Chemical Genetics

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Mixed Isotope Photoaffinity Reagents for Identification of Small-Molecule Targets by Mass Spectrometry**

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Identifying the protein targets of biologically active small molecules is often the rate-determining step in the process of elucidating protein function through chemical genetics.[1] A

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classical approach for target identification is chromatography with immobilized ligands. Affinity chromatography approaches are often unsuccessful for identifying binding proteins with low affinity and low abundance because of contaminating nonspecifically purified proteins. These limitations have spurred the development of new methods for the discovery of target ligands.

Photoaffinity labeling (PAL) is an attractive alternative to affinity chromatography for the discovery of target ligands[2] since matrix and avidity effects of immobilized ligands are avoided. PAL methods using soluble probes are more amenable for the identification of low-affinity targets since the ligand concentration can be controlled. Photoaffinity probes also have the potential for use inside live cells, thus enabling affinity-based profiling of the entire proteome in its native state. However, biochemical purification and identification of labeled proteins, often facilitated by the addition of biotin or radioisotopes, remains a significant challenge in PAL. Here we describe the synthesis and application of target identification probe (TIP) reagents for addressing these issues. These reagents contain affinity, photoaffinity, and mixed isotope labels to facilitate the identification of binding proteins by mass spectrometry (MS).

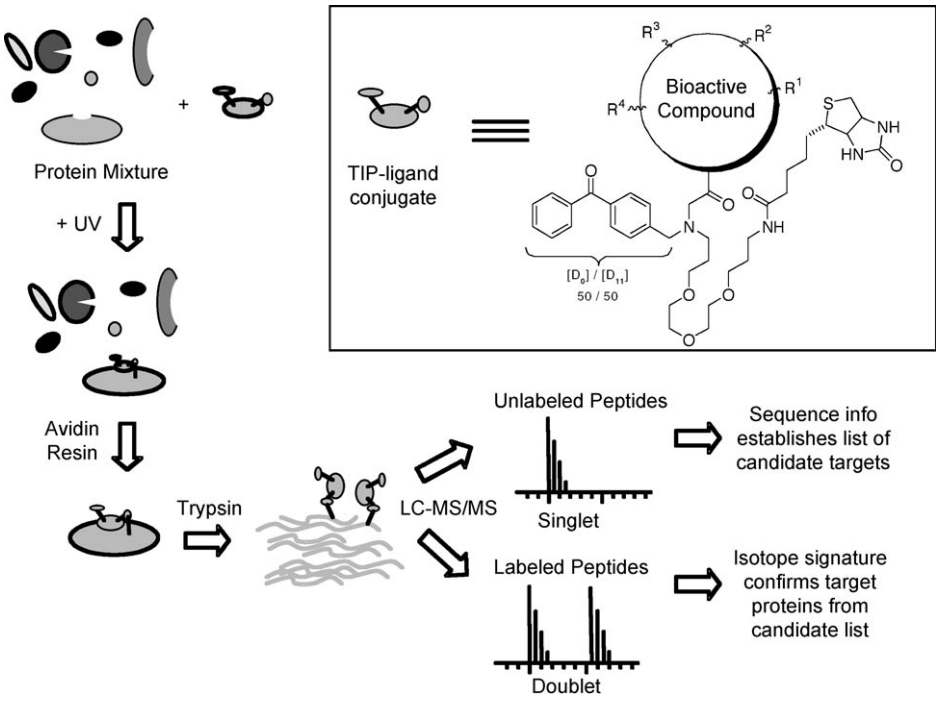


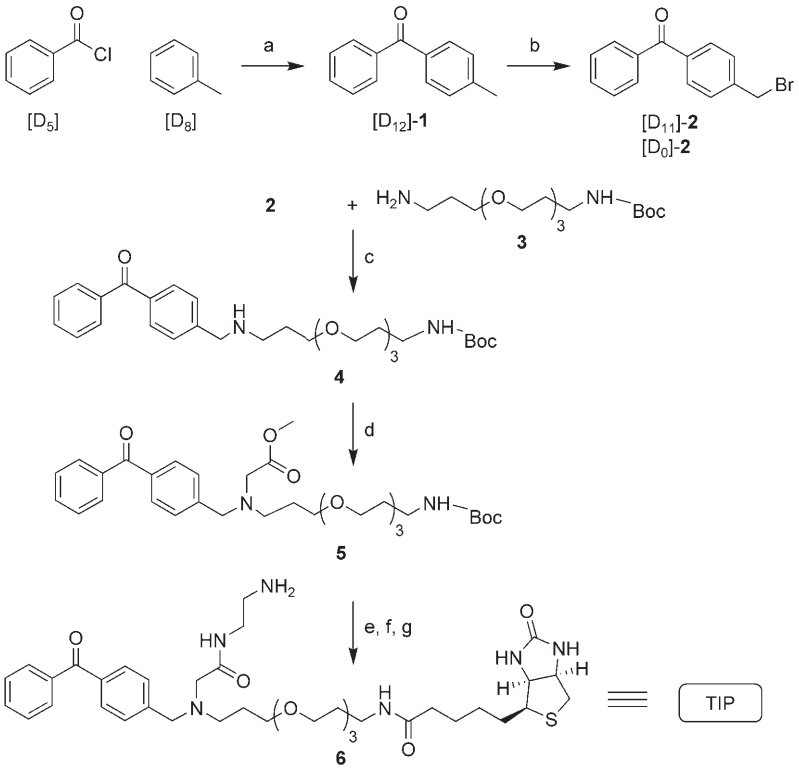
Figure 1. Strategy for determining the identity of small-molecule binding proteins.

