DEREPICATION OF PENICILLIUM BIOACTIVES USING MULTIDETECTOR HPLC AND BIOACTIVITY PROFILES

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ABSTRACT

The NCI drug discovery program has, among >150,000 natural products extracts, over 18,000 extracts obtained from fungal fermentations. Over 80 different species of Penicillium are included in the collection. Members of the diverse genus Penicillium are known producers of bioactive substances, such as the natural penicillins, penicillic acid, wortmannin, and others, which will be repeatedly detected in screening programs. Thus, the natural products chemist in a drug discovery program will be presented with the same bioactive extracts, as detected with different bioassays, and faced with the challenge of determining whether the active substance is a known molecule or a new chemical entity.

For example, 28 toxic extracts from Penicillium sp. were “fingerprinted” using a multidetector HPLC system which included diode array, laser light scattering, fluorescence, and mass spectrometer. Column eluate was collected in microtiter format for bioassay. Comparisons of both physical and biological data can then be made to assess the presence or absence of known bioactive compounds in the crude extract.

INTRODUCTION

The NCI drug discovery program at FCRDC includes a search for new lead compounds from plant, marine and fungal extracts as anticancer, antiviral and antimicrobial agents. Among the library of >150,000 crude natural products extracts are >18,900 extracts of fungal origin, and among these are 1,987 extracts from cultures identified as genus = Penicillium. Ninety different viable cultures identified as Penicillium are represented in the DTP repository.

The genus Penicillium has about 150 described species, and these various organisms are well known as producers of a variety of bioactive substances (i.e., the natural penicillins, penicillic acid, wortmannin, gliotoxin, duclauxin, cyclopiazonic acid, etc.) that will be detected by many different sorts of bioactivity screens. The Berdy Antibiotic Database lists 602 bioactive compounds with Penicillium as the producing organism. But even when taxonomy has determined that two fungal isolates are identical to the species level, they do not necessarily produce the same secondary metabolites. Possible genetic differences within the species, variable culture conditions, and composition of the growth media all affect the mixture of metabolites produced by the fungus. Thus, the natural products chemist in a drug discovery program will be repeatedly presented with bioactive extracts from Penicillium cultures, and be faced with the challenge of determining whether the active substance is a known molecule or a new chemical entity. Previously, chemists have utilized UV profiles of bioactive substances (1), HPLC retention tables (2), and chromatographic plus bioactivity (3,4) methods to address the identification of compounds found in fungal extracts.

The technique we have been developing for dereplication of extracts is construction of a “fingerprint” of the extract composed of both spectral and biological data through use of a multidetector HPLC system which includes diode array, evaporative laser light scattering, fluorescence and mass spectrometer. Physical data relating to retention time, UV absorption profile, weight of component in the extract, and mass of the molecule are collected simultaneously. Acquiring these measurements consumes 5% of the column eluate with the remaining 95% being collected into microtiter plates. Microtiter plates are replicated with each well (fraction) tested for biological activity. Biological and physical information are then correlated and determinations can be made involving the presence (or absence) of known bioactive compounds in these extracts.
MATERIALS AND METHODS

a) PRODUCTION OF FUNGAL EXTRACTS

In the NCI screening program fungal cultures are typically grown in three media: potato dextrose broth (PDB) stationary, soy-glucose-starch (SGSM) agitated, and glucose-sucrose-fructose (GSF) agitated. Whole broth is homogenized and extracted by partitioning against dichloromethane. Following drying and weighing, the extract is prepared for screening in microtiter plate format, with either 50 µg/well (turbidometric measurement of growth inhibition) or 500 µg (filter paper disc) for disc diffusion bioassay.

b) CHROMATOGRAPHIC ANALYSIS

The multidetector HPLC system consists of a Waters 600 pump controlled by Waters Millennium 32 software and a Waters 2700 autosampler which allows for samples to be loaded in either vials or microtiter plate format. Eluate from the column is split initially 115:1 with a high precision LCP splitter which directs 0.8% volume to a Kratos fluorescence detector, a Waters 996 Photodiode Array Detector, and a Waters Micromass API Electrospray Mass Spectrometer. The bulk of the flow goes to a 25:1 LCP splitter, with the major flow directed to an ISCO Foxy fraction collector which collects in microtiter plate format. The remainder of the flow is directed into a Varex Evaporative Light Scattering Detector. This arrangement gives 95% sample recovery.

