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# Quantitative Profiling of Protein-Derived Electrophilic Cofactors in Bacterial Cells with a Hydrazine-Derived Probe

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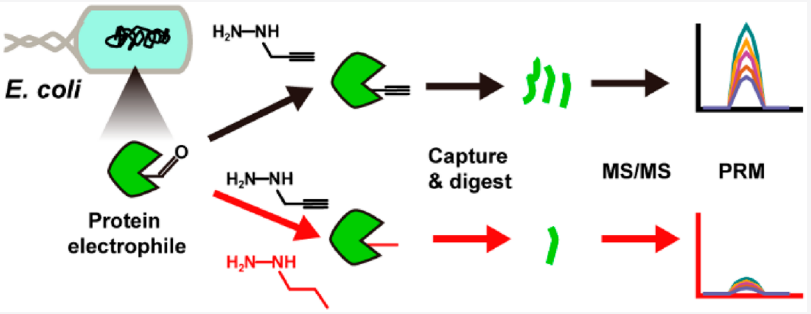


**ABSTRACT:** Post-translational modification of proteins can form electrophilic cofactors that serve as a catalytic center. The derived electrophilic cofactors greatly expand protein activities and functions. However, there are few studies concerning how to profile the electrophiles in bacteria. Herein, we utilized a clickable probe called propargyl hydrazine to profile the protein-derived electrophilic cofactors in *Escherichia coli* (*E. coli*) cells. Since the cofactors are mostly carbonyl groups, the hydrazine-based probe can specifically react with the cofactors to form a Schiff base. The labeled proteins were then pulled down for mass spectrometry (MS) analysis. Fourteen proteins were shown to undergo enrichment by the probe and competitive binding by its analogue, propyl hydrazine. The identified proteins were further analyzed with targeted proteomics based on parallel reaction monitoring (PRM). Using this strategy, we obtained a global portrait of protein electrophiles in bacterial cells, among which the proteins of speD and panD were previously reported to derive pyruvoyl group as an electrophilic center while lpp can retain N-terminal formyl methionine. This quantitative chemical proteomics strategy can be used to find out protein electrophiles in bacteria and holds great potential to further characterize the protein functions.



## INTRODUCTION

Proteins typically exert catalytic functions through active amino acid residues that act as a nucleophilic center.[1] As certain critical reactions require electrophile for catalysis, proteins generally recruit exogenous cofactors to accomplish the catalytic functions.[2] These cofactors include inorganic ions (such as copper ion, zinc ion), organic molecules (such as nicotinamide adenine dinucleotide, and pyridoxal phosphate), and organometallic compounds (like heme).[3] The exogenous cofactors regulate protein structures and expand protein functions. It has been found that proteins also derive endogenous cofactors from post-translational modification of amino acid residues.[4,5] The modified residues endow the proteins with novel functions and empower bacteria with new adaptations even in the absence of exogenous cofactors.[6,7] However, it still lacks methods to profile the endogenous cofactors in bacteria; there is thus a great need to develop effective approaches to exploit the derived electrophiles and to unveil their catalytic functions.

The protein-derived cofactors mostly comprise carbonyl groups that are formed through either autocatalytic cleavage or enzymatic catalysis; however, the electrophilic cofactors cannot be readily predicted from protein sequence.[8] There are few studies regarding the endogenous cofactors in bacterial cells, mainly due to the lack of analytical methods. Methods based on electrochemistry, Western blot, and Raman spectrometry enable analysis of the overall protein carbonylation level, but they are not yet able to identify the carbonylated proteins.[9−11] Recently, high-resolution mass spectrometry has been applied to profile the proteins with N-terminal formylation, an electrophilic modification specific to bacteria and organelles.[12,13] Nucleophilic probes were employed to capture low abundant electrophiles for MS analysis. For instance, two hydrazine-derived probes were developed to analyze the electrophilic cofactors and localize the stress-induced carbony-

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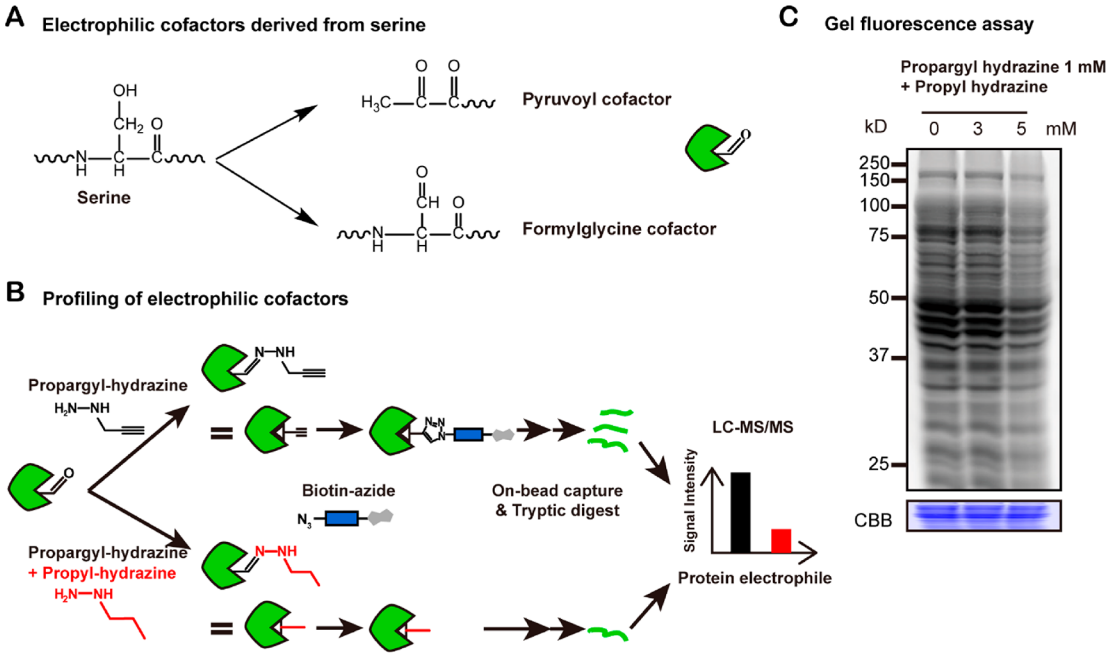


**Figure 1.** Formation of protein-derived electrophilic cofactors and profiling of the electrophiles by propargyl hydrazine. (A) Derivatization of serine residue to form pyruvoyl or formylglycine cofactor. (B) Schematic of the protocol for MS profiling of protein-derived electrophiles with the propargyl hydrazine in the presence or absence of propyl hydrazine. (C) Gel fluorescence analysis of proteins labeled by 1 mM of propargyl hydrazine and competed by a serial concentration of propyl hydrazine (0, 3, and 5 mM, respectively), and its corresponding Coomassie blue staining is marked as CBB.

lated proteins in human cells.[14,15] Probes derived from aniline or aminooxy group were also utilized to identify proteins modified with lipid-derived carbonyls.[16,17] These nucleophilic probes can undergo chemo-selective ligation with carbonyl group to form a covalent adduct, which can be used to enrich the carbonyl-containing proteins before detection with MS.[18] By using activity-based protein profiling (ABPP) strategy, Chen et al. employed an aniline-derived probe to analyze the protein carbonylation induced by ferroptosis.[19] Tian et al. applied a hydrazine-based probe to profile the protein carbonylation induced by oxidation stress.[20] The probes are mostly utilized to profile the proteins conjugated with lipid-derived carbonyls, and there is still a lack of research to investigate the protein-derived electrophiles in bacteria.

In this paper, we utilized a clickable probe called propargyl hydrazine to profile the protein-derived electrophilic cofactors in *E. coli* cells. The labeled cofactors are biotinylated and then enriched by streptavidin beads before MS analysis. We also employed the probe along with its analog called propyl hydrazine to exclude the proteins of nonspecific binding. Fourteen proteins were observed to get enriched after the probe treatment and their labeling effects by the probe were all decreased by the probe analog. These proteins were considered as candidates that can derive the carbonyl-containing electrophiles. Five of the proteins were validated to possess N-formyl, pyruvoyl, or formylglycine groups according to previous studies.[21−23] We further employed targeted proteomics based on PRM mode, which can increase detection sensitivity and reduce false positives, to further verify the identified protein-derived electrophiles. The present study demonstrates a viable strategy that utilizes a hydrazine-derived probe and targeted proteomics to profile protein-derived electrophiles in bacterial proteins. It also underscores the need of further investigation into the biological effects that these protein electrophiles can exert.

