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High-Throughput Profiling of Microbial Extracts

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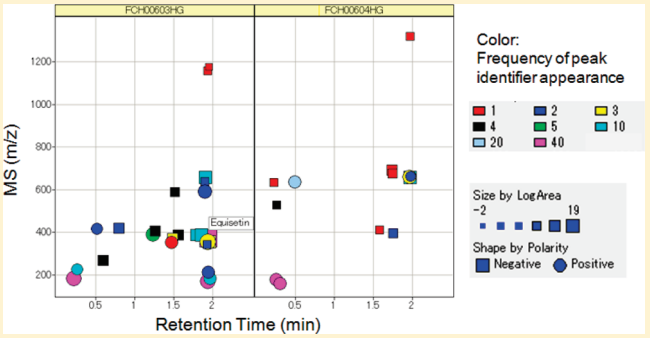
Supporting Information

ABSTRACT: Microbial culture extracts are used for natural products screening in the drug lead discovery process. An extract of a microbial culture is a complex mixture of organic compounds, making it difficult to evaluate the diversity and redundancy of the compounds. However, having a diverse extract library is a key to success in generating a lead for drug discovery. We have developed a high-throughput and robust LC-MS analysis and data processing method for visualizing sample profile and diversity. In the LC-MS analysis of 16 025 microbial culture extracts, positive and negative ions were acquired simultaneously with an electrospray ionization source. The raw data were processed using ACD IntelliXtract, and peak lists of each extract were generated and stored in the database. The peak list data were binned by nominal mass (m/z 150−1350) and retention time. How frequently the binned peaks, termed peak identifiers, appeared in the extracts library was calculated, and these data were visualized by scattered plots in Spotfire. From 4 to 115 peaks were observed in each sample. As it is easy to eliminate the ubiquitous peaks shown in most samples and simplify the plots, the unique or redundant compounds could be detected.



Natural products play an important role in the drug lead discovery process.[1,2] Microbes are a proven resource for new compounds,[3,4] and many groups collect and evaluate microbes, especially fungi and actinomycetes strains. The strains are cultured in various fermentation conditions, and the crude extracts and/or fractionated extracts are stored as libraries for natural products screening. A large number of natural product samples are used for high-throughput screening (HTS) in the same way as compound libraries.[5] However, natural product samples differ greatly from the synthesized compound libraries in the following ways: they have much more diverse carbon skeletons and stereochemistries; they are mixtures of unknown compounds; the concentrations of the compounds are unknown; they are sometimes redundant with no drug-like components in the sample; and finally, some compounds with potent nonselective activities frequently appear during screening.

To overcome these problems, various improvements to the basic screening method have already been made.[6] After obtaining a hit in primary screening, the active compounds must be purified in bioassay-guided steps and their structures elucidated, an extremely laborious and time-consuming process. Many of the compounds are already known and of no real interest, and dereplication processes have been reported in which known nuisance compounds can be eliminated.[7,8] Combining LC-UV-MS with micro-scale fractionation is a general procedure for dereplication,[9,10] and detailed dereplication data of 472 fungal metabolites have been reported.[11] Combining ion exchange solid-phase extraction with other methods was also developed for dereplication.[12] Finally, a rapid extract dereplication method using LC-SPE-NMR was developed for the analysis of isoflavonoids.[13]

Nevertheless, when natural product samples are used for HTS, the number of active samples in some cases is several percent of those tested and the dereplication process including fractionation and bioassay of fractions requires significant resources.

For this reason, we wanted to analyze all samples in our natural product library before screening. Using the data generated, we could judge compound duplication and in some cases identify compounds, and store this information in a database to support the rational selection of natural product samples for primary screening. Using the data generated from the screening process and information stored in the database, we could focus on only those samples with the greatest promise.

