference plate as they appeared. Reference plates were heated thoroughly to remove all traces of acid and then photographed and digitized. Multiple plates were developed simultaneously in large solvent chambers and spotted, developed, dried, scored, photographed, and digitized in less than 1 h. Comparisons of secondary metabolite profiles for each extract were made from reference plates as indicated by the occurrence and location of UV-active and/or unusual acid-charring compounds. Photoaccessory pigments, triglycerides, steroids, and polar lipids were located and used as internal chromatographic standards. An example is provided in the Supporting Information.

Pathogen Production and Inoculum Preparation. Filamentous fungi of the genus *Colletotrichum* and its teleomorph *Glomerella* are major plant pathogens worldwide. *Colletotrichum* species cause typical disease symptoms known as anthracnose, which is characterized by sunken necrotic lesions often bounded by a red margin.[13,14] Anthracnose diseases of strawberry (*Fragaria* x *ananassa* Duch.) are serious problems for fruit and plant production in many areas of the world.[15,16] The pathogens *Colletotrichum acutatum* J. H. Simmonds, *C. gloeosporioides* (Penz.) Penz., *C. fragariae* A. N. Brooks, and *Phomopsis* sp. can occur singly or in combination and can infect strawberry flowers, fruit, leaves, petioles, stolons, and crowns.[17]

Isolates of *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, and *Phomopsis* sp. were obtained from B. J. Smith, USDA, ARS, Small Fruit Research Station, Poplarville, MS. Conidia were harvested from two-week-old cultures by flooding plates with 5 mL of sterile distilled water and dislodging conidia from acervuli using an L-shaped glass rod. Conidial suspensions were filtered through sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) to remove mycelia. Conidia concentrations were determined photometrically[3,18] from a standard curve, and suspensions were then adjusted with sterile distilled water to a concentration of 1.0 × 10^6 conidia/mL. Subsequently, 25−50 mL of inoculum spray

solution (ca. 3.0×10^5) was prepared for each test fungus with liquid potato dextrose broth (PDB) containing 12 g/500 mL PDB, 0.1% bacto agar, and 0.1% Tween-80.

Bioautography. Inhibition of fungal growth on chromatographic plates was evaluated by specifically adapting 2D-TLC analysis to a modified TLC bioautography system.[9-11] To detect biological activity directly on the TLC plate, Si gel plates were inoculated with a spore suspension. Aliquats of 25−50 mL of inoculum spray solution (ca. 3×10^5 conidia/mL) were prepared for each test fungus with liquid potato dextrose broth (PDB) containing 12 g/500 mL of (PDB), 0.1% bacto agar, and 0.1% Tween-80. Using a 50 mL chromatographic sprayer, each plate was sprayed lightly (to a damp appearance) three times with the conidial suspension. Inoculated plates were placed in a 30 × 13 × 7.5 cm moisture chamber (398-C, Pioneer Plastics, Inc., Dixon, KY) and incubated in a growth chamber at 24 ± 1 °C and 12 h photoperiod under 60 ± 5 μmol light. Inhibition of fungal growth for each test fungus was measured 4 days after treatment. Sensitivity of each fungal isolate to test compounds and a fungicide standard at 1 or 2 μg was determined by comparing the dimensions of antifungal zones. Typically, thiophanate-methyl (**13**) was spotted in the upper right-hand corner of each plate prior to inoculation.

Bioautography of Commercial Fungicide Standards. Commercial agricultural fungicides and antifungal drugs were used to evaluate the sensitivity and resolving power of the assay. Axenic cultures of plant pathogenic fungi were examined for their sensitivity to commercial agricultural fungicides (compounds **1**-**16**) by direct 1D-TLC bioautography (Table 1). Antifungal standards were applied (2 μg) of 5 μM ethanolic solutions (1×10^{-5} μmol of each standard/spot). Glass-backed Si gel plates, as described in the 2D-TLC method, were developed in a chamber saturated with 1:1 v/v EtOAc in hexanes.

Mixtures consisting of combinations of agricultural fungicides and antifungal drugs were used to examine the resolving power of the 2D-TLC system on structurally dissimilar chemical standards (Figure 1). The agricultural fungicides tested were azoxystrobin (**1**), captan (**2**), carbendazim (**3**), chlorothalonil (**4**), dichloran (**5**), maneb (**10**), thiabendazole (**12**), triadimefon (**14**), and triforine (**15**). Antifungal drugs tested were amphotericin B (**17**) and ketoconazole (**18**). Antifungal standards were applied in various combinations to form a single spot on TLC plates, as described for crude extracts. The TLC plates were then developed according to the two-dimensional protocol as described, air-dried, inoculated with conidial suspensions, and incubated. The plates were removed after 4 days, dried, photographed, and digitized. Zones of antifungal activity associated with each of the standards were recorded for each fungus strain tested.

Bioautography of Extracts from Aquatic and Marine Cyanobacteria, Algae, and Plants. Natural product-rich lipophilic and methanolic extracts of plants and marine organisms that were found to contain complex mixtures of biologically active secondary metabolites were evaluated for antifungal activity using the described 2D-TLC bioautography method (e.g., Figure 2). Replicate plates were each inoculated with three different strains of fungal pathogens and incubated. Differences in pathogen sensitivity (fungicide resistance) were observed by direct comparison of zones of fungal inhibition produced by active metabolites in each extract against each pathogen strain tested.

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Supporting Information Available: Comparison of a chemically complex crude extract from a collection of a tropical marine alga by 2D-TLC analysis and direct bioautography. This material is available free of charge via the Internet at http://pubs.acs.org.

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