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NMR Spectroscopy

Differential Analysis of 2D NMR Spectra: New Natural Products from a Pilot-Scale Fungal Extract Library**

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The efficient analysis of small-molecule mixtures underlies many endeavors in chemical biology. The sensitivity of mass spectrometry (MS) has resulted in its widespread adoption for such analyses, and today rapid automated LC-MS analyses are widely used. Several recent studies have demonstrated the feasibility of NMR spectroscopic analyses of complex small-molecule mixtures, including the use of diffusion-ordered spectroscopy (DOSY)[1] or principal component analysis (PCA) in metabolomics,[2] as well as the characterization of crude unfractionated natural product extracts using routine two-dimensional NMR spectra.[3] Compared to MS analyses, 2D NMR spectroscopic investigations of small-molecule mixtures offer the benefit of more detailed structural information, which is of particular relevance for the detection of novel chemotypes. However, the complexity of 2D spectra typically obtained for small-molecule mixtures has limited a broader implementation of NMR spectroscopy for their characterization. Herein, we describe a simple procedure for the differential analysis of arrays of 2D NMR spectra and demonstrate its utility for the detection of new natural products from a small library of fungal extracts.

Fungi are prolific producers of natural products derived from terpene,[4] polyketide,[5] and nonribosomal peptide pathways.[6] Several lines of evidence indicate that only a fraction of the biosynthetic capabilities of fungi (and other cultured organisms) are discovered in traditional screening operations, as most secondary metabolite pathways are not expressed under the culture conditions used. Various approaches are being pursued to increase the accessible fraction of fungal metabolomes, and anecdotal evidence suggests that fungi respond to even small variations in their culturing protocol by starting (or stopping) the biosynthesis of specific natural products.[7–11] Clearly, a more systematic exploration of factors modulating secondary metabolite biosynthesis in fungi (and, by the same token, in bacteria) would be highly desirable. In a pilot study, we used differential analyses of 2D NMR spectra for the characterization of a small library of fungal extracts derived from a *Tolypocladium cylindrosporum* strain, cultured with a variety of protocols, which quickly revealed two new terpenoid indole alkaloids.

T. cylindrosporum strain TC705 was selected from a group of insect-pathogenic fungi[12] because it has a number of nonribosomal peptide and polyketide biosynthetic genes that suggest a high metabolic potential for the production of secondary metabolites.[13] For our studies, TC705 cultures were grown using seven different protocols, based on four different media (YM, SDY, mEM, and diEM; see Supporting Information for full details). Three protocols (YM-SDY, YM-mEM, and YM-diEM) included growing cultures in a two-step fermentation procedure, whereby each culture is initiated using a nutrient-rich medium and then transferred to a minimal or partially nutrient-deficient medium.[14] For subsequent NMR spectroscopic analyses, ethyl acetate extracts of the fungal broths were used.[14]

The initial NMR spectroscopic analysis of the unfractionated extracts was based on double quantum filtered correlation spectroscopy (DQF-COSY), as previous experience had shown that a single DQF-COSY spectrum often provides sufficient information to recognize the presence of significant quantities of any unusual small molecules.[3] DQF-COSY spectra were acquired for 25 extracts derived from three repetitions of the seven culturing protocols and four media controls, using a set of acquisition parameters optimized for high resolution in both frequency dimensions. As expected, the resulting DQF-COSY spectra were extremely complex, and a detailed cross-peak-by-cross-peak analysis of all 25 spectra was not feasible. To address this challenge, we developed a simple two-step protocol for a differential analysis of the DQF-COSY spectra (Scheme 1).