Stable mixed isotopes have been applied as selective identification tags in mass spectrometry. For example, drug metabolites can be easily identified, even in very complex mixtures, if isotopes are incorporated to give a unique isotopic pattern.[3] This approach has been used successfully in the identification of modification sites on proteins from covalently modifying inhibitors[4] and protein–protein cross-linking reagents.[5] The incorporation of mixed isotopes and photoaffinity labels into peptide ligands has been shown to aid the identification of receptor binding pockets[6] and has been suggested as an approach to aid the identification of small-molecule PAL targets.[7] To generalize this approach we employ mixed isotopes within a modular photoaffinity-labeling reagent to clearly identify labeled proteins in the presence of contaminants commonly observed in affinity purification and MS.

Our approach is outlined in Figure 1. After suitable attachment of the TIP to a bioactive small molecule, the conjugate is incubated in a protein mixture and covalently photoincorporated into target proteins. Avidin affinity chromatography is used to purify intact labeled proteins, which are proteolyzed and analyzed by mass spectrometry. Sequence information from MS/MS analysis of purified unlabeled peptides enables a list of candidate binding proteins to be established. Conclusive determination of labeled proteins is made possible by the presence of peptides bearing the unique isotopic signature (M, $M+11$) of the probe.

This strategy for characterizing the labeled and unlabeled peptides of chemically modified proteins is advantageous for identification. Relying on a probe-labeled peptide alone to identify a protein can be problematic,[8] particularly for photo-cross-linked peptides.[9] Photo-cross-linking reactions generally modify peptides with little site specificity, thereby producing isobaric peptides representing a number of chemical species.[10] Indeed, the MS fragmentation of the probe itself often results in MS/MS data that are difficult to interpret.[11] This current approach obviates the need to sequence labeled peptides, while providing high sequence coverage and confidence in identifying labeled proteins even in the presence of contaminants.

The synthesis of the probe began with the preparation of deuterated benzophenone $[D_{12}]$-**1** by a Friedel–Crafts acylation using commercially available $[D_8]$toluene and $[D_5]$benzoyl chloride (Scheme 1). In this way, a large isotopic mass difference ($[D_0]$ versus $[D_{11}]$) can be incorporated in a compact and economical manner. Radical bromination of $[D_{12}]$-**1** with NBS in the presence of a catalytic amount of AIBN yielded the benzophenone $[D_{11}]$-**2**. The commercially available benzophenone $[D_0]$-**2** was used to prepare the unlabeled series of compounds. Primary amine **3** was alkylated with $[D_{11}]$-**2** or $[D_0]$-**2** to produce secondary amines $[D_{11}]$-**4** and $[D_0]$-**4**, respectively. These were further alkylated with methyl bromoacetate to afford $[D_{11}]$-**5** and $[D_0]$-**5**. These compounds were converted into probes $[D_{11}]$-**6** and $[D_0]$-**6** by sequential cleavage of the Boc protecting groups, coupling to biotin (DIC/HOBt/DIPEA), and amidation with neat ethylene diamine. The resulting compounds $[D_{11}]$-**6** and $[D_0]$-**6**



