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Isotope-coded, fluorous photoaffinity labeling reagents†

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A pair of isotope-coded, fluorous photoaffinity labeling reagents has been developed and coupled with a peptide. The modified peptides form adducts with methanol upon light illumination, which show characteristic isotope labeling patterns in mass spectra and can be separated from other peptides through fluorous silica.

Identifying cellular targets of bioactive small molecules from endogenous and synthetic chemical libraries is essential for their further applications as drug candidates or chemical probes.[1] Various techniques, including affinity-based chemical proteomics,[2] genetics-based cDNA complementation and RNA interference (RNAi),[3] and transcriptional profiling-based approaches[4] have accordingly been developed. Among these techniques, the affinity-based chemical proteomics is the most popular strategy for target identification because the entire proteome can be scanned without bias for the tightest binder. However, the biochemical pulldown experiments typically rely on the reversible interactions between ligands and their targets. Consequently, it remains challenging when the target is a low-abundant protein, or the small molecule–protein interaction is weak, or the target protein is one member of a family of proteins with similar functions. Moreover, it is difficult to distinguish the non-specific small molecule binding proteins from the context-specific targets.

Isotope labeling of partial or entire proteome for protein profiling has gained wide popularity because of its quantification accuracy and potential to distinguish real from nonspecific interactions.[5] The isotopes can be introduced through either chemical or metabolic labeling methods. In chemical methods, isotopes are introduced through tags that specifically react with functional groups in proteins. The first such isotope-coded affinity tag (ICAT) reacts with the sulfhydryl groups of cysteine residues.[6] Other commonly used tags for peptide or protein labeling include isobaric tags for relative and absolute quantification (iTRAQ)[7] and tandem mass tags (TMT);[8] both react specifically with primary amine groups in the N-termini or the side chains of lysine residues. In metabolic labeling approaches, the most widely used method is stable isotope labeling with amino acids in cell culture (SILAC).[9] Briefly, the heavy isotope-labeled amino acids are added to the cell culture medium and incorporated into proteins through metabolic interference after several cell doublings. Equal amounts of the heavy isotope-labeled proteome and the normal proteome are then mixed for analysis. Because the intensities of the normal and heavy isotope-labeled peptides correlate with the corresponding protein abundance ratios, SILAC has been used to quantify *in vivo* changes of different proteins in the proteome.[10] Recently, SILAC has also been used in combination with biochemical pulldown experiments to identify cellular targets of small molecule probes.[11] However, a potential problem with SILAC is that the cells need to be efficiently labeled through metabolic interference, which typically takes several cell doublings (1–2 weeks for mammalian cells).

We have previously developed fluorous photoaffinity labeling reagents in efforts to capture low affinity, low abundant or transient proteins through light induced crosslinking of small molecules with their interacting proteins.[12] The fluorous tags are introduced to enrich proteins with low abundance through fluorous solid phase extraction (FSPE) over fluorous silica,[13] while the diazirine is incorporated to label interacting partners through covalent interactions upon light illumination. However, the photoaffinity labeling has the tendency to crosslink with nonspecific targets as well. Coupling the isotope labeling strategy with fluorous photoaffinity approach could minimize the non-specific interactions, and enhance the chances to identify cellular targets of small molecule probes. Instead of tagging the proteins with isotope-labeled tags, we chose to develop reagents that are isotope-coded for quantitative proteomic analysis to identify interacting proteins. As shown in Fig. 1A, both normal and heavy isotope-labeled fluorous diazirines are attached to the small molecule (SM) of interest. Equal amounts of the attached probes will be used to treat cells, one with cell state 1 while the

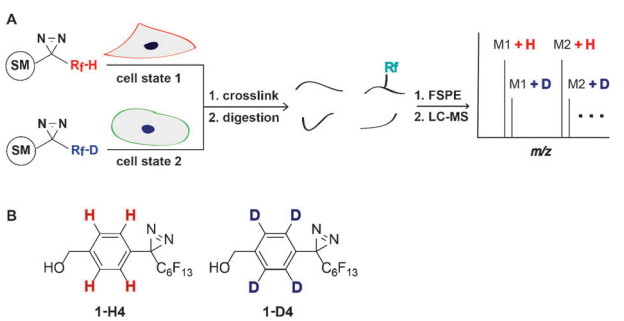

Fig. 1 Fluorous isotope-coded affinity labeling reagents. (A) ICAT reagents are used for quantifying protein levels in 2 cellular states. (B) Chemical structures of the ICAT reagents.