The first step consisted of a graphical analysis based on multiplicative stacking of bitmaps derived from magnitude mode versions of the DQF-COSY spectra.[15] This technique clearly distinguished signals present in only one spectrum from signals common to several spectra. For example, overlay

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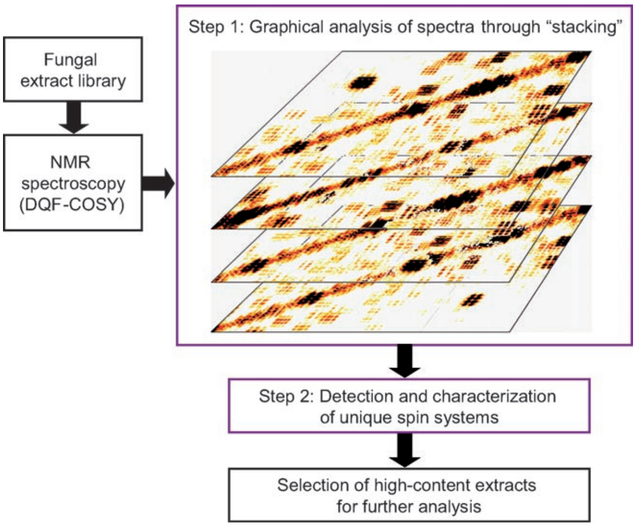


Scheme 1. Two-step differential analysis of DQF-COSY spectra obtained for a library of fungal extracts.

of the spectrum obtained for the YM-SDY protocol (Figure 1 a) with the three spectra obtained for the SDY-only protocol, the YM medium control, and the SDY medium control showed that most signals present in the YM-SDY spectrum correspond to compounds also present in the media controls or the SDY-only extract, as indicated by partial extinction and strong color shifts of these signals (Figure 1 b). Only a small subset of signals in the YM-SDY spectrum remained unaffected by superposition of the three other spectra. These signals correspond to compound(s) present only in the YM-SDY extract. In this manner, a simple graphical manipulation of the COSY-derived bitmaps, which can be accomplished using commonly available image editing software, clearly distinguished signals corresponding to compounds produced only under a specific culturing protocol from signals of compounds produced under most conditions. The graphical manipulation of the bitmap spectra described here is significantly more efficient than subtraction of spectra, because it results in obvious color shifts and partial extinction of signals common to two spectra even in cases where the concentration of the corresponding compound in the two extracts being compared is vastly different. In fact, the efficacy of this comparison method is limited primarily by the dynamic range and sensitivity of the NMR spectrometer.[15]

The second step consisted of a more detailed analysis of the signals representing unique or unusual metabolites in a specific extract. The corresponding spin systems were characterized based on the phase-sensitive originals of the DQF-COSY spectra (Step 2 in Scheme 1; Figure 1 c). For extracts containing structurally intriguing components, additional heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra were acquired.

Subjecting the DQF-COSY spectra of the extracts derived from the seven culturing protocols and media controls to this evaluation protocol immediately revealed significant differences. Extracts derived from protocols using mEM or diEM

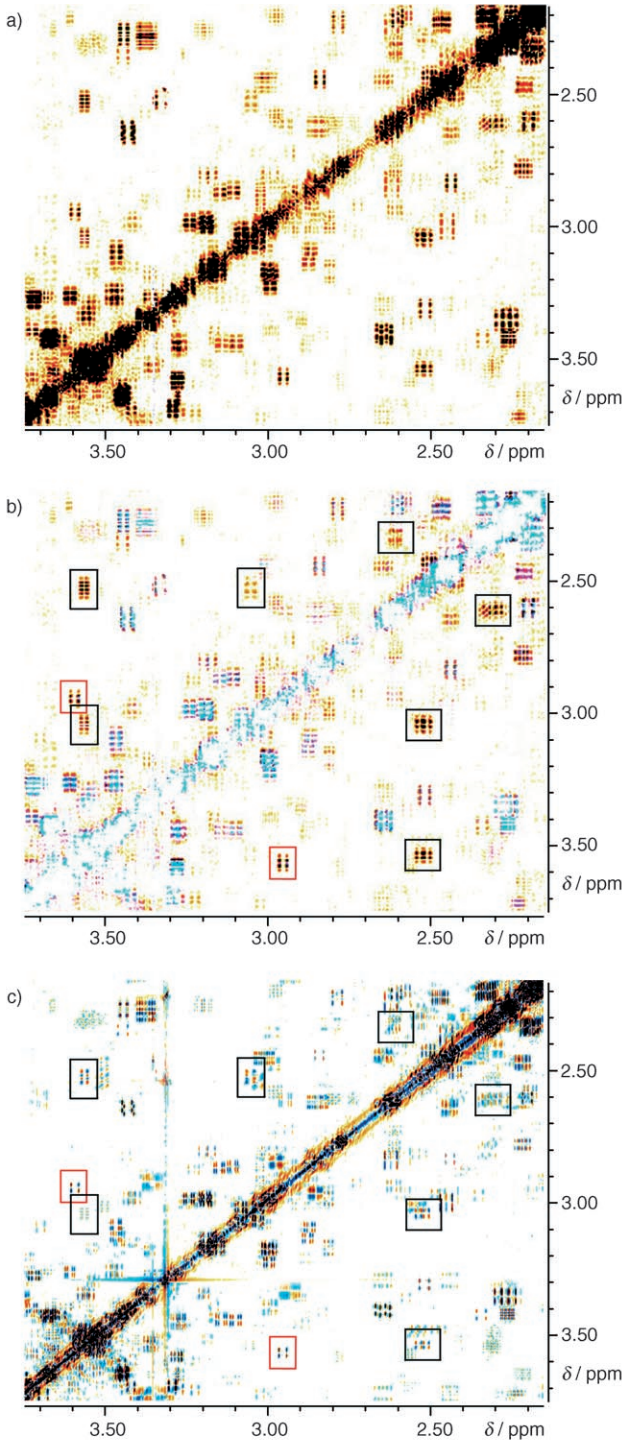
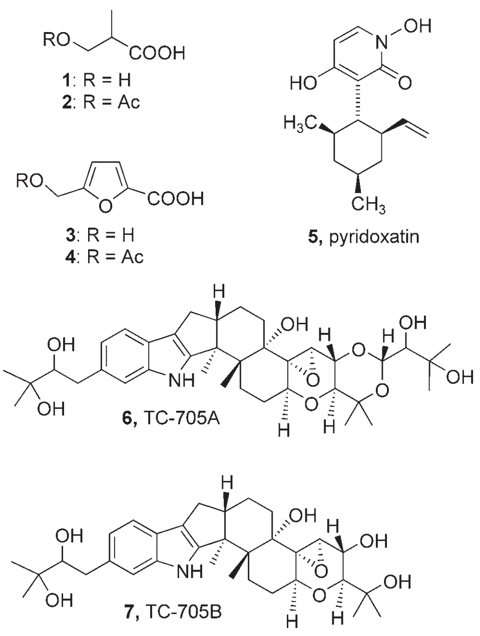


Figure 1. a) Section of the magnitude-mode DQF-COSY spectrum obtained for the YM-SDY extract. b) Same spectrum after multiplicative stacking with spectra for the YM extract, SDY extract, and SDY medium control, showing partial extinction and strong color shifts for signals that are present in the YM-SDY spectrum *and* in at least one of the YM, SDY, and SDY medium spectra. Cross-peaks unaffected by the multiplication layers represent compounds present only in the YM-SDY extract. Cross-peaks marked with black rectangles correspond to compounds **6** and **7**, whereas those marked with red rectangles represent fatty acid ethanolamides. c) Phase-sensitive representation of the DQF-COSY spectrum used for detailed characterization of the spin systems of **6** and **7**.

media contained large amounts of 3-hydroxyisobutyric acid (**1**) and its acetyl derivative **2**, whereas the furan derivatives **3** and **4** were produced only under the two protocols using mEM medium (Scheme 2).[16] The analyses of compounds specific to the three two-media combination protocols were particularly interesting. Under the YM-mEM protocol, large amounts of compounds represented by several spin systems in the aromatic and aliphatic regions were produced. These compounds were also detected in extracts derived from the YM-SDY protocol, although in smaller amounts (Figure 1 b). Analysis of the YM-mEM DQF-COSY spectra and additional HSQC, HMBC, and NOESY spectra of the unfractioned extracts suggested structures **6** and **7**, which represent two previously unreported terpenoid indole alkaloids (Scheme 2).[17–19] As these alkaloids constitute new natural products, we subsequently isolated **6** and **7** from the best-producing YM-mEM media combination by reversed-phase HPLC and confirmed the structural assignments shown.[20]



Scheme 2.

