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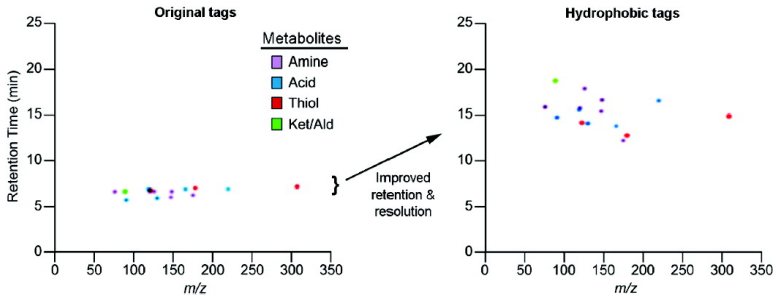

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Enrichment Tags for Enhanced-Resolution Profiling of the Polar Metabolome

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A principal goal of post-genomic research is to map the complex array of metabolic and signaling networks that regulate health and disease. These efforts require the integrated application of large-scale profiling methods, such as genomics,[1] proteomics,[2] and metabolomics.[3] Metabolomics, or the study of the full complement of endogenous small molecules in biological systems, has facilitated important discoveries, including, for example, the identification of biomarkers[4] and enzymatic pathways[5] linked to human disease. One of the major challenges in metabolomics is obtaining adequate resolution of compounds with similar physicochemical properties.[3,6,7] The resolution of polar metabolites can be exceptionally problematic as these compounds are often poorly retained with reverse phase matrices and require the use of additional techniques such as hydrophilic interaction chromatography, which offer only moderate separation capacity.[6−8] Recently, we introduced a new technology for metabolite profiling, termed metabolite enrichment by tagging and proteolytic release (METPR).[9] Here, we report an advanced version of METPR that greatly improves the resolution and structural characterization of polar metabolites from complex biological systems.

In METPR, metabolites are captured onto a solid support by conjugation to resin-bound reactive groups that target distinct classes of small molecules bearing common chemical functionalities (e.g., amines, acids, thiols, ketones/aldehydes). After enrichment, captured metabolites are liberated from the solid support using a "chemo-orthogonal" cleavage step promoted by the protease trypsin to yield solutions of metabolites that are appended to a permanent tag. These metabolites are then detected by untargeted liquid chromatography−mass spectrometry (LC−MS) analysis. We previously applied METPR to the breast cancer cell line, MDA-MB-231, resulting in the detection of more than 300 putative metabolites.[9] We noted that the captured metabolites tended to cluster into two retention time windows (4.5−7 and 15−20 min) following separation by reverse-phase LC. We observed good resolution of compounds eluting in the later time period (15−20 min). However, many other metabolites migrated at the solvent front and were poorly resolved (4.5−7 min). Identification of several of these compounds confirmed that they were highly polar metabolites (e.g., pyruvate, glutathione, cysteine). The residual tags appended to captured metabolites in these initial studies were derivatives of lysine or the dipeptide, glutamate-valine (Figure 1A). We therefore asked whether addition of a more hydrophobic residue might improve polar metabolite retention and resolution. To test this premise, we designed METPR tags that incorporated a *p*-chlorophenylalanine (Cl-Phe) moiety (Figure 1B).

We selected the Cl-Phe derivative because the chlorine atom displays a distinct isotopic abundance pattern (3.1:1 ratio for ^{35}Cl and ^{37}Cl, respectively), that could be used as a mass signature to distinguish tagged metabolites from background peaks (i.e., false positive ions).[10] This strategy offers an advantage over our original tags, which required visualization of a diagnostic peak in the fragmentation data of each metabolite.[9] The presence of multiply tagged species (e.g., polyamines and acids, such as spermidine and succinate, respectively) should also be discernible by examination of the isotopic distribution pattern. Finally, the unique isotope pattern afforded by chlorine could be utilized as a selection parameter during automated data analysis.[11]

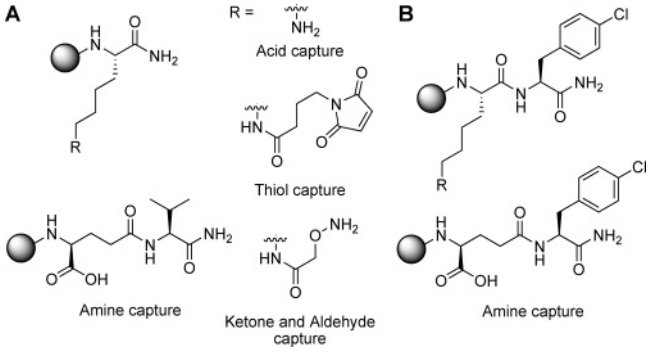

Figure 1. Structures of METPR tags. (A) Original tag designs. Carboxylic acid-, thiol-, and ketone/aldehyde-containing metabolites are conjugated to a lysine derivative. Amine-functionalized metabolites are covalently attached to a glutamate-valine derivative. (B) Cl-Phe tags designed to increase the hydrophobicity of labeled small molecules and improve chromatographic retention and resolution of polar metabolites.

To assess whether the Cl-Phe tag increased the retention and resolution of polar metabolites, a mixture of small molecule standards (1 μmol of each compound) was captured with both the original and Cl-Phe-functionalized tags, released by trypsin, and analyzed by untargeted LC−MS. Notable improvements in retention and resolution were observed for polar metabolites conjugated to the Cl-Phe tag and separated using a modified LC gradient (Figure 2A and B). With the original tag, polar metabolites eluted at or near the solvent front (t_R = 4.66−5.70 min, total elution time of ~1 min, Figure 2A). When conjugated to the Cl-Phe tag, these compounds were well-retained on the reverse phase resin, enabling significantly better resolution (t_R = 11.83−17.60 min, total elution time of ~6 min, Figure 2B). Compounds that only differ by one carbon unit, such as acetic acid and propionic acid, were clearly resolved by the Cl-Phe tag (acetic acid t_R = 15.34 min, propionic acid t_R = 17.60 min). Positively charged polar metabolites, such as the quaternary amine, acetyl carnitine, were also retained and resolved from the other polar metabolites (t_R = 11.83 min). These data also demonstrated that addition of a hydrophobic residue does not cause polar metabolites to coelute with more hydrophobic small molecules. As illustrated in Figure 2A, hydrophobic compounds, which were well resolved from both polar metabolites and each other when conjugated to the original tag (ethyl itoconate t_R = 13.96 min, Fmoc-Ala-OH t_R = 22.39 min), continue to be well separated after addition of the Cl-Phe moiety (ethyl itoconate t_R = 22.65 min, Fmoc-Ala-OH t_R = 32.94 min). The peak broadening seen upon conjugation of the Cl-Phe tag to the most hydrophobic compound,

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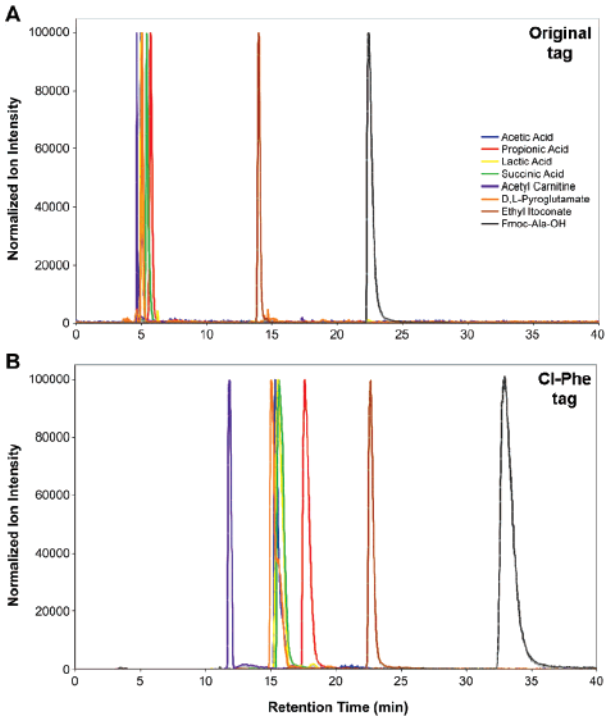

Figure 2. Comparison of the LC elution profiles for small molecules conjugated to the original (A) or Cl-Phe (B) acid-capture tags. The six polar molecules are poorly retained and resolved by the original tag using C18 media: acetyl carnitine t_R = 4.66 min, acetic acid t_R = 4.98 min, lactic acid t_R = 5.00 min, D,L-pyroglutamate t_R = 5.06 min, succinic acid t_R = 5.39 min, propionic acid t_R = 5.70 min. Retention and resolution of these polar compounds were markedly improved upon conjugation to the Cl-Phe tag (B; elution with a modified LC gradient, see Supporting Information for details): acetyl carnitine t_R = 11.83 min, acetic acid t_R = 15.34 min, lactic acid t_R = 15.54 min, D,L-pyroglutamate t_R = 15.05 min, succinic acid t_R = 15.66 min, propionic acid t_R = 17.60 min.

Fmoc-Ala-OH, suggests that the hydrophobic tagging strategy may be most suitable for polar metabolite analysis, making it complementary to the original METPR method.

We next asked whether the Cl-Phe tag could improve the resolution of endogenous metabolites derived from mammalian cells. To explore this issue, we first sought to identify 15 of the putative polar metabolites (i.e., compounds that eluted at the solvent front) observed in our original study of MDA-MB-231 cells.[9] We identified representative metabolites for each of the four targeted functional group classes (i.e., amines, acids, thiols, ketones/aldehydes). These compounds ranged from small organic acids such as lactic acid and pantothenic acid to amino acids, taurine, and the small peptides, cysteinyl-glycine and glutathione (full list provided in Supporting Information).

With the original tagging strategy, the polar metabolites eluted in a very short time window, resulting in poor resolution (Figure 3A, t_R = 5.68−7.07 min). The Cl-Phe-functionalized tags produced striking increases in the retention and resolution of these metabolites (Figure 3B, t_R = 12.16−18.74 min, ∼5 fold increase in separation). Notably, despite their differences in mass, the three thiol-containing metabolites (cysteine, cysteinyl-glycine, and glutathione), when conjugated to the original tag, eluted over a time range of less than 30 s (red signals in Figure 3A, t_R = 6.69, 6.93, 7.07 min, respectively). In contrast, labeling of these compounds with the Cl-Phe tag provided a separation window of more than 2 min (Figure 3B, t_R = 14.17, 12.77, 14.87 min, respectively). Similar results were obtained with polar amine metabolites, which displayed shifts in migration from t_R = 6.01−6.64 min to t_R = 12.16−17.86 min when labeled with the original and Cl-Phe tags, respectively

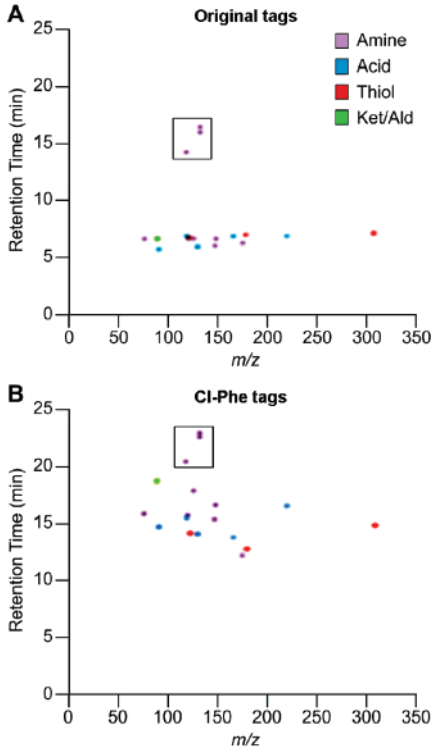

Figure 3. Comparison of the LC elution profiles for endogenous polar metabolites from MDA-MB-231 cells tagged with either the original (A) or Cl-Phe (B) METPR tags. Fifteen polar and three nonpolar (indicated by box) metabolites were characterized. Metabolites are plotted based on LC retention time (*y*-axis) and predicted mass values after subtraction of the tag (*x*-axis). Mass signals are color coded with the spot size and intensity correlating with signal strength.

(Figure 3, purple signals). These metabolites included amino acids that coeluted using the original tag (Gly t_R = 6.62 min, Thr t_R = 6.63 min, Glu t_R = 6.63 min), but were readily resolved after conjugation to the Cl-Phe tag (Gly t_R = 15.81 min, Thr t_R = 15.70 min, Glu t_R = 16.58 min). Finally, we also monitored signals for three hydrophobic metabolites, valine, isoleucine, and leucine (boxed in Figure 3), to confirm that addition of the Cl-Phe tag did not significantly impair their resolution.

The dramatic increase in metabolite resolution afforded by the Cl-Phe tags confers several advantages. First, it minimizes the potential for ion suppression effects caused by the coelution of multiple molecular species.[12,13] Additionally, it improves the quality of data needed for structural characterization of metabolites. In the current study, metabolite structures were determined by acquisition of accurate mass and fragmentation data using an LTQ-Orbitrap Hybrid mass spectrometer.[14] After assignment of a putative structure, the tandem mass spectral data from endogenous tagged metabolites were compared with synthetic standards to confirm metabolite identities (see Supporting Information). We found that, due to the poor resolution of hydrophilic compounds labeled with the original METPR tags, in six of the fifteen cases, we were unable to obtain adequate fragmentation data for these compounds due to the presence of coeluting species with similar *m*/*z* values (the LTQ is only capable of unit mass resolution and thus cannot distinguish between coeluting compounds within ±1 unit of the target molecule; see data obtained for succinic acid, phenylalanine, pantothenic acid, threonine, cysteine, and pyruvate in Supporting Information). In contrast, labeling with the Cl-Phe tags provided sufficient resolution of these metabolites to enable acquisition of high-quality fragmentation data for all but one compound (lactic acid; see Supporting Information).

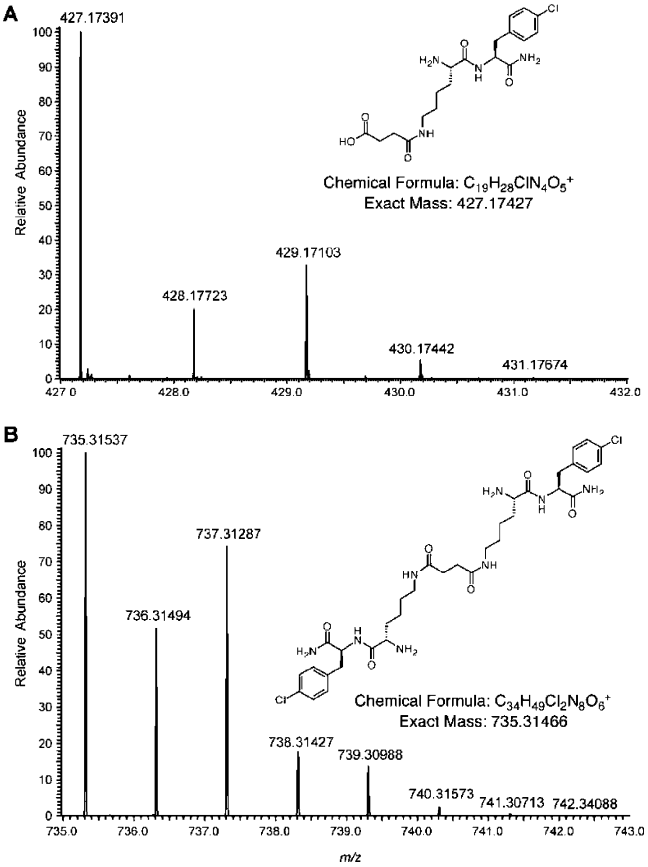

Figure 4. Distinct isotopic abundance patterns due to the presence of a chlorine atom in the Cl-Phe tag. Shown are the isotopic distribution patterns for singly (A) and doubly (B) tagged forms of succinic acid.

Another key advantage of the Cl-Phe tags is that they can distinguish untagged (i.e., false positives) from singly and multiply tagged species. Because of the isotopic distribution of chlorine, the M + 2 ion is more prevalent than the M + 1 ion, providing a signature that is easily discernible by MS. To illustrate this distinct isotopic distribution, we have shown the abundance pattern observed for endogenous succinic acid enriched from MDA-MB-231 cells by the acid-capture Cl-Phe tag (Figure 4). Signals with the expected *m*/*z* ratios and isotopic distribution patterns for singly and doubly tagged succinic acid were detected, confirming the ability of the Cl-Phe tag to identify metabolites with multiple copies of the same functional group. The observed abundance patterns match closely to theoretical isotopic distributions for these compounds (see Supporting Information).

In summary, we have developed a new set of hydrophobic METPR tags for enhanced-resolution profiling of polar metabolites. Capture of a mixture of small molecule standards revealed substantial improvements in retention and resolution of polar metabolites upon coupling to hydrophobic tags. Similar improvements were observed upon profiling endogenous metabolites from breast cancer cells. Notably, the enhanced resolution afforded by hydrophobic METPR tags minimized overlap in tandem MS profiles for polar metabolites, thereby facilitating structure determination. We have also shown that inclusion of a chlorine atom in the hydrophobic tags permits MS-based discrimination of tagged metabolites from background peaks (i.e., false positives) and identification of multiply tagged species (e.g., succinic acid). The latter feature could facilitate the structural characterization of unknown metabolites by designating the quantity of a specific functional group that they possess. Collectively, these results showcase the value of chemoselective metabolite tags, which, through their ability to manipulate the physicochemical properties of endogenous small molecules, offer a versatile way to enrich and profile challenging fractions of the metabolome.

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Supporting Information Available: Synthesis of the Cl-Phe capture reagents, methods for metabolite capture and elution, table of identified metabolites, fragmentation data for identified metabolites, and theoretical isotopic distribution spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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