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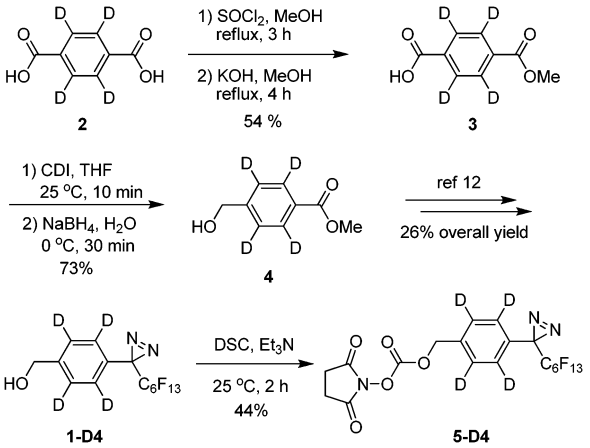

Scheme 1 Synthesis of deuterated **5-D$_4$**.

other with cell state 2. Light illumination will initiate crosslinking of the tagged small molecule with its interacting proteins. The cell lysates are then combined and digested with proteases to generate peptides. The fluorous-tagged peptides are enriched by FSPE and analyzed by liquid chromatography–mass spectrometry (LC-MS). Because equal amounts of normal and heavy isotope-labeled small molecules are used, the mass spectra of interacting proteins will show isotope-labeling patterns (Fig. 1A), *i.e.*, the intensities of the M + H and M + D ions will correlate with the ratio of proteins in the two states.

We chose **1-H$_4$** and **1-D$_4$** (Fig. 1B) as the first pair of isotope-coded, fluorous photoaffinity labeling reagents to develop because of our previous success in synthesizing **1-H$_4$**.[12] The synthesis of **1-D$_4$** starts with the commercially available terephthalic-2,3,5,6-d4 acid **2**. Activation of the acid with thionyl chloride ($SOCl_2$) followed by esterification with MeOH[14] provided the monoester **3** in 54% yield. The carboxylic acid group in **3** was then activated with 1,1′-carbonyldiimidazole (CDI) and subsequently reduced by sodium borohydride ($NaBH_4$)[15] to generate alcohol **4** in 73% yield. Following the literature protocol,[12] the alcohol **4** was converted to the fluorous deuterated diazirine **1-D$_4$**. Subsequent coupling of **1-D$_4$** with *N*,*N*′-disuccinimidyl carbonate (DSC) then provided **5-D$_4$** (Scheme 1). Similarly, **5-H$_4$** was prepared from **1-H$_4$** in an analogous manner.

The peptide RKRSRAE **6** serves as the substrate of protein kinase G (PKG)[16] and is selected as a model compound for attaching to **5-H$_4$** and **5-D$_4$**. To test whether the isotope-coded pair can be used for quantification, a 1 : 1 mixture of **5-H$_4$** and **5-D$_4$** reacted with the peptide to generate the corresponding conjugates **7-H$_4$** and **7-D$_4$**. As shown in the mass spectrum of the reaction mixture (Fig. 2A), the desired coupling products were generated along with several unidentified species. The structures of **7-H$_4$** and **7-D$_4$** were further confirmed by tandem mass spectrometry analysis (Fig. S1, ESI†). As expected, the isotope patterns of **7-H$_4$** and **7-D$_4$** were also observed in Fig. 2A and the intensities of M (normal) and M + 4 (heavy isotope-labeled) ions reflected the ratio of **5-H$_4$** to **5-D$_4$**. The molecular ion of the original peptide was also identified, suggesting that the conversion of **6** to **7-H$_4$** and **7-D$_4$** was not complete. We did not attempt to further improve the reaction conversion or characterize the unknown species because the goal of this work is to demonstrate that the isotope-coded,

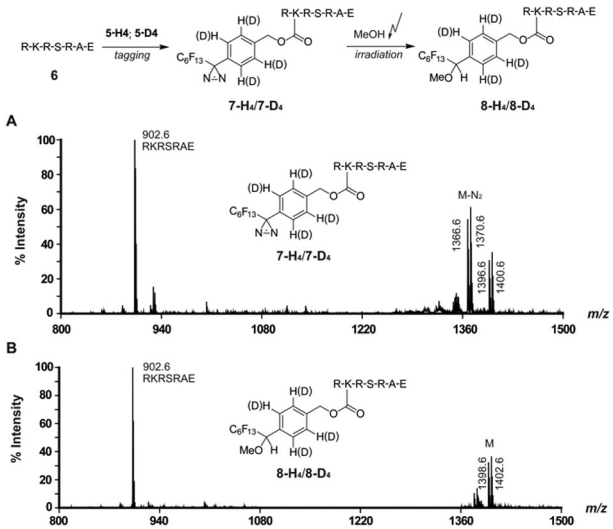

Fig. 2 The coupling of peptide RKRSRAE with **5-H$_4$**/**5-D$_4$** and photolysis of the resulting conjugate. MS spectra of the reaction mixtures that contain **7-H$_4$**/**7-D$_4$** (A) and **8-H$_4$**/**8-D$_4$** (B), respectively.

fluorous photoaffinity labeling reagents have photoaffinity labeling capacity, and the resulting products can be separated from other non-fluorous components in the mixture. Consequently, we used the reaction mixture that contains **7-H$_4$** and **7-D$_4$** for the subsequent experiments.