Profiling methods for crude extracts had been reported in the area of untargeted metabolomics study.[14] Gas chromatography coupled with mass spectrometry (GC-MS),[15,16] liquid chromatography−mass spectrometry (LC-MS),[17−19] and NMR[20,21] technologies were developed for these studies. Microbial extracts are diverse, comprising compounds that are highly polar to those that are highly hydrophobic, have a broad metabolite concentration range, and are sometimes complex mixtures. Reversed-phase LC-MS is a suitable method for profiling the metabolites for natural products screening, having the advantage of sensitivity, robustness, and wide applicability.

Furthermore, ultrahigh-performance liquid chromatography coupled mass spectrometry (UHPLC-MS) has recently been developed and has become widely used.[22−24] The small particle size of a

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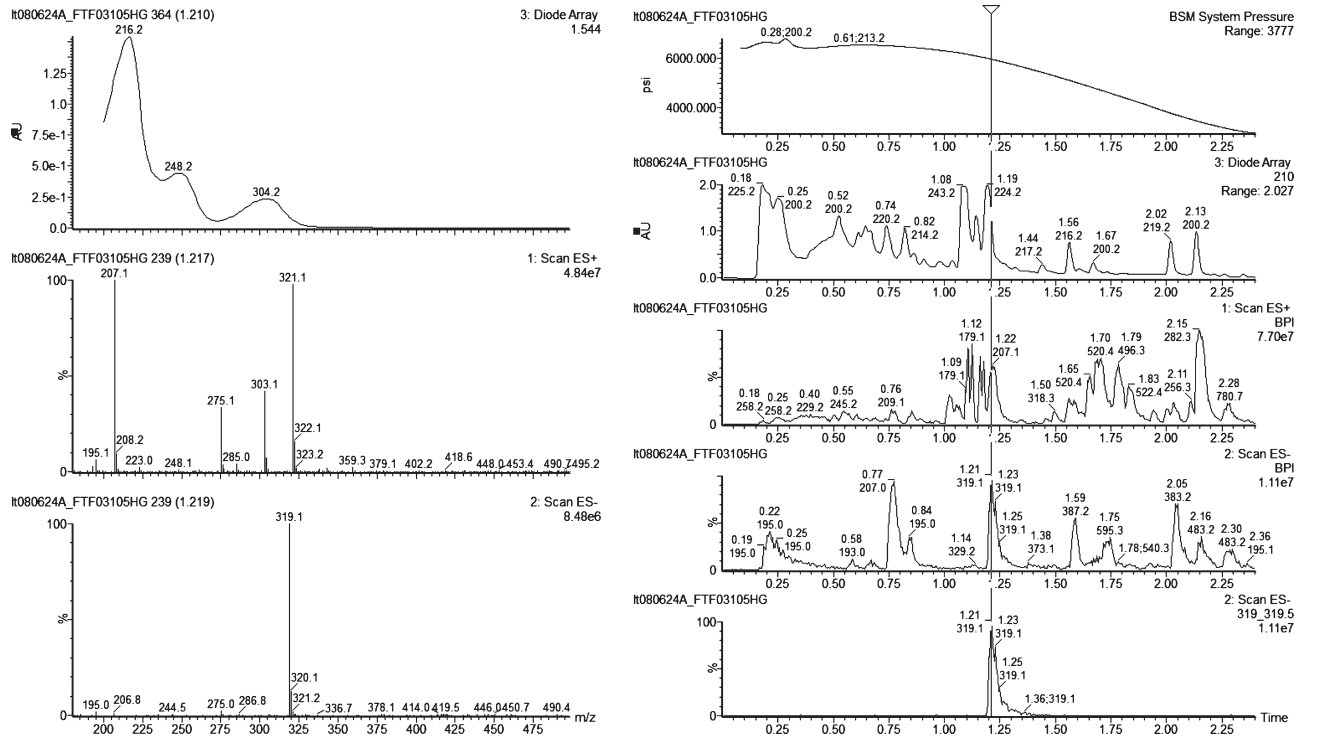

Figure 1. Typical chromatogram and spectrum of a fungal extract recorded by UHPLC-MS. The right panel shows (from top to bottom) system pressure, UV chromatogram at 210 nm, ESI positive and negative base peak chromatograms, and MS chromatogram at m/z 321 (negative). Mycophenolic acid was detected at 1.21 min, and the left panel shows UV and ESI positive and negative of the metabolite at a retention time of 1.21 min.