## EXPERIMENTAL SECTION

**Chemicals and Reagents.** *E. coli* K12 cell was purchased from Yale *E. coli* genetic stock center. Propyl hydrazine dihydrochloride was provided by Fluorochem Ltd., UK. Propargyl hydrazine was obtained from Aldlab Chemicals, Woburn, MA. Rhodamine-azide, sodium ascorbate, tris-(benzyltriazolylmethyl)amine (THPTA), and sodium cyanoborohydride ($NaBH_3CN$) were from Sigma-Aldrich. Biotin-azide was from Click Chemistry Tool. NAP-10 columns were from GE Health Care, Chicago, IL. Sequencing-grade trypsin was from Promega. Streptavidin magnetic beads, streptavidin-HRP, and SuperSignal West Femto Kit were purchased from Thermo Scientific. N-pyruvoyl HICVHTYPESHPEGGLCTFR was synthesized by Hybio Pharmaceutical Company, China.

**Gel Fluorescence Analysis of Probe-Labeled Proteins.** For probe labeling assay, proteins extracted from *E. coli* were treated with propargyl hydrazine (0, 0.02, 0.1, 0.5, and 2 mM, respectively) for 1 h. For competition assay, proteins were treated with propyl hydrazine (0, 3, 5 mM, respectively) for 1 h before being labeled with 1 mM propargyl hydrazine at RT for 1 h. Click chemistry reaction was initiated with 0.2 mM rhodamine-azide, 1% sodium dodecyl sulfate (SDS), 1 mM sodium ascorbate, 0.5 mM THPTA, and 1 mM $CuSO_4$. After incubation in darkness for 1 h, the proteins were mixed with loading buffer for gel electrophoresis with 12% SDS-PAGE gel (polyacrylamide gel electrophoresis). The electrophoresis was run for additional 15 min after the blue dye moved out of the gel. Gels underwent fluorescence scanning using Typhoon FLA9500 (GE Healthcare). The emission filter was 580 nm. Then the gel was stained with Coomassie blue and scanned.


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**Evaluation of Probe Labeling Efficiency with N-Pyruvoyl Peptide.** A synthesized peptide, N-pyruvoyl HICVHTYPESHPEGGLCTFR, was dissolved in phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 0.2 mM and then reacted with 1 mM propargyl hydrazine for 1 h at RT. The sample was treated with or without 20 mM $NaBH_3CN$ for 30 min, followed by being desalted with C18 StageTip. After reaction with 20 mM iodoacetamide (IAA) and 10 mM dithiothreitol (DTT), the peptides were desalted by C18 StageTip prior to LC-MS/MS analysis.

**Profiling of Protein-Derived Electrophiles Using Mass Spectrometry.** The detailed experimental steps and data processing procedures are described in the Supporting Information (SI). We had three groups of proteins named as "Blank", "Probe", and "Compete", respectively. In "Blank" group, proteins were not treated by probe. In "Probe" group, proteins were labeled with 1 mM propargyl hydrazine. In "Compete" group, proteins were labeled with 1 mM probe after treatment with 10 mM propyl hydrazine. After reaction for 1 h, the proteins were precipitated by acetone to remove the probe and its analog. Then biotin was ligated to the probe-labeled proteins with click chemistry reaction. The proteins were purified by NAP-10 columns and reconstituted in 1.4 mL of 1% IGEPAL, 0.1% SDS in PBS. The proteins were mixed with streptavidin beads to capture the biotin-labeled proteins. The captured proteins were reduced by DTT, treated by IAA, and digested by trypsin into peptides. The peptides were desalted by C18 StageTip for nanoLC-MS/MS analysis. The acquired MS data from data-dependent acquisition (DDA) mode were analyzed with MaxQuant for label-free quantification (LFQ) of proteins. Fold changes were calculated for each protein including the Enrichment ratio (protein intensity in "Probe" group divided by that in "Blank" group) and the Competition ratio (protein intensity in "Probe" group divided by that in "Compete" group).