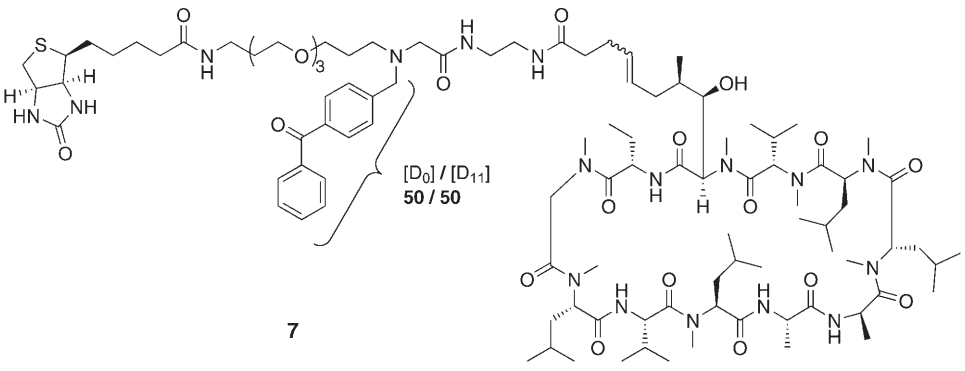
Scheme 1. a) [D_8]Toluene, [D_5]benzoyl chloride, $AlCl_3$; b) NBS, AIBN, CCl_4, reflux 3 h; c) **3**, K_2CO_3, CH_3CN/CH_2Cl_2 (5:2); d) methyl bromoacetate, DIPEA, CH_2Cl_2; e) 40% TFA in CH_2Cl_2; f) biotin, DIC, HOBt, DIPEA, DMF; g) neat ethylenediamine. Unlabeled compounds are derived from commercially available bromomethylbenzophenone ([D_0]-**2**). Boc = *tert*-butoxycarbonyl, NBS = *N*-bromosuccinimide, AIBN = azobisisobutyronitrile, DIPEA = *N*,*N*-diisopropylethylamine, DIC = diisopropylcarbodiimide, HOBt = 1-hydroxy-1*H*-benzotriazole, TFA = trifluoroacetic acid.

represent our modular multifunctional scaffold for preparing photoaffinity reagents.

For a proof of principle experiment, we chose the immunosuppressive drug CsA, whose biological activity is mediated through binding to cyclophilin A (CypA).[12] We derivatized CsA by olefin metathesis to its *N*-methyl-(4*R*)-4-[(*E*)-2-butenyl)-4-methyl]-L-threonine (MeBmt) residue, as previously described.[13] The modified CsA was appended onto [D_{11}]-**6** and [D_0]-**6** to give the heavy and light CsA–TIP conjugates [D_{11}]-**7** and [D_0]-**7**, respectively (Scheme 2). These were used as a 1:1 mixture for subsequent labeling experiments.

The photo-cross-linking specificity of the CsA conjugate was assessed within a protein mixture containing CypA. Upon UV irradiation, the reagent predominantly labeled CypA, as indicated by Western blot analysis (Figure 2b, lane 4). The addition of the photolabel produced an expected shift of the protein band to about 22 kDa. Photolabeling in the presence of excess CsA drastically reduced CypA labeling and slightly increased the labeling of bystander proteins (Figure 2b, lane 5). The results indicate that the photo-cross-linking is highly specific for CypA and is dependent on the ligand-binding interaction.

To demonstrate the applicability of the approach, labeled protein was purified from the protein mixture by using avidin resin (Figure 3, lane 2). Tryptic digests of eluted protein were analyzed directly by MS. A SEQUEST search of the database against the 4 protein sequences with MS/MS data identified 11 peptides from CypA corresponding to 59% sequence coverage. Single peptides from both FKBP and ovalbumin were also identified. A search of the full human database similarly identified CypA and a number of spurious proteins common to such searches, including ubiquitin and keratin. Only two peptides containing the unique isotopic signature were found in the mass spectra, and these were easily identified among many nonlabeled peptides (Figure 4). These corresponded in mass to labeled CypA peptides 92–118 and 56–82 (Figure 4c) and did not match with the theoretical labeled peptides from any of the other proteins identified. Both of the identified peptides are surface exposed and in proximity to the MeBmt side chain of the CypA–CsA complex.[14] Our results demonstrate that the presence of mixed-label peptides allows nonspecific proteins from both the affinity chromatography and MS analysis to be ignored, thereby allowing the target to be confidently determined. The incorporation of a large 11-Da mass difference permitted easy visual recognition of labeled peptides in the mass spectra. This mass difference enables the resolution of doublets for large peptides and even whole proteins (ca. 40 kDa), despite the presence of isotope manifolds (from natural abundance heavy isotopes) and thus could find utility in top-down proteomics applications. Further investigations with these reagents will include applications within live cells. In the event that the reagents are not



Scheme 2. Cyclosporin (CsA)/TIP conjugate.