UHPLC column makes it possible to analyze complex mixtures of microbial extracts in minutes. In response to narrow LC peaks, an instrument able to scan the mass at high speed was developed, and UHPLC-MS was investigated as a method to profile natural product samples.[25,26]

For the purpose of processing the large number of samples (>1000) the method used for untargeted metabolomics study[27] was considered. With this method, LC-MS analysis is conducted, and retention time, m/z, and intensity data are captured; the retention time and m/z values are aligned across all samples.[28] In the alignment process, the fluctuation of retention time and m/z between analyses is corrected by various algorithms, and the aligned data are subjected to various multivariate analyses. However, trying to use this alignment process and multivariate analyses across the data of up to several tens of thousands of different samples was not feasible when profiling a natural products library. To overcome this, we used the binning method, which, although it is not used in LC-MS study, is used in ^{1}H NMR metabolomics to analyze mixtures.[29] Each NMR spectrum is binned into 225 regions over a range of 0.5 to 9.5 ppm, and each region is integrated. We thought that the binning method would be simple and easy to use for a large number of LC-MS data and would be appropriate for evaluating the uniqueness of crude extract contents.

In this study, we developed a robust high-throughput analysis method using ACD MS Manager with add-in software IntelliXtract[30] for the purpose of evaluating the natural products samples and used it to analyze 16 025 microbial samples. This profiling method provides an effective tool that chemically clarifies the contents of microbial extracts and could be applied to other natural product samples including those of plant origin.

RESULTS AND DISCUSSION

Development of UHPLC-MS Analysis. A UHPLC analytical method using a 2.1 × 30 mm C18 UHPLC column in which analytical time was 2.4 min (cycle time 3.1 min) was developed (Figure 1).

Because we expected clogging and rapid depletion of the UHPLC column, we centrifuged samples to remove insoluble particles. In addition, a precolumn filter and guard column were used, and four column lines were exchanged every 88 samples, which made the analytical system more robust and made it possible to continue analysis of 1320 samples in a single 72 h experiment. Reproducibility of the retention time and mass are important for the later binning process, and therefore a quality control (QC) sample containing eight typical natural compounds was injected every 44 samples, and the retention time of all QC peaks was checked, allowing 0.03 min and m/z 0.3 as margins for error. During the QC check, the system pressure and shape of chromatographic peaks were also monitored. If some abnormality was detected (typically a blocked guard column), the data from that batch were discarded and the samples reanalyzed after the problem had been addressed.

A Waters single quadrupole mass detector (SQD) was used because of its fast polarity switching. Scan speed was tuned to maximum speed in this instrument, and 3.5 scans per second in each polarity (total 7 scans per second in both polarities) were performed, while the average of full width at half-maximum (fwhm) of peaks in our study was around 1.2 s. The sensitivity to natural compounds was diverse, with some compounds being detected only in positive mode (ESI+), other compounds detected only in negative mode (ESI−), while some compounds could be detected at both polarities (Figure S1). Previously, the

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results of a multiple ionization study of the human serum extract found that ESI in both positive and negative mode is most comprehensive compared with APCI and mixed mode ionization,[31] and in our experiments we employed both polarities in ESI for comprehensive compound detection.

Peak Picking with ACD IntelliXtract and Data Processing. The work flow of the data processing is summarized in Figure 2. Peak picking from the raw LC/MS data was performed using ACD IntelliXtract, an add-in software of ACD MS Manager. MassLynx files were converted to ACD MS Manager native files using Splitter.exe (an additive program of ACD MS Manager). Next, ACD MS Manager files were processed by IntelliXtract, using the Group Macro function of ACD MS Manager, into positive and negative. The thresholds of peak picking in positive and negative mode (ESI+ 1 000 000; ESI− 100 000) were restricted by Dynamic Filter, a function of IntelliXtract. The threshold was optimized for the visible base peak ion (BPI) chromatogram. From 4 to 115 peaks (the sum of positive and negative modes) were extracted from each sample. The average number of peaks picked was 34.5.

The output of ACD IntelliXtract is a simple text file (Table S2) containing the integer value of m/z, retention time, peak area, peak height, base peak, notation of ions (M + H, M + Na, M + NH^4, 2M + H, M − H, M + HCOO), existence of halogen (A+2 column), and so on. By limiting the polarity and notation as M + H or M − H in this way, we expected to obtain one peak per compound.

The UHPLC analysis time of 2.4 min was divided into 48 fractions. One fraction is 0.05 min (3 s), which is almost double the time of the general peak fwhm (1.2 s), so, although the possibility of a peak being spread over three fractions is low, it is more probable for one peak to be registered in two fractions. Nominal mass values exported from IntelliXtract were used ranging from 150 to 1350 m/z.

Peak identifier (PI) is the key text variable in this processing. The PI was constructed from four values: analytical method (fixed: UpSQD in this study), polarity (positive or negative), nominal mass (4 digits), and fraction (2 digits, calculated from retention time). These four values were concatenated with "_", using the function of Microsoft Excel 2003 or Microsoft Access 2003. As an example of PI, "UpSQD_Positive_0381_25" indicates the peak shown in positive mode, m/z 381, and retention time 1.20 to 1.25 min (Figure S3).

In this study, 16 025 samples were analyzed, and all peak data were stored in a single table of Microsoft Access. The frequency with which each PI appeared was tallied up using the query function of Microsoft Access, and this summarized information of PIs was referred to from the peak table using PIs as key.

We used Spotfire Decisionsite for data visualization (Figure 3). Peak information was plotted in one panel for every sample, using the vertical axis for MS, horizontal axis for retention time, symbol shape as polarity, and symbol size as peak intensity, with the frequency of PI represented by the symbol color. The peaks caused by the medium ingredient or primary metabolite of microbes were eliminated from the plot (Table S3, Figure S4). Graphs of samples of an arbitrary number were displayed on one screen using the Trellis function.

Results of Analyzing 16 025 Samples. Five groups of the fungal extract library and two groups of the actinomycetes extract library, making a total of 16 025 samples, were analyzed (Table 1). Group A, consisting of 5924 samples, displayed a total of 255 094 peaks, with an average of 43.1 peaks per sample. The number of PIs, representing the total when duplicate peaks had been omitted, was 24 235, suggesting that approximately 90% of observed peaks were redundant.

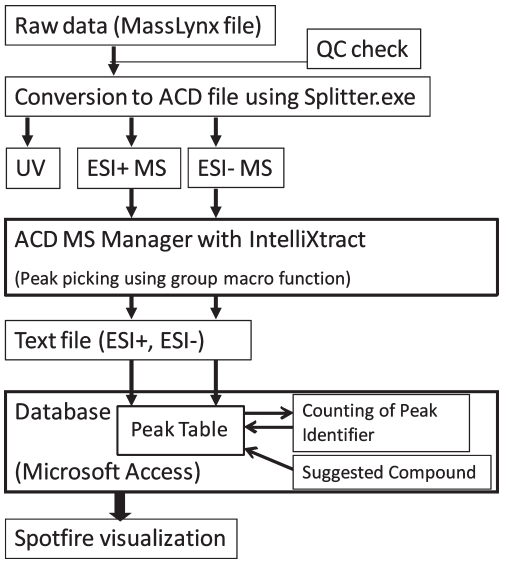

Figure 2. Work flow of data processing from MassLynx raw file to Spotfire visualization. ACD IntelliXtract was used for peak picking.

