Development of an isotope-coded activity-based probe for the quantitative profiling of cysteine proteases

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Abstract—Quantification studies of complex protein mixtures have been restricted mainly to whole cell extracts. Here we describe the synthesis of two sets of isotope-coded activity-based probes that allow quantitative functional proteomics experiments on the cathepsins.

Proteomics research aims at the study of the expression levels and functioning of (subsets of) the proteins present in a biological sample. Proteomics research presents a number of challenges to the researchers. The protein content in a cell is dynamic and cannot be amplified easily. Further, there is a difference of several orders of magnitude between the most and the least abundant protein. Traditionally, 2D-gel electrophoresis is used to separate mixtures of proteins, allowing identification of the proteins, originally using Edman degradation. Nowadays, protein sequencing is normally performed using mass spectrometry techniques, as introduced by Watanabe and co-workers in their groundbreaking report.1

In chemical proteomics approaches, a complex biological mixture of proteins is simplified before analysis by labeling a specific set of related proteins with an affinity—or fluorescence tag.2–5 For instance, broad spectrum, irreversible protease inhibitors have been used in the profiling of serine proteases,6 cysteine proteases,7 and the catalytically active subunits of the proteasome.8,9 A relevant example of a chemical proteomics probe is represented by 1a (DCG-04), developed by Bogyo and co-workers as an irreversible cysteine protease inhibitor, and applied by us to monitor the proteolytic activity of maturing phagosomes in live antigen-presenting cells.10,11 Compound 1a consists of three functionalities: (1) an electrophilic epoxysuccinate, that alkylates the active site cysteine residue, (2) a short peptide sequence that allows recognition of the probe by the cathepsin family of cysteine proteases, and (3) biotin, for the detection and isolation of the modified proteins (Fig. 1). The biotin is connected to the peptide epoxysuccinate by an aminohexanoic acid residue.

We reasoned that incorporation of an isotopic encoded entity, in analogy to the isotope-coded affinity tag (ICAT) reagent developed by Aebersold and co-workers for the quantitative analysis of cysteine containing proteins,12,13 would allow for both quantitative and functional assessment of the cathepsin family of cysteine proteases from complex biological samples.14 The ICAT strategy is based on the presence (heavy) or absence

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The synthesis of FmocAhx-$d_0$ (14a) and N$_3$Aoh-$d_8$ (14b) were accomplished as follows (Scheme 2). Upon tritylation of deuterated 8, monotrityl ether 9 was obtained in 23% and deuterated butanediol was recovered easily (68%). Tosylation of the primary alcohol followed by the replacement of the tosylate by azide gave protected azidoalcohol 11. Detritylation (TFA/TES in CH$_2$Cl$_2$) afforded azidoalcohol 12b. Both known 4-azido-1-butanoal 12a and deuterated 12b were alkylated under phase transfer conditions with tert-butyl bromoacetate to furnish the corresponding tert-butyl ester 13a and 13b. Subsequent acidolysis of the tert-butyl esters employing 50% v/v TFA/CH$_2$Cl$_2$ yielded, respectively, azido acid 14a in 49% over two steps and 14b in an overall yield of 38% over six steps.

The incorporation of spacers 7b and 14ab into the respective cysteine protease inhibitors 1b and 2ab is shown in Scheme 3. Immobilization of biocytin on a Rink linker was accomplished as described earlier. Standard solid phase peptide synthesis (SPPS) with the sequential addition of a spacer (7, 14a, and 14b, respectively), FmocTyr(OtBu)OH, FmocLeuOH, and ethyl (2S,3S)oxirane-2,3-dicarboxylate was achieved by repeated precipitation from the resin and purification by repeated precipitation afforded target compounds 1b, 2a and 2b in 34%, 29%, and 40%, respectively, in 80–90% purity as judged by LCMS. A small portion of each product was purified to homogeneity by HPLC.

The synthesis of FmocAhx-$d_0$ (7) (Scheme 1) starts with a copper(I) catalyzed oxidative Glaser coupling of Fmoc-protected propargylamine (3) and benzyl propiolate (4) to furnish dyne 5. Upon reduction of dyne 5 with deuterium gas and palladium on carbon, the benzyl ester remained intact, yielding deuterated 6. Contrary to the results of reduction of several analogs of 5, no isotopic scrambling had taken place. Finally, the benzyl ester was hydrolyzed using DCl in deuterium oxide and dioxane to give isotopically coded Fmoc-AhxOH-$d_8$ (7).

The syntheses of N$_3$Aoh-$d_0$ (14a) and N$_3$Aoh-$d_8$ (14b) were accomplished as follows (Scheme 2). Upon tritylation of deuterated 8, monotrityl ether 9 was obtained in 23% and deuterated butanediol was recovered easily (68%). Tosylation of the primary alcohol followed by the replacement of the tosylate by azide gave protected azidoalcohol 11. Detritylation (TFA/TES in CH$_2$Cl$_2$) afforded azidoalcohol 12b. Both known 4-azido-1-butanoal 12a and deuterated 12b were alkylated under phase transfer conditions with tert-butyl bromoacetate to furnish the corresponding tert-butyl ester 13a and 13b. Subsequent acidolysis of the tert-butyl esters employing 50% v/v TFA/CH$_2$Cl$_2$ yielded, respectively, azido acid 14a in 49% over two steps and 14b in an overall yield of 38% over six steps.

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To establish the inhibition profile of the newly synthesized probes, we performed a set of labeