Chapter 6

The Chemical Identification and Analysis of *Aspergillus nidulans* Secondary Metabolites

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Abstract

Filamentous fungi have long been recognized to be a rich source of secondary metabolites with potential medicinal applications. The recent genomic sequencing of several *Aspergillus* species has revealed that many secondary metabolite gene clusters are apparently silent under standard laboratory conditions. Several successful approaches have been utilized to upregulate these genes and unearth the corresponding natural products. A straightforward, reliable method to purify and characterize new metabolites therefore should be useful. Details are provided herein on the cultivation of *Aspergillus nidulans* and the LC/MS analysis of the metabolic profile. Following is an explanation of silica gel chromatography, HPLC, and preparative TLC. Finally, the NMR characterization of previously unknown *A. nidulans* metabolites is detailed.

Key words: Secondary metabolites, Natural products, Polyketides, Purification methods, *Aspergillus nidulans*

1. Introduction

Fungal genera such as *Aspergillus* are well known to generate a number of structurally complex secondary metabolites. Many of them have been observed to be significantly relevant to human health, including lovastatin, a well-known antihypercholesterolemic agent produced by *A. terreus*, and aflatoxin, a carcinogen produced by *A. flavus* (1, 2).

The recent sequencing of a number of fungal genomes has revealed, surprisingly, that fungi have many more secondary metabolism pathways than previously thought (3–6). As one example,

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Aspergillus nidulans contains 27 polyketide synthase (PKS) and 14 nonribosomal peptide synthetase (NRPS) genes (3), but fewer than ten secondary metabolites had been regularly observed. Since far fewer secondary metabolites have been identified from this heavily studied species, the products of many of these biosynthetic pathways are still unknown and are presumably silent under standard laboratory conditions. There is thus great opportunity to discover new metabolites that could potentially be new therapeutics or important mycotoxins.

A variety of methods have been developed to turn on silent gene clusters. Deletion of *A. nidulans* CclA, a Bre2 ortholog involved in histone 3 lysine 4 methylation, activated the expression of hidden secondary metabolite clusters. One novel cluster generated monodictyphenone, emodin, and emodin derivatives, while a second encoded two anti-osteoporosis polyketides, F9775A and F9775B (7). The latter polyketides were also revealed from a study of over 20 separate culture conditions, based on the understanding that different conditions can yield different products, an approach known as One Strain Many Compounds (OSMAC) (8).

Other targets besides histone proteins may be linked to the regulation of some secondary metabolites. In *A. nidulans* the single sumoylation gene *sumO* was demonstrated to influence the production of several metabolites (9). Deletion of this gene led to a decrease in austinol and dehydroaustinol production and also a substantial increase in the formation of the polyketide asperthecin. The significant amount of asperthecin allowed researchers to determine the genetic cluster and propose its biosynthesis.

As another approach, the native promoter of a transcriptional activator gene, *afoA*, was replaced by an inducible promoter, allowing the expression of the corresponding PKS and concurrent production of the polyketide asperfuranone (10).

With the generation of an increasing number of secondary metabolites, a reliable protocol is needed for their purification and identification. Here we describe the methods the Wang group carries out to isolate and characterize *A. nidulans* secondary metabolites, primarily polyketides, although the methods should be applicable to other fungal secondary metabolites.

2. Materials

2.1. Compositions of Trace Metal Solution and Common Media

1. Trace metal solution: $ZnSO_4 \cdot 7H_2O$, 2.2 g, H_3BO_3, 1.1 g, $MnCl_2 \cdot 4H_2O$, 0.5 g, $FeSO_4 \cdot 7H_2O$, 0.5 g, $CoCl_2 \cdot 5H_2O$, 0.16 g, $CuSO_4 \cdot 5H_2O$, 0.16 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.11 g, Na_4EDTA, 5.0 g. Add the solids in the listed order to 80 mL

of H_2O, dissolving each completely before adding the next. Heat the solution to boiling, cool to 60°C, and adjust the pH to 6.5 with saturated KOH. Cool to room temperature and adjust the volume to 100 mL with H_2O (see Note 1). Autoclave and store at room temperature.

2. 20× salts solution: $NaNO_3$, 120 g, KCl, 10.4 g, $MgSO_4 \cdot 7H_2O$, 10.4 g, KH_2PO_4, 30.4 g, H_2O, 1 L.
3. Glucose minimal media: Glucose (dextrose), 10 g, 20× salt solution, 50 mL, trace metal solution, 1 mL, H_2O, 1 L.
4. Czapek's minimal media: Sucrose, 30 g, 20× salt solution, 50 mL, trace metal solution, 1 mL, H_2O, 1 L.
5. Lactose minimal media: Lactose, 20 g, glucose (dextrose), 10 g, 20× salt solution, 50 mL, trace metal solution, 1 mL, H_2O, 1 L.

   For all liquid media, adjust the pH to 6.5 using concentrated KOH or HCl. Be sure to include any necessary supplements that the strain requires (e.g., pyridoxine). Autoclave and store at room temperature. The trace metal solution and supplements should also be autoclaved. The supplement uridine cannot be autoclaved and must instead be introduced to the autoclaved media through a sterile syringe filter.
6. Yeast Agar Glucose (YAG) Plates: Yeast extract, 5 g, glucose (dextrose), 20 g, agar, 15 g, trace metal solution, 2 mL, H_2O, 1 L. Be sure to include any necessary supplements.

   However, the yeast extract may already contain enough of the supplement in question. Our experience has shown that it is not necessary to add pyridoxine, but it is still necessary to add riboflavin or uridine and uracil. Autoclave and cool until it can be handled properly (but before it solidifies). Again, the supplement uridine cannot be autoclaved and must instead be introduced to the autoclaved media through a sterile syringe filter. Pour the liquid YAG media into Petri dishes (10 cm diameter), approximately halfway to the top. After they solidify, store them at 4°C.

2.2. Solutions for LC/MS Analysis and Purification

1. LC/MS "A" buffer: 950 mL H_2O, 50 mL acetonitrile (see Note 2), 500 μL formic acid.
2. LC/MS "B" buffer: 1 L acetonitrile, 500 μL formic acid.
3. HPLC "A" buffer: 950 mL H_2O, 50 mL acetonitrile (HPLC grade), 500 μL trifluoroacetic acid.
4. HPLC "B" buffer: 1 L acetonitrile, 500 μL trifluoroacetic acid.

3. Methods

3.1. Acquisition of Spore Suspension

1. Transfer a small amount of the strain of interest (it need not be quantified) from a "stock solution" (typically from glycerol or silica stock) onto a new YAG plate under sterile conditions.
2. Spread the strain around on the plate using a sterile bacterial cell spreader. Adding 1 mL of sterile water beforehand will make spreading easier.
3. This YAG plate then requires 2–3 days in a 37°C incubator for conidia to cover the top of a plate. Place 7 mL of sterile water on top of the plate, followed by gentle scraping with a spreader. The spore suspension should then be transferred to a sterile Falcon tube (see Note 3).
4. Measure the concentration of the suspension; this can be accomplished with a standard hemocytometer. A typical spore concentration for *A. nidulans* is approximately 1×10^6 spores/μL (see Note 4). Accordingly, a small amount of spore suspension will need to be diluted ~1,000-fold with water in order to count a reasonable number of spores.

3.2. Small-Scale Cultivation for Initial Analysis

1. Under sterile conditions, transfer 50 mL of the liquid media of choice into a sterile 125-mL flask. Enough spore suspension should then be transferred into the same flask such that the spore concentration is 5×10^6 spores/mL (or 2.5×10^8 spores in total).
2. Typically, place the flask in a shaker at ~100 rpm for 4 days at 37°C. By this time, the mycelia should have grown and appear as light-colored spheres.
3. If an inducible promoter is involved, the inducer should be added 18 h after initial cultivation. For *alcA* promoters, 133 μL of cyclopentanone is added to a 50 mL cultivation. The volume can be scaled accordingly.
4. After cultivation, 25 mL of filtered medium is typically collected in a 50 mL Falcon tube, using a piece of Miracloth for filtration. The media is then to be extracted twice with an equal amount of ethyl acetate (this can be done within the Falcon tube), and the ethyl acetate layer is transferred by pipette to tared vessels.
5. After evaporation, the mass of the residue can be determined (see Note 5). It can be dissolved in methanol at a concentration of 1 mg/mL to prepare it for LC/MS analysis (see Note 6).
6. As an alternative to liquid media, a solid agar plate can be used instead (see Note 7). The result may be a different metabolite profile. A total of 1×10^7 spores should be added to a plate of

10 cm diameter, spread onto the solid medium of choice. Place the plate in a 37°C incubator for 5 days.

7. Afterwards, the agar must be chopped into ~1 cm^2 pieces and collected into a flask. Add methanol until it covers all of the pieces. The flask is then sonicated for 1 h.
8. Collect the filtrate (again using Miracloth) and add a 1:1 mixture of methanol:dichloromethane to the chopped agar until the solvent reaches the top of the pieces. Sonicate again for an hour.
9. Filter again, this time using KimWipes instead of Miracloth. The combined solvent is then evaporated until water is the only solvent (see Note 8), which is then extracted as before.
10. When necessary (see Note 9) add concentrated HCl to the filtered media (whether from a liquid or solid source) until a pH of 2 is reached (pH paper can be used). Extract and evaporate as usual.
11. In all cases the solution should be syringe-filtered to remove particulates that can clog an LC/MS column. Place ~100 μL of solution into a disposable 1 mL syringe fitted with a 13 mm filter with a PTFE membrane. With the syringe plunger, push the solvent through the filter and into an LC/MS vial. The sample is then ready to be submitted to the LC/MS.
12. LC/MS typically uses two buffers, a polar "A" buffer and a nonpolar "B" buffer (see Subheading 2.2).
13. A gradient, an increasing amount of the "B" buffer over time, is used to separate and elute the molecules. A typical gradient in the Wang lab is 0% B from 0 to 5 min, 0–100% B from 5 to 35 min, 100% B from 35 to 40 min, 100–0% B from 40 to 45 min, and re-equilibration with 0% B from 45 to 50 min. The flow rate is 125 μL/min.

3.3. LC/MS Analysis

The most straightforward situation is when the metabolite is naturally secreted in abundant amounts by the organism under standard cultivation conditions (such as Glucose Minimal Media, 37°C, 4 days) and can be easily detected. This is true of sterigmatocystin and terrequinone A, both of which are produced by *A. nidulans* in standard cultivations and are UV-active, making them detectable by analytical chromatography.

Fortunately, a modern LC/MS machine (see Note 10) will allow one to detect non-UV-active compounds as well through a Total Ion Current (TIC) chromatogram. The *y*-axis of this chromatogram measures signal intensity as the molecules at any given point of the chromatogram are ionized for mass spectrometric analysis. As a result, compounds that escape detection under UV-based conditions, such as the emericellamides, reveal themselves as peaks in a TIC chromatogram. One important caveat to

keep in mind is that this mode of molecule detection relies on its ability to be ionized, and this ability varies from molecule to molecule. However, a mass spectrometer can be programmed to form positively charged ions ("positive mode") or negatively charged ones ("negative mode"), and it is rare that a secondary metabolite will fail to be ionized in both cases. Some molecules, such as the emericellamides, are apparent in either positive or negative mode. Others are only detectable in one of the two modes. Therefore, if the object is to search for new metabolites whose properties are unknown, it is beneficial to examine three chromatograms: the UV chromatogram, the TIC chromatogram in positive mode, and the TIC chromatogram in negative mode.

If an unknown molecule emerges, it is unlikely that the structure can be solved at this point; however, important clues can be gleaned from the data provided. Aside from the mass, it can be determined if the compound is polar or nonpolar, depending on what time it elutes from the column. Also, the UV spectra can offer hints. For example, xanthones exhibit a maxima at ~380 nm, and this was helpful in locating xanthones from *A. nidulans* using the UV chromatogram.

3.4. Large-Scale Cultivation for Isolation of Metabolite

1. It will likely be necessary to grow the strain again in a larger scale to acquire enough material for NMR analysis. The amount of medium that is necessary will depend on the titer of the metabolite. A good starting point is to use about 2 L of medium.
2. For liquid culture, the researcher can simply use larger flasks and larger volumes of media. However, he or she may need to experiment with various parameters before the scale-up is successful. For instance, if a metabolite was acquired from 50 mL of media in a 125-mL flask, it does not automatically follow that the same result will come from using 1 L of media in a 2-L flask. The experimenter may instead need to use a number of smaller flasks.
3. For solid media it is normally not a problem to use larger plates (15 cm diameter) in place of smaller plates (10 cm diameter) for convenience. More sterile water (3 mL) should be added on top of each of these plates to facilitate spreading, and 2.25×10^7 μL of spore solution should be transferred.
4. The volumes of ethyl acetate involved will mean that separatory funnels must be used in the place of Falcon tubes, and the organic material should be collected in a large round-bottom flask. Extract at least twice; four times may be optimal.
5. A small amount (~0.5 mg) of dried material can be dissolved in methanol and submitted to the LC/MS to determine if the scaled-up cultivation was successful. Remember to syringe-filter the solution.

3.5. Silica Gel Column Chromatography for Semi-purification

The next step is to isolate the metabolite in question. Because a crude mixture is likely to contain a number of different products, multiple purification steps may be necessary, and careful planning is needed. The first round of purification is often standard organic chemistry silica gel chromatography. Although in some instances it is possible to isolate a metabolite at this step, the sheer number of chemicals in a crude mixture means that often we use the silica column step to obtain a semi-pure isolate that can be further purified in later stages using other methods.

1. Weigh out two portions of silica (particle size 0.063–0.200 mm). One portion will be 2–3 times the mass of the crude material. The other portion will be 30 times the crude mass.
2. Dissolve the crude material. If common organic solvents such as dichloromethane and methanol fail to dissolve the material, try adding water and then sonicate.
3. Put the small amount of silica into the dissolved material and evaporate the solvents completely. A substance resembling sand should be achieved. Remove any clumps with a spatula.
4. Put the large amount of silica in a large Erlenmeyer flask. Add in enough hexanes to form a slurry. ("Hexanes" is a common, commercially provided mixture of isomers of hexane.)
5. Pour the slurry into a glass chromatography column (see Note 11). Make sure there is a small amount of cotton taken from a cotton ball at the bottom of the column to prevent the silica from leaking out. Do not use too much cotton as it will significantly slow the flow of the solvent. The Wang group prefers not to use columns with frits at the bottom because an air bubble may form. It will be necessary to rinse the Erlenmeyer flask several times with hexanes to transfer all of the silica.
6. Allow solvent to drain out of the column. Using air pressure via an adapter with a regulator and release valve will greatly facilitate the running of the column, but always be careful that the valve at the bottom of the column is open before applying pressure. Stop when there is no longer any solvent that is above the silica.
7. Lightly tap the edge of the column with a blunt object such as the back of a hammer. This will cause the silica to settle. Some solvent will emerge above the level of the silica. Drain until again there is no solvent above the silica. Tap the edge of the column again to further pack the column. Drain.
8. Repeat tapping and draining until the silica settles no more (at which point no solvent will emerge above the silica). The column is now packed.
9. Add more hexanes to the top of the silica so that it is ~1 cm in height. Be careful not to disrupt the silica. It is a good idea to

use a pipette and slowly release the solvent onto the inner side of the column in order to preserve the integrity of the packed silica.

10. Pour in the small amount of silica that was mixed with the crude fungal extract. Make sure that all of this material is off the sides of the column. Extra hexanes can be used to completely transfer the material, if necessary. If so, drain the solvent until it is at the top of the material (see Note 12).
11. Add standard, commercially available sand so that it is ~1 cm in height. The sand serves as a physical barrier and will help prevent disruption of the packed silica when solvent is added.
12. Elute with the solvent systems that are necessary to drain the metabolite(s) of interest. For every 1.5 g of crude material, use 1 L of solvent mixture. A suitable solvent system is a mixture of ethyl acetate and hexanes. The most nonpolar metabolites will be eluted from a mixture of 10% ethyl acetate in hexanes. The more polar the metabolite, the more ethyl acetate will be needed, all the way up to 100% ethyl acetate (see Note 13).
13. For especially polar metabolites, it may be necessary to use methanol in ethyl acetate. Do not exceed 10% methanol and start gradually (see Note 14). If the experimenter is unsure which solvent composition is appropriate, it is advised to start with 10% ethyl acetate/hexanes, and then follow to 20% ethyl acetate/hexanes, and so forth. Each fraction should be collected separately and evaporated.
14. A small amount of material can be prepared for LC/MS analysis to keep track of the status of the purification.

3.6. Final, HPLC Purification of the Metabolite

1. Provided that the metabolite was eluted, the researcher can then proceed to HPLC purification. The evaporated fraction(s) from silica gel chromatography should be completely dissolved in methanol (see Note 15). Try to keep solvent volumes to a minimum. DMSO can also be added if the material fails to readily dissolve in the first solvent (see Note 16).
2. The solution is then filtered through a glass syringe into a vial for HPLC, the syringe fitted with the same filter used for LC/MS.
3. Refer to Subheading 2.2 for the composition of HPLC "A" and "B" buffers. A flow rate of 5.0 mL is used, with a UV detector at 250 nm.
4. The gradient to be chosen depends on the polarity on the compound to be purified (see Note 17).
5. Fractions containing the product (see Note 18) should be combined and evaporated, but first a sample should be submitted to the LC/MS to verify that it is indeed the product in question.