After adding the 1376 samples of group B to group A, we observed 9654 extra PIs, but this was reduced to 3629 PIs when we omitted duplicates of those observed in group A. The total number of unique PIs for groups A and B thus became 27 864. The number of PIs accumulated in this manner is shown in Figure 4. By this method we could evaluate how much the new samples had enhanced the peak diversity of the existing library. In total, 38 753 PIs were observed, which, when divided by the total number of samples, suggests that, on the whole, 2 to 3 unique peaks existed in each sample (38 753/16 025 = 2.42).

Groups E and G, which were samples from actinomycetes, had fewer PIs compared to the fungal extracts, leading us to think that the average content of compounds in the actinomycetes extracts was lower than in the fungal ones.

To visualize the contribution of each group to the library, a comparison of group-specific PIs to other groups can be made. An example for group A is shown in Figure 5 with information taken from Table 1. The value of group-specific PI per sample was spread from 0.44 to 1.73, and this value suggests the uniqueness of sample groups compared to the library as a whole. The average was 1.15 and was different from the accumulation of PIs (2.42), because some common PIs in the accumulation process were counted that were not counted when comparing the individual groups to the whole library. Analysis of PI by this method is a simple and powerful way to evaluate the compound diversity in the mixture samples and sample group.

Suggested Compound and Problem of Binning. A total of 115 200 PIs were calculated from the range of acquired mass (1200), 48 fractions, and two polarities, which we decided was sufficient to cover the number of known microbial natural products, recorded as 37 191 in Antibase 2010.[32] If the known compounds were analyzed beforehand, and PIs of such compounds were accumulated, a simple compound identification might become possible. We made a table of suggested compounds that contained the field of PIs as a key. Referring to this table, the suggested compound name was shown in the Spotfire visualization (Figure 3).