**Verification of Protein Electrophiles Using PRM Mode.** The detailed PRM methods were included in SI. First, the DDA data were analyzed by Proteome Discoverer and imported into Skyline. The proteins of interest were listed in Skyline, and only their unique peptides were selected for PRM analysis. PRM samples were treated in the same way to the DDA samples. In PRM acquisition, the peptides of interest were selectively fragmented, and their precursor and fragment ions were both determined. The acquired data were directly imported into Skyline to integrate the precursor ions with their first three isotopic peaks and summed up the fragment ions (y ions or b ions) that ranked top five in signal intensity. The integrated peak area of the fragment ions was summed up to represent the peptide. The selected peptides were summed up to quantify the protein.

## RESULTS AND DISCUSSION

**Analysis of Probe Labeling Effects with Biotin Assay and Gel Fluorescence Assay.** Certain proteins could derive an electrophilic group to expand their functional range (Figure 1A).

For example, S-adenosylmethionine decarboxylase can spontaneously derive pyruvoyl from serine to accomplish its decarboxylation activity, while sulfatases derive formylglycine as functional group.[24,25] The derived functional groups mostly possess reactive carbonyls that can be captured by nucleophilic probes.[26,27] Therefore, we applied propargyl hydrazine to react with the protein-derived cofactors to form a Schiff base, which was then used for MS identification (Figure 1B). Before MS identification, a biotin assay was used to analyze the probe labeling effects and profile the protein electrophiles. The probe-labeled proteins were conjugated with biotin-azide, followed by gel electrophoresis and being blotted onto nitrocellulose membrane for chemiluminescence analysis. There were numerous bands in the probe treatment sample; moreover, addition of propyl hydrazine, an analog of the probe, was observed to decrease the signal of proteins with masses of ~40, 50, and 62 kDa (SI Figure S-1). The results indicated that the probe was reactive in labeling protein electrophiles and the probe analog could compete with the probe to label the protein electrophiles. It is much likely that these proteins had carbonyl groups that can react with both of the probe and its analog. However, naturally biotinylated proteins in the *E. coli* cells could introduce background signals to the biotin assay, which could account for the finding that the bands were not significantly decreased after adding propyl hydrazine that was 10-fold amount of the probe.

To circumvent the background issue, we further utilized gel fluorescence assays to verify the probe labeling efficiency. The probe-labeled proteins were conjugated with rhodamine-azide instead of biotin-azide to reduce the background signals (SI Figure S-2A). The results showed that the probe labeled numerous proteins in a clear concentration-dependent manner (SI Figure S-2B). The labeling was nearly saturated when the probe concentration reached 0.5 mM, so it was sufficient to use 1 mM of the probe to label the protein electrophiles for our following experiments. Moreover, the probe labeling effects were significantly reduced when propyl hydrazine was added to 5-fold of the probe (Figure 1C). The background signals were not as apparent as the biotin assay, but the competition between the probe and its analog was consistent with the biotin assay. These results indicated the feasibility of using the probe and its analog as a competitor to profile the protein-derived cofactors.