6. Frequently, the metabolite is now pure and, if a sufficient quantity was obtained, it is ready to be analyzed by NMR. However, sometimes the LC/MS and/or NMR reveal the presence of impurities, and further purification is necessary. Two strategies can be followed. One is to reuse the HPLC, this time employing a slower gradient, or even no gradient at all (isocratic). Another is to use preparative TLC (see next section).

3.7. Preparative TLC

1. Preparative TLC is appropriate for compounds that can be visualized under a UV light. The sample is dissolved in a small amount of methanol. A bit of cotton is taken from a cotton ball and packed down partly through the end of a glass pipette. This "paint brush" is used to apply the material as a line about 2 cm above the bottom of a pre-coated 20×20 cm TLC plate. No more than 10 mg of material should be applied to one plate.
2. If the appropriate solvent system is unknown, take a small amount of sample and run an analytical TLC. (Consult an organic chemistry laboratory textbook for instructions.) The right solvent composition will be similar to, but not necessarily identical to, the composition used to elute the product in column chromatography.
3. Place the plate in the developing chamber. Remove the plate when the solvent is about 2 cm from the top of the plate. This will take about an hour. Let dry for another hour.
4. If the product is colorless, it will be necessary to place the plate under a UV lamp (~250 nm) to visualize the location of the product. With a pencil, trace the outside of the product, then remove the plate from the UV lamp.
5. Using a razor blade, scrape off the silica that contains the product and collect in a flask. Pour in 10 mL of 95:5 dichloromethane:methanol. Add in a stir bar and stir for 1 h.
6. Collect the product by pouring the solvent over a filter atop a funnel and into a tared flask. Evaporate the solvent.

3.8. NMR Characterization of the Metabolite

Once pure product is obtained in sufficient quantity (as much as 5 mg may be needed), it is ready to be analyzed by NMR. The types of NMR experiments that should be performed depend on the nature of the product.

1. A proton NMR should be run first, and preferably also a carbon NMR. Be sure that the NMR tubes are clean and dry and that deuterated solvent is used. Consult an organic chemistry laboratory textbook if the operation of this machine is unclear.
2. If the researcher has an idea of the metabolite's identity (see Note 19), he or she can compare the acquired spectra with the metabolite's spectral data published in a journal article or a book (if such data exists).

3. If the metabolite's identity is still unknown, additional experiments, such as DEPT, HSQC, HMBC, COSY, and ROESY, may be needed to deduce the structure. The analysis of NMR data is beyond the scope of this chapter, and the reader is advised to consult a suitable text from a chemistry library.
4. It is often not necessary to fully solve the structure from these data. Rather, the researcher may solve only part of that structure, but that part is enough for him or her to enter into such programs as SciFinder Scholar and ascertain whether any published compounds also feature that part of the structure.
5. If the researcher is still unable to solve the structure, he or she may make use of a software program, such as ACD/Labs that can assist in this regard. Such programs can either compare the acquired NMR data with those in their databases or attempt to logically solve the structure. Success will rely on the amount of data that the experimenter is able to provide to the program.
6. Some computer programs also will predict the NMR spectra for a given chemical, so if the experimenter has an idea of the structure, he or she can see if the data match the computer prediction. A close match does not guarantee that the structure is correct, but it may help the researcher develop an understanding of the identity of the molecule.
7. Solving a structure through NMR can be a "back and forth" process. That is, the NMR data may provide some clues about the structure. The researcher can then consult journal articles, use software programs, or consult with peers, which may provide inspiration for the choice of further NMR experiments to advance in solving the structure (see Note 20).

4. Notes

1. The Wang group uses Barnstead's "DIamond RO" purification system whenever water is required, except for LC/MS, in which HPLC grade water is needed.
2. Be aware that many solvents are commercially sold in various "grades," which refers to the purity of the solvent. Solvent from different vendors with comparable "grades" should be interchangeable. Solvent prices are correlated with purity and because solvent is one of the major costs in these type of experiments, our choice of solvent grade is based on necessity. We reserve the use of ultra high purity solvents only when required. Acetonitrile should be HPLC grade. The other solvents we use are high purity (99.9%).