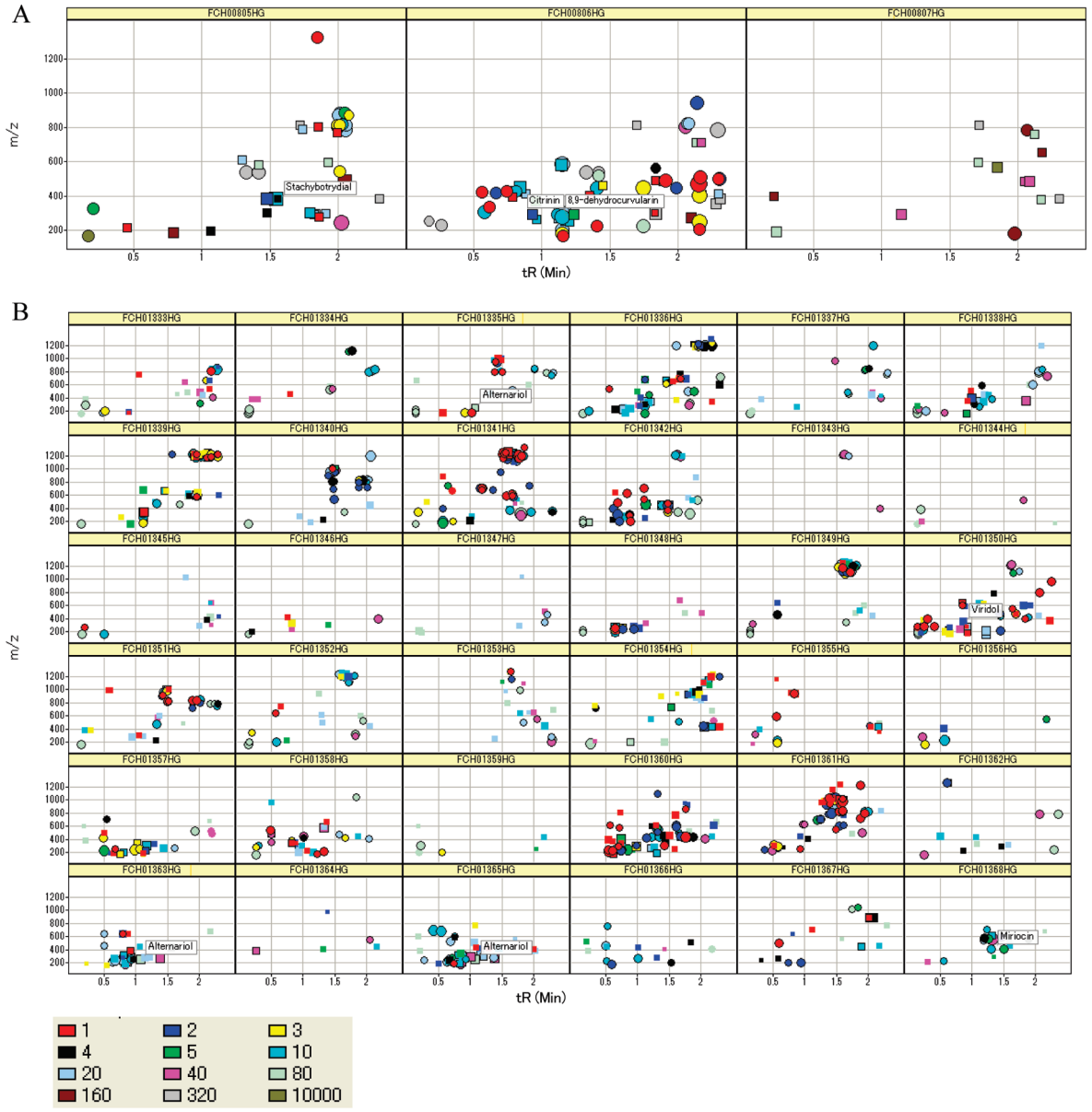

Figure 3. Visualization of peak uniqueness using Spotfire Decisionsite (A: detailed graph, B: multiple visualization using Trellis function). Horizontal axis is retention time (min); vertical axis is mass (m/z). Each cell shows one sample. Squares indicate positive ion and circles indicate negative ion of mass. Symbol size represents peak intensity. Frequency of PI was indicated by color as shown at bottom of figure. Ubiquitous peaks (frequency >80) are hidden in B.

Table 1. Analytical Results of 16 025 Samples

group	microbe	number of samples	number of peaks	number of peaks/sample	peak identifiers (PIs)	accumulation of PIs	group-specific PIs	group-specific PIs/sample
A	fungi	5924	255 094	43.1	24 235	24 235	10 257	1.73
B	fungi	1376	47 342	34.4	9654	27 864	2120	1.54
C	fungi	1624	56 254	34.6	9759	30 892	1850	1.14
D	fungi	1587	46 380	29.2	9013	33 620	2190	1.38
E	actinomycete	854	24 480	28.7	2025	34 302	379	0.44
F	fungi	2474	70 749	28.6	11 858	37 013	2575	1.04
G	actinomycete	2186	52 320	23.9	5063	38 753	1740	0.80
total		16 025	552 619	34.5	71 607	38 753	21 111	1.15[a]

[a] Average of group-specific PIs/sample.