**Probe Labeling Outcomes with and Without $NaBH_3CN$.** The reaction between propargyl hydrazine and carbonyl group is reversible and can form an unstable Schiff base. Previous studies indicated that $NaBH_3CN$ can transform the Schiff base to a stable product.[14,28] However, it remains uncertain whether $NaBH_3CN$ can also transform the electrophilic cofactors to byproducts. Our biotin assay also showed that $NaBH_3CN$ decreased the labeling effects of proteins, such as proteins of ~35 and 50 kDa (SI Figure S-1). To unravel the reaction mechanisms, we used a synthesized peptide with N-terminal pyruvoyl group, N-pyruvoyl HICVHTYPESHPEGGLCTFR, to react with propargyl hydrazine. The products were then treated with and without $NaBH_3CN$, followed by IAA treatment. In Figure 2A, the reaction without $NaBH_3CN$ showed that the N-end pyruvoyl group was ligated with the probe, which resulted in changing the N-end pyruvoyl to $m/z$ = 123.0558. In addition, the probe-labeled peptide showed a fragment ion pattern that was consistent with theoretical calculation (SI Figure S-3A). This reaction verified it is feasible to use propargyl hydrazine to label the carbonyl-containing peptides. The chromatogram in Figure 2A showed the three primary peptides were the original peptide, the peptide modified by IAA, and the peptide labeled by the probe and modified by IAA. We then calculated the area-under-a-curve (AUC) of all the three peptides, which showed that the probe-labeled peptide accounted for only 5.0% of all the three peptides without considering their difference in ionization


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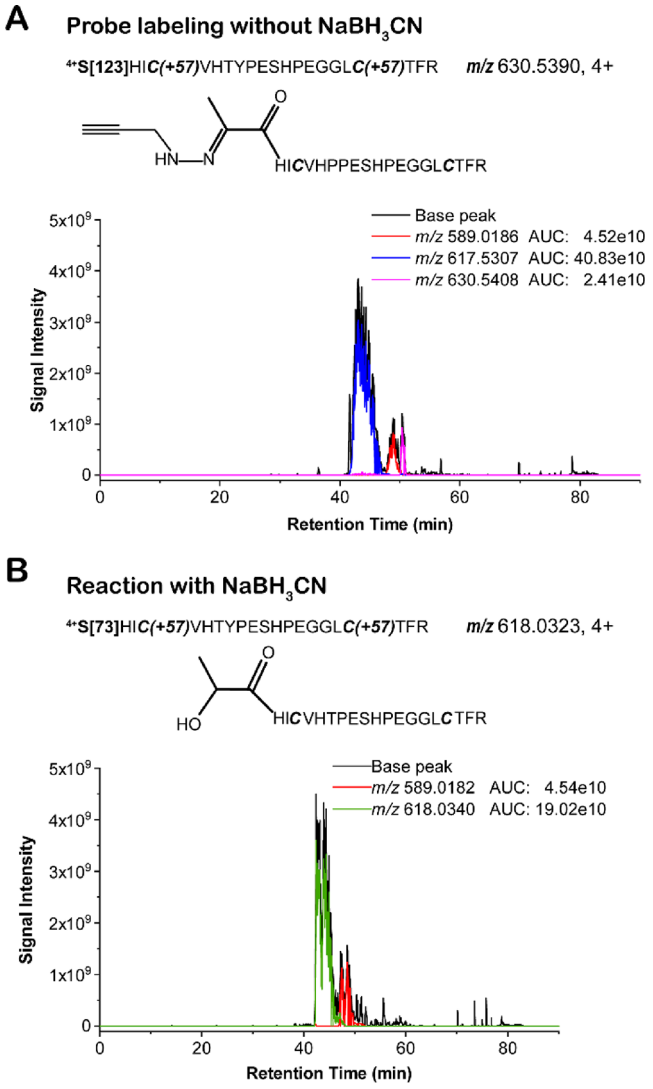
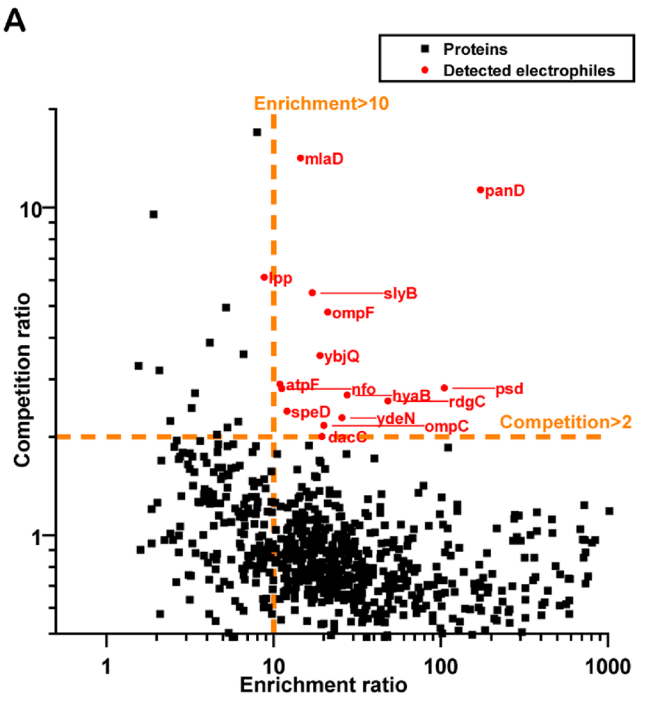



**Figure 2.** MS analysis of the peptide of pyruvol-HICVHTYPESHPEGGLCTFR by labeling it with propargyl hydrazine only (A) or followed by adding $NaBH_3CN$ (B). (A) The chromatogram of the three major peptides, including the synthesized peptide ($m/z$ = 589.0186, 4+), the peptide with two cysteines modified with IAA ($m/z$ = 617.5307, 4+), and the peptide modified with IAA and labeled by propargyl hydrazine ($m/z$ = 630.5408, 4+) and their corresponding AUCs. (B) The extracted chromatogram of the original peptide ($m/z$ = 589.0182, 4+) and the peptide that is reduced by $NaBH_3CN$ and labeled with IAA ($m/z$ = 618.0340, 4+) and their AUCs. The pyruvoyl is substituted with "S" after reactions.

efficiency. The low yield of the probe-labeled peptide was also consistent with the fact that the Schiff reaction was reversible.[29]

When $NaBH_3CN$ was added, it was observed that most of carbonyl group in the N-pyruvoyl peptide was reduced to hydroxyl group, resulting in a mass shift of +2 for the N-end pyruvoyl group ($m/z$ = 73.0290) (Figure 2B). The fragment ion pattern verified that the major product was a reduced form of the peptide (SI Figure S-3B). Using the calculation method mentioned above, it was found that the reduced peptide accounted for 80.7% of the two major peptides. The results showed that $NaBH_3CN$ reduced pyruvoyl group instead of stabilizing Schiff base. It was consistent with the biotin assay result showing that $NaBH_3CN$ could decrease the probe labeling effects (SI Figure S-1). The same side reactions might occur for other carbonyl-containing electrophiles. To address this issue, we chose to directly apply propargyl hydrazine to label the protein-derived electrophiles without using the reductant for the following experiments.

**MS Identification of Proteins That Can Derive Electrophilic Cofactors.** Three groups of proteins were used to identify the bacterial protein-derived electrophiles. "Blank" group without probe treatment was used as a negative control. "Probe" group used propargyl hydrazine to label the protein electrophiles. Moreover, "Compete" group utilized propyl hydrazine, an analogue of the probe, to compete with the probe and exclude nonspecific protein binding. Enrichment ratio and Competition ratio were then calculated to screen out the proteins that can be labeled by the probe, and the labeling can be competed by the probe analog. Using stringent cutoffs of Enrichment ratio >10 and Competition ratio >2, we identified 13 proteins that met the criteria and were thus likely to possess electrophilic cofactors (Figure 3A).




B

| Protein ID | Protein name | Gene name | Eletrophile |
|---|---|---|---|
| P0A790 | Aspartate 1-decarboxylase | panD | pyruvoyl |
| P0A8K1 | Phosphatidylserine decarboxylase | psd | pyruvoyl |
| P77318 | Uncharacterized sulfatase | ydeN | N-formyl; formylglycine |
| P0A7F6 | S-adenosylmethionine decarboxylase | speD | pyruvoyl |
| P69776 | Major outer membrane lipoprotein | lpp | N-formyl |