3. Fungal suspensions can be stored in a refrigerator for up to a month. After that, we tend to prepare a fresh suspension.
4. Some suspensions of mutant strains may be at least an order of magnitude less concentrated than wild-type suspensions.
5. Using a rotary evaporator is a convenient method to evaporate solvent. Its operation is straightforward, and instructions can be found in a number of organic chemistry laboratory texts. We use rotary evaporators from Buchi. For small scale evaporation we use the Turbovap system from Caliper Life Sciences that is able to dry down large number of samples in parallel unattended. One can either use nitrogen or compressed air to dry the samples. We found that the use of compressed air is sufficient and cost effective.
6. The final LC/MS concentration of 1 mg/mL need not be rigorously adhered to if the researcher finds it beneficial to increase or decrease the concentration somewhat.
7. Relative to the extract from liquid media, the extract from solid media is frequently larger in mass and more difficult to dissolve. The crude extract from solid media can be dissolved in DMSO at a concentration of 5 mg/mL, and then diluted fivefold with methanol.
8. It can be difficult to use a rotary evaporator on a mixture of water and organic solvents, because the mixture vigorously bubbles under vacuum and threatens to spill out of the flask. Evaporation will be easier if the flask is not more than a quarter full.
9. In many instances the secondary metabolite will be charged or otherwise be polar and cannot be extracted with an organic solvent. This problem can sometimes be circumvented by adjusting the pH of the aqueous solution so that previously charged species become neutral and thus more lipophilic. Lowering the pH to ~2 using concentrated HCl neutralizes negatively charged molecules. It might also be helpful to raise the pH of another portion of media to about 12, in case there are positively charged metabolites.
10. The Wang group maintains its own LC/MS. A ThermoFinnigan ion trap mass spectrometer with an electrospray source is used for initial screening. An Agilent time-of-flight mass spectrometer to obtain high resolution masses is also utilized. A sample can also be sent to a core facility for analysis. Often, the sample can be dry, and the facility will take care of sample preparation.
11. Columns come in a variety of sizes. Choose one that will result in the large portion of silica reaching a height of ~10 cm when it is poured in. ChemGlass is an excellent source of glassware.

12. There are two main ways to prepare a column, "dry loading" and "wet loading," and variations within these methods. A version of dry loading is described above. Although some people will insist on the superiority of a certain method, the "best" method is likely to be the one you are most comfortable with. We refer the interested reader to a seminal paper (11).
13. Often in the course of silica gel chromatography the product of interest will elute in more than one fraction. For instance, a product may elute in the 10% ethyl acetate fraction and the 20% ethyl acetate fraction. Since it is often desired to collect as much product as possible, it is a good idea to run multiple fractions of increasing polarity (such as 10, 20, and 30% ethyl acetate) to ensure that all of the product has eluted.
14. Small changes in the amount of methanol in an eluent will have a dramatic effect on the eluent's polarity, so measure carefully: An eluent with 5% methanol in ethyl acetate, for example, may have a dramatically different effect from one with 2% methanol.
15. When dissolving the material for HPLC, if there is a significant amount of insoluble material which the researcher suspects is not the product, it may be better to first centrifuge the solution and transfer the supernatant to the syringe. Otherwise, the insoluble particulates may quickly clog the filter.
16. When dissolving samples for the LC/MS or the HPLC, do not use solvents other than water, acetonitrile, methanol, and DMSO, as other solvents may damage the instruments.
17. Choosing the right gradient is a bit of an art and may require some trial and error. It may be useful to consider the LC/MS retention time and start with a gradient that was successful in purifying a compound with a similar retention time, based on your group's experience or the literature.
18. Depending on the size of your HPLC column, you may need to inject less than the entire volume of the solution and run the HPLC multiple times until all the solution has been purified, so that the column is not overloaded. It is nevertheless a good idea to first inject a small amount of solution first, to make sure that the gradient is appropriate and other parameters are acceptable. The column that the Wang lab uses is Phenomenex Luna 5 μm C18 (2), 250×21.2 mm.
19. It is advantageous to be aware of not only the secondary metabolites that are produced by the species in question but also many of those that are produced by other filamentous fungi. In our experience, a metabolite that is new to *A. nidulans* has much of the time been previously observed in another species. There is obviously a benefit, then, to be aware of many fungal metabolites and to think about whether there is any

possibility that one of them is the same as the metabolite that you have purified.

20. Infrared (IR) spectroscopy can also be used in conjunction with NMR to characterize the molecule (and some journals will require IR characterization). Although it is typically not as effective as NMR in illuminating the structure, it can be useful in determining the presence of various functional groups. The reader is advised to consult an organic chemistry textbook for more information. Many institutions will have their own IR spectrometers that are available to its members or to visitors upon request.

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