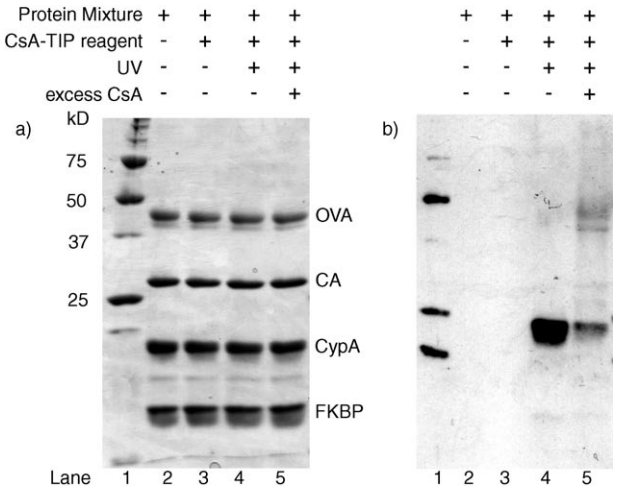


Figure 2. Detection of CsA binding proteins in a protein mixture. A protein mixture consisting of ovalbumin (OVA), carbonic anhydrase (CA), CypA, and FK binding protein (FKBP; 70 $\mu g\,mL^{-1}$ each) in phosphate-buffered saline (PBS) was incubated with CsA–TIP reagent (3.5 μM) and irradiated. Excess CsA (50 μM) was added where indicated. Portions of 15 μL were subjected to sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis. a) Coomassie Blue stained gel. b) Duplicate Western blot visualized with streptavidin-alkaline phosphatase.

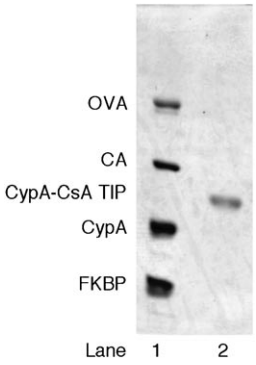


Figure 3. Purification of the CsA binding protein from the protein mixture. A protein mixture consisting of ovalbumin (OVA), carbonic anhydrase (CA), CypA, and FKBP (70 $\mu g\,mL^{-1}$ each) in PBS was incubated with CsA–TIP reagent (3.5 μM) and irradiated. Labeled species were purified with monomeric avidin resin (500 μL) and analyzed by SDS-PAGE. Lane 1: 15 μL of protein mixture, lane 2: elution from avidin stationary phase.

cell permeable, our modular design should readily enable modifications to increase cell permeability such as replacement of the purification tag and/or linkage to the bioactive molecule.

In summary, we have prepared a multifunctional reagent for the identification of small-molecule binding proteins through photoaffinity labeling and mass spectrometry. A facile and economic route for the incorporation of deuterated benzophenones (a widely used photoaffinity tag) into probes has been developed. Conjugates to the ligand CsA were prepared and used to identify a known target of CsA from a mixture of proteins. We have described a novel strategy for the high-confidence identification of photoaffinity-labeled

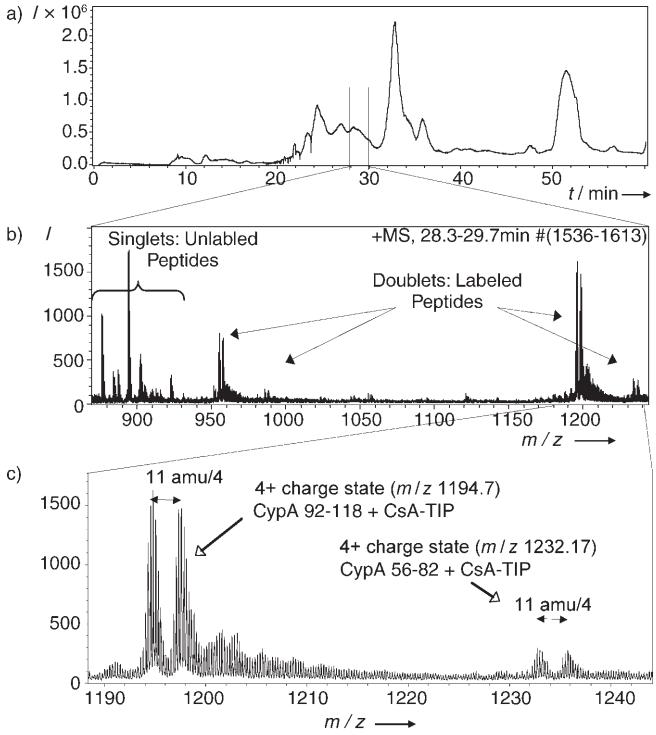


Figure 4. Identification of the two peptides containing the unique isotopic signature in the mass spectra. a) Chromatogram showing the total ion current; b,c) ms data.

proteins that combines unique isotope signatures with broad sequence coverage of purified, unlabeled peptide fragments. These approaches should be broadly applicable to MS analysis of chemically modified proteins.

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