The photolysis of the diazirine group is next investigated. The peptide conjugates **7-H$_4$** and **7-D$_4$** were illuminated at 350 nm for 10 min and 254 nm for 5 min in MeOH. As shown in the mass spectrum of the reaction mixture, the molecular ion of the expected adducts with MeOH were obtained (Fig. 2B). The tandem MS results of **8-H$_4$** and **8-D$_4$** were consistent with this conclusion (Fig. S1, ESI†), further confirming that the photoaffinity labeling group was attached to the lysine residue. Again, the products formed the isotope labeling patterns, and the intensities of M and M + 4 ions reflected the ratio of **5-H$_4$** to **5-D$_4$**.

To test the separation efficiency, the reaction mixture containing **8-H$_4$** and **8-D$_4$** was mixed with the trypsin digest of bovine serum albumin (BSA) to form a relatively complex mixture with its mass spectrum shown in Fig. 3A. Compared to other peptides in the mixture, the intensities of the fluorous peptides are low. The mixture was then loaded on a column with fluorous silica. Elution of the column with 60% methanol in 10 mM ammonium formate removed the non-fluorous peptides, while the fluorous peptides were obtained after elution with methanol (Fig. 3B). These results demonstrated that FSPE effectively enriched fluorous peptides from a complex mixture. We also generated **8-H$_4$**/**8-D$_4$** from a 2 : 1 mixture of **5-H$_4$**/**5-D$_4$** and mixed it with the trypsin digest of BSA (Fig. 3C) for FSPE. As expected, the fluorous peptides were enriched after FSPE (Fig. 3D). Furthermore, the ratio of the normal to deuterated fluorous peptides changed from 1 : 1 in Fig. 3B to approximate 2 : 1 in Fig. 3D, suggesting that the intensities of the deuterated to non-deuterated peptides were dictated by the ratio **5-H$_4$**/**5-D$_4$**.

In summary, we have developed a novel pair of isotope-coded, fluorous photoaffinity labeling reagents. The photoactive diazirine group will capture the transient and/or weakly interacting

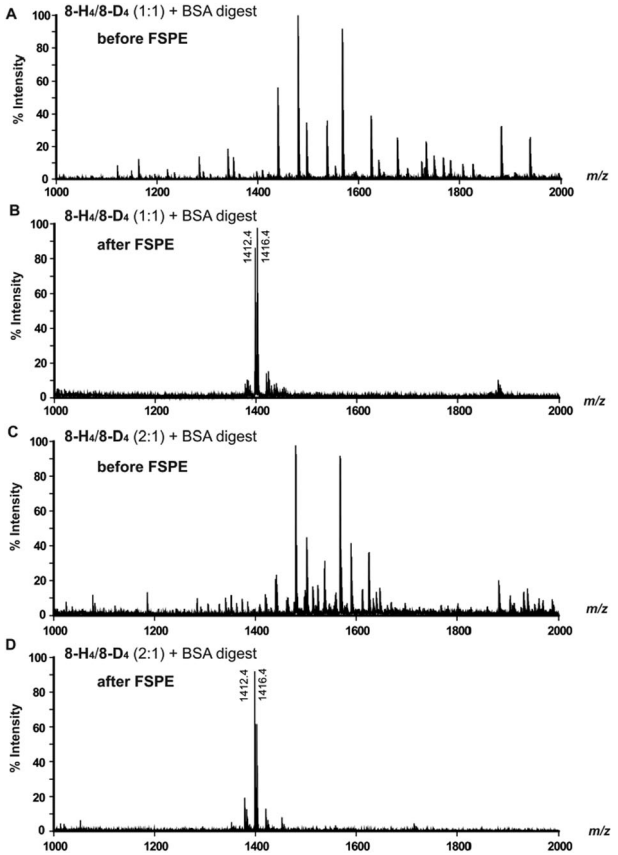

Fig. 3 Separation of fluorous tagged peptides from a complex mixture. The mass spectrum of a mixture that contains **8-H$_4$**/**8-D$_4$** (1 μL, derived from 10 μM **6**) and BSA digest (5 μL, 7.5 μM): (A) before FSPE, **8-H$_4$**/**8-D$_4$** = 1 : 1; (B) after FSPE, **8-H$_4$**/**8-D$_4$** = 1 : 1; (C) before FSPE, **8-H$_4$**/**8-D$_4$** = 2 : 1; (D) after FSPE, **8-H$_4$**/**8-D$_4$** = 2 : 1.

proteins while the fluorous tag will enable the enrichment of the labeled peptides. The isotope-coded information on the new reagents could be used to quantify the interacting proteins (like SILAC) but without the necessity to label the proteome through metabolic interference (unlike SILAC). These features will enable the reagents developed in this work suitable to capture low abundance, low affinity, and context-specific interactions in the cells. Although peptide is used for a demonstration, the isotope-coded, fluorous photoaffinity groups can also be incorporated into drug candidates, oligonucleotides, or proteins to identify novel small molecule–protein, protein–nucleotide, or protein–protein interactions.

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