**Figure 3.** MaxQuant analysis of proteins that are enriched by propargyl hydrazine and competed by propyl hydrazine. (A) Statistical analysis of the enrichment ratio and competition ratio of the identified proteins. Each group has four biological replicates. Two cutoffs (Enrichment ratio >10 and Competition ratio >2) are annotated as the orange dashed line, and the identified protein electrophiles are highlighted in red. (B) List of the identified protein electrophiles and their reported electrophilic cofactors.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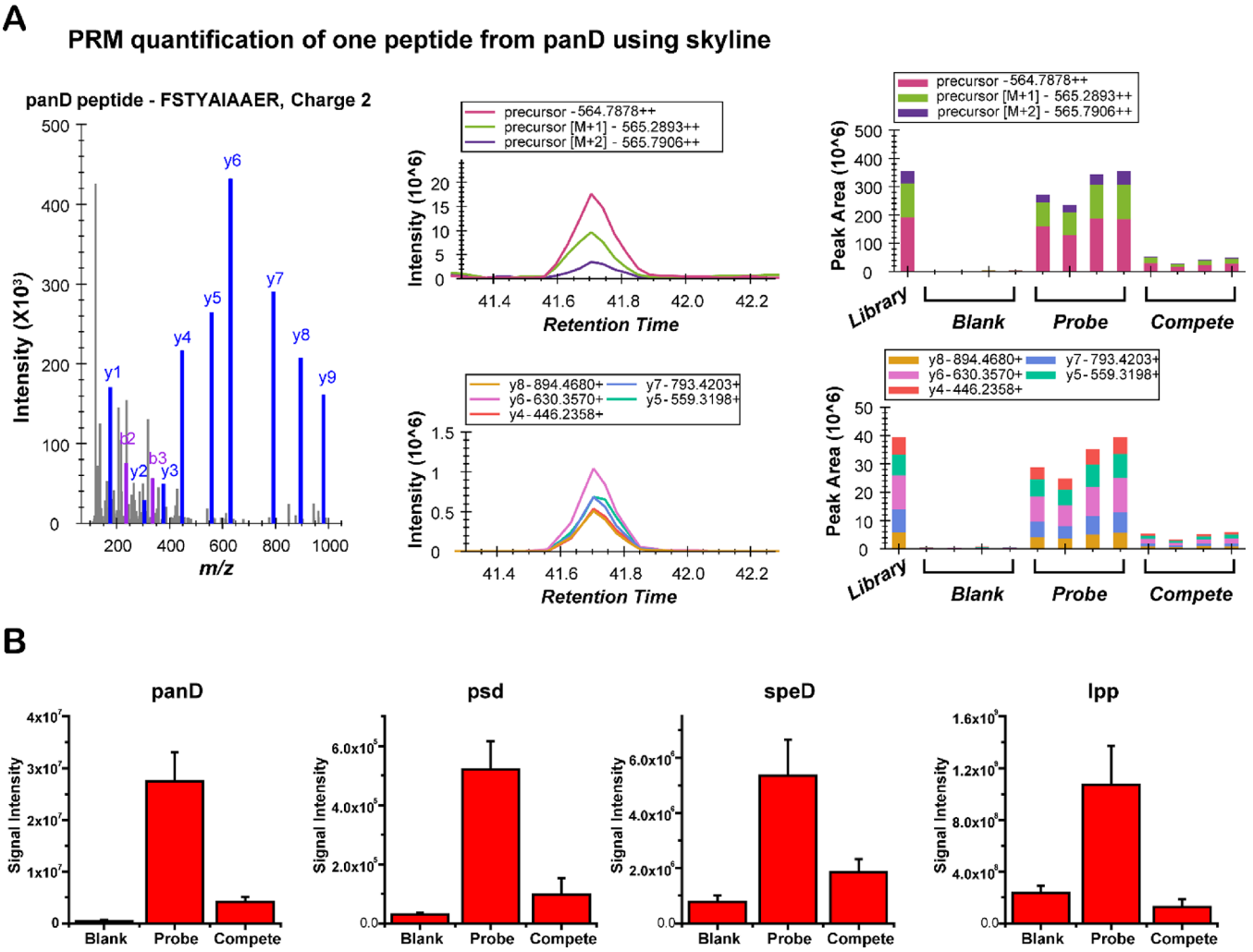


**Figure 4.** PRM quantification of the identified protein electrophiles with Skyline. (A) PRM analysis of one peptide from panD, FSTYAIAAER++, including (left) its fragment ion pattern, (middle) its precursor ions and fragment ions, and (right) quanfication of the peptide in "Blank", "Probe", and "Compete" groups by using precursor ions (upper right) and fragment ions (lower right), respectively. (B) PRM quantification of four proteins in "Blank", "Probe", and "Compete" groups, respectively. Each group has four biological replicates.

Moreover, we found that lpp was close to the cutoffs so it was also included as an identified protein. After consulting protein databases and literatures, we found that five of the identified proteins were reported to have protein electrophiles.[30] The identified proteins are speD, pad, panD, lpp, and ydeN (Figure 3B). SpeD, psd, and panD could derive pyruvoyl groups as an electrophiles.[31−33] Lpp and psd were reported to have N-formyl methionine, which is a unique protein cap for bacteria and organelles.[34] YdeN could derive serine-derived formylglycine as an electrophile, which empowers the sulfatase with catalytic capabilities.[35] The results showed that it was feasible to use propargyl hydrazine as probe and propyl hydrazine as competitor to identify the protein electrophiles in bacteria. The remaining nine protein candidates have not been reported to have protein-derived electrophiles; thus it still needs further confirmation whether they can derive electrophiles or only undergo carbonylation induced by oxidative stress. To answer that question, further research could be conducted by using biotin-azide with cleavable linkers to obtain the probe-labeled peptides.[36] The results showed the feasibility of this platform to profile the carbonyl-containing electrophiles, but still requires further identification of the amino acids that could derive the specific protein electrophilic cofactors or modifications.

**Targeted Proteomics Based on PRM to Verify the Identified Proteins.** PRM has been used as a quantitative strategy in metabolomics and proteomics studies, mainly because it can improve specificity and sensitivity.[37,38] Therefore, we applied PRM-based proteomics to analyze the proteins that were identified by the DDA method, aiming to confirm the quantification results. As shown in Figure 4A, we applied PRM-based strategy to calculate the protein abundance of panD.

One unique peptide of panD was selected from DDA result and its precursor ions and fragment ions were monitored in PRM mode. The PRM results showed that the peptide abundance was increased in "Probe" group when compared with "Blank" group, and the peptide signal was then reduced in "Compete" group when compared with "Probe" group (Figure 4A). Moreover, it showed a consistency to quantify the peptide using precursor ions and fragment ions, indicating the robustness of using PRM to quantify the peptide of interest. The protein abundance of panD was then quantified by summing up the selected peptides, and the PRM quantification


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result was consistent with the DDA result (Figure 4B). The similar patterns were also observed for peptides from the proteins of psd, speD, and lpp. The PRM analysis further verified that combination of propargyl hydrazine and propyl hydrazine could be applied to exploit the protein-derived electrophilic cofactors.

## CONCLUSION

In this study, a quantitative chemical proteomics strategy was first applied to profile the proteins that can derive electrophilic cofactors in *E. coli* cells. The strategy utilized propargyl hydrazine as a nucleophilic probe and propyl hydrazine as its competitor to analyze the carbonyl-containing protein electrophiles. Untargeted proteomics studies identified 14 protein as candidates that can derive electrophilic cofactors. Five of the identified proteins were reported to possess the electrophilic cofactors according to previous reports, including speD, panD, psd, lpp, and ydeN. Targeted proteomics studies verified that four of the five identified proteins were consistent in untargeted and targeted proteomics analysis. This platform was based on chemical proteomics and targeted proteomics strategies, and it provided a promising tool to identify protein-derived electrophilic cofactors. Further investigation is required to find out the amino acid sites that can derive the electrophilic cofactors or modifications, which is beneficial for characterization of protein functions and could be accomplished by using biotin-azide with cleavable linkers or desthiobiotin-azide. This quantitative chemical proteomics platform holds great potential to find out unknown protein-derived electrophilic cofactors and analyze the functions related with the derived cofactors.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b05607.

Supplemental Text provides detailed information about protein extraction method, biotin assay, MS analysis using DDA mode and PRM mode, as well as Figures S-1−S-3 (PDF)

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### Notes

The authors declare no competing financial interest.

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