Detailed analysis of the DQF-COSY spectra derived from the seven fermentation protocols showed that **6** and **7** are consistently produced in high yields only in the YM-mEM protocol, while the amounts produced under the YM-SDY protocol varied. Furthermore, analysis of the extracts by both NMR spectroscopy and HPLC-UV confirmed that extracts derived from single-medium mEM or SDY cultures contained none or only trace amounts of **6** and **7**.[14]

Only one of the major components in these extracts was produced under all seven protocols. This compound was identified as the known fungal metabolite pyridoxatin (**5**), which is produced as a major component under all but one of seven protocols.[21] The exception was the YM-mEM media combination, in which case only trace amounts of **5** were found.

To validate the results of our NMR-based analyses, all media extracts were subjected to additional HPLC/electrospray ionization (ESI) MS analyses, which showed significant differences between the various extracts as well. However, the ESI mass spectra alone provided little structural information compared to the NMR spectroscopic analyses. Furthermore, positive electrospray ionization efficiencies of secondary metabolites identical to "secondary metabolites", such as pyridoxatin (**5**) or the terpenoids **6** and **7**, were orders of magnitude lower than those for peptides and other amino acid derivatives, which resulted in a strongly skewed representation of the actual compositions. Accordingly, the strongest peaks in the HPLC/ESI-MS analyses represented amino acids and several series of oligopeptides, whereas the NMR spectra indicated that peptides account for only a small fraction of the total extracts. The major peptide components as identified by HPLC/ESI-MS were a series of efrapeptins,[22] variable amounts of which were produced under all protocols. In addition, two series of as-yet unidentified peptides were produced under the YM-mEM protocol. In this regard, HPLC/ESI-MS and NMR spectroscopic analyses complement each other.

These results show that, as predicted by earlier PCR analysis,[13] the metabolism of TC705 is highly variable and responds strongly to changes in culturing conditions. The two-stage differential analysis of NMR spectra obtained for the unfractionated extracts allowed the rapid detection of two new natural products.[23] The scope of such NMR spectroscopic characterization of largely unfractionated extracts from fungal, bacterial, and other sources could be easily extended. The analyses described here are primarily limited by the finite dynamic range of NMR spectroscopy, and as a consequence, most components accounting for less than a few percent of the total extracts cannot be reliably characterized because signal-to-noise ratios for the corresponding signals are too low. Compounds missed by the NMR spectroscopic analysis included the various oligopeptides that were detected by LC-MS. Detection limits could be lowered considerably by including a coarse prefractionation of the extracts prior to NMR spectroscopic analysis. As graphical comparison of the DQF-COSY spectra is fast, the corresponding increase in the number of spectra could be easily addressed. Acquisition of larger numbers of spectra could be accomplished for example by using recently introduced capillary NMR technology (CapNMR).[24]

In comparing our analyses to other NMR-based approaches for characterizing complex mixtures of small molecules,[1,2] it should be noted that our primary goal was the detection and characterization of novel metabolites. Our approach thus focuses on extracting structural information (connectivity information) instead of determining characteristic quantitative differences in integrated signal intensity.

Differential analysis of NMR spectra provides a useful tool for a non-discriminatory characterization of small-molecule mixtures, with many potential applications in metabolomics and natural products chemistry. Among these, the possibility of complementing bacterial and fungal genetics with NMR-based differential analysis of corresponding changes in secondary metabolite production is particularly intriguing.

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