For routine analysis, 50 mg of crude extract is dissolved in acetonitrile/water (1:1) at 10 mg/ml and injected onto a 250 mm x 21 mm Dynamax 60A C-18 phase bonded column eluted with acetonitrile/20 mM ammonium acetate pH 4.0 at 15 ml/min under gradient conditions 0-5 min (30:70), 25 min (50:50), 45-60 min (100:0). A typical total volume of eluate is 645 mls, which is collected into 4 x 24 well microtiter plates. These are reformatted by a TECAN High Speed Robotic Liquid Handling System into two 96 well master microtiter plates with 2 ml in each well, plus 15 replicate daughterplates at 250 µl in each well. Three of these daughter plates are 96 well polystyrene flat bottom containing one 5 mm filter paper disc in each well. All plates are dried for several hours in a Savant Speed Vac to remove the majority of the organic solvent, then placed into dry ice, and when solidly frozen, into a freeze drier. Once thoroughly dried, these fractions are suitable for biological evaluation, while the bulk of the sample mass is retained in 96 well plate format for further chromatography or spectroscopy. All the data is captured electronically, and following biological evaluation, a combined physical/biological data chart is assembled.

c) BIOASSAY

1) Of 297 organic solvent extracts from fermentations of Penicillia which were evaluated during a high throughput screen against an azole resistant strain of Candida albicans, 44 showed toxicity. A subset (20) of these were selected for chromatographic analysis to test the applicability of HPLC/bioactivity fingerprinting to the task of dereplication.

2) As stated above, an aliquot of the column eluate is transferred directly into microtiter plates. Thus, the exact mass applied to each test disc is not known. Disc diffusion assays were performed by transferring each 5 mm filter disc from the well of the vacuum dried polypropylene plate onto the surface of a 245 x 245 mm Petri dish to which one of three wild type organisms had been added prior to pouring: Candida albicans (fungus), Bacillus subtilis (gm+), or Escherichia coli (gm–). Typically, the paper discs from one 96 well microtiter plate were placed onto one 245 x 245 agar plate in the same 8 x 12 format as found on the plate. Appropriate antibiotic containing standard discs were also applied. After 24 hrs incubation at 30°C, the zones of inhibition were scored manually, and normalized to the standards. Additionally, selected fractions were evaluated in an azole resistant strain of Candida albicans 99-778 and a methicillin resistant strain of Staphlococcus aureus grown in liquid culture in microtiter plate format, with inhibition of growth determined by optical density after ~20 hrs.

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<tr>
<th>Compound</th>
<th>MF/MW</th>
<th>Test Organism</th>
<th>C. albicans 99-778</th>
<th>C. albicans W</th>
<th>E. coli W</th>
<th>B. subtilis W</th>
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*Internal Standard, not a Penicillium compound
STANDARDS

The chromatographic and biological profiles of several frequently encountered bioactive compounds known to be produced by the genus *Penicillium* were assembled. Always included in the analysis of extracts from microbial fermentations is a chromatogram of the extract obtained from un-inoculated medium.

EXTRACTS CONTAINING KNOWN TOXIC COMPOUNDS

Selected for special analysis were several extracts obtained from fungal strains known to be producers of toxic substances. Penicillic acid is a compound frequently detected in large quantity. An example is shown of *Penicillium duclauxi*, a producer of wortmannin.
TOXIC EXTRACTS OF UNDETERMINED COMPOSITION

Chromatographic resolution of extract components has often revealed multiple zones of bioactivity. In the example below a large amount of patulin is responsible for the observed toxicity to *E. coli*, while a second bioactive component in well 46 has a separate activity against *C. albicans*.

In the second example, the culture had tentatively been identified as a Penicillium, but the retention time, spectra, and bioactivity of a major component did not correspond to a known Penicillium toxin. Following isolation the structure was determined to be 2,3 dimethoxy 5,6 dimethyl benzophenone, which had been reported from *Gliocladium roseum*. Re-examination of the culture confirmed that it is a Gliocladium.

CONCLUSIONS

The HPLC/multidetector/bioactivity fingerprint can confirm the presence of a number of known Penicillium-produced toxic natural products from a crude extract in a single chromatographic step without additional purification. In one instance detection of a known compound not anticipated from a Penicillium provided a key bit of chemotaxonomic information which aided in the identification of the culture as a Gliocladium sp.

More interestingly, the presence of two or more zones of bioactivity, sometimes with toxicities toward different test organisms, is often detected with this approach to analysis. The chemist can then focus on the specific bioactivity of interest.

As hundreds of spectral plus bioactivity fingerprints are acquired and added to a searchable database, the value of that data in speeding the dereplication process will increase.

REFERENCES

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