Because of a problem peculiar to the binning technique, the peaks situated in the vicinity of a bin boundary are divided into different PIs. The reproducibility of the UHPLC-MS analysis in this study using a QC check guarantees a mass error less than m/z 0.3 and retention time error less than 0.03 min. Theoretically therefore, it is possible that one compound appears as anywhere

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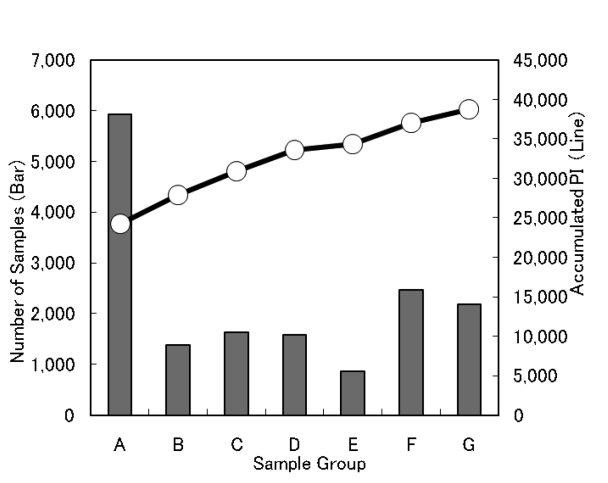

Figure 4. Accumulation of PIs during the addition of sample groups to the natural products library. Bars represent the number of samples in the group. Line represents the accumulation of PIs. Group A originally has 24 235 PIs, but as each subsequent group (B−G) is added, this amount increases, finally totaling 38 753 PIs.

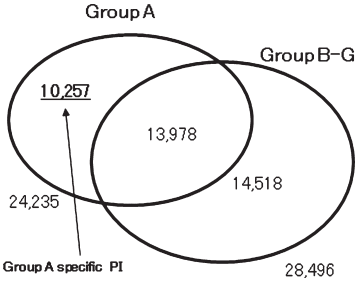

Figure 5. Group-specific PIs: Group A in comparison to other groups.

from two to six different PIs in one polarity. For example, actinomycin D (monoisotopic mass 1254.6, experimental retention time 1.69 min) was registered as having eight different PIs in both positive and negative data, but it was actually observed as having only three PIs (Figure S5).

The above-mentioned binning problem means that when evaluating the novelty of the peaks we should consider the possibility that the peaks of adjoining different PIs are actually the same compound. On the other hand, compounds at the same molecular weight and the same retention time could also overlap by chance and be shown as the same PI. Therefore, the relationship between the compound and the PI is not always absolute, and the number of peaks therefore approximates the number of compounds to some degree, at this time.

EXPERIMENTAL SECTION

General Experimental Procedures. LC-MS grade acetonitrile and formic acid was purchased from Wako Pure Chemical (Osaka, Japan). Water was obtained from Milli-Q system from Millipore (Millerica, MA).

Actinomycin D, antimycin A, and erythromycin were purchased from Sigma-Aldrich (St. Louis, MO). Chloramphenicol, daunorubicin, genistein, griseofulvin, and mycophenolic acid were purchased from Wako Pure Chemical. Alternariol, curvularin, piericidin A1, radicicol, and rugulosin were stock-purified samples from our laboratory.

Microbial Fermentation Extracts Library. Fungal and actinomycetal strains were cultured from frozen stock in our strain library. After appropriate seed culture (e.g., 1.5% glycerol, 2.0% glucose, 1.0% potato starch, 0.3% NaCl, 0.35% yeast extract, 0.25% polypeptone, 0.5% $CaCO_3$, 1.0% Toast soya, 0.005% $ZnSO_4$, and 0.0005% $CuSO_4$) had been performed, 1% to 10% of seed was transferred to the various conditions to produce cultures. We used both solid and liquid cultures. Solid culture (8 g wet weight; e.g., 5 g brown rice, 3 mg yeast extract, 1.5 mg sodium tartrate, 1.5 mg KH_2PO_4, and 3 mL distilled water) was extracted with 13 mL of methanol, while liquid culture (e.g., same as seed culture) was extracted with an equal volume of butanol. These extracted solutions were evaporated using Genevac HT-12 centrifugal evaporator (Ipswich, England) and dissolved in DMSO (concentrated 3-fold).

UHPLC-MS Instrument. The UHPLC was performed on a Waters ACQUITY UPLC system (Waters, Milford, MA), which was equipped with a binary solvent delivery manager, sample organizer (22 microtiter plate capacity), column manager (four column switching), and photodiode array detector. MS detection was performed on a Waters SQD. The instrument was fitted with four Acquity UPLC BEH C18 columns, 1.7 μm, 2.1 × 30 mm (Waters), operated at 40 °C.

Linear gradient analyses with mobile phase A, H_2O (0.1% formic acid), and mobile phase B, acetonitrile (0.1% formic acid), were performed with a flow rate of 0.6 mL/min. Gradient conditions were as follows: 0−0.05 min hold at 10% B, 0.05−2 min linear gradient 10% to 98% B, 2−2.4 min hold at 98%, 2.4−2.42 min 98% to 10%, 2.42−3.1 min hold at 10%.

Library samples were diluted up to 6-fold with a 1:1 DMSO/MeOH mixture and centrifuged at 1500*g* for 10 min. A 4 μL amount of supernatants was injected for UHPLC.

The MS range scanned was m/z 150−1350 at an estimated scan speed of 10 000 amu/s. Positive and negative ions were acquired simultaneously (rapid switching). During the 2.4 min acquisition phase, 500 scanned data were obtained at each polarity. All flow from the column and PDA detector was directly introduced to the ESI source (no splitter used).

All acquisition and data collecting were performed by MassLynx software. Approximately 3.5 MB data including ESI+, ESI−, and PDA were stored in each sample. The blank suppression was not performed in this study.

For QC of the UHPLC-MS analysis, a mixture solution (0.125 mg/mL each) of antimycin A, actinomycin D, erythromycin, genistein, griseofulvin, and mycophenolic acid was injected every 44 samples.

Using UHPLC-MS, around 400 samples per day can be analyzed. This analytical method can be said to be robust because there has been no fatal trouble other than mechanical malfunction of a UHPLC-MS sample organizer (auto sampler), despite having analyzed 52 000 microbial extracts so far. As for data processing, the speed is faster than UHPLC-MS acquisition. Processed data were stored in a database such as Microsoft Access, so it can easily be used again.

Peak Picking of ACD IntelliXtract and Data Processing. ACD MS Manager and ACD IntelliXtract (version 11.1, Advanced Chemistry Development, ACD, Toronto, Canada) were purchased from and technically supported by Fujitsu (Tokyo, Japan). This software was installed on a Windows XP PC that has a 2.83 GHz Core 2 Duo processor and 3 GB RAM.

MassLynx data files were converted to ACD native files of ESI+, ESI−, and PDA using the batch program of Splitter.exe (ACD additive software). The batch program was coded by Excel VBA. We used the Group Macro function of ACD MS Manager to control the number of peaks picked by IntelliXtract. The IntelliXtract settings were changed from the default setting as follows: fwhm deviation to 30%, mass accuracy to integer, CODA window width to three scans, peak picking to the three most abundant peaks. The peak table generated by IntelliXtract was processed by Dynamic Filter. After low-intensity peaks (ESI+ <1 000 000; ESI− <100 000) had been eliminated, the data were exported as a text file. Each exported text file (2 files/sample) was

assembled in one table by the VB Script program. The retention time of LC analysis (2.4 min) was divided into 48 fractions using the following formula.

$$\text{Fraction} = \text{integer}(RT) \times 20 + 1$$

The analytical method, polarity, integer mass, and fraction were concatenated as Figure S3. Spotfire Decisionsite (TIBCO, Palo Alto, CA) was used for the data visualization.

Known compounds (0.125 μg/mL, 4 μL) were analyzed by UHPLC-MS. The data were confirmed by ACD IntelliXtract peak picking and by direct observation of chromatograms on a MassLynx. Monoisotopic mass value was used from Antibase 2010 (Wiley-VCH) and Dictionary of Natural Products (Chapman & Hall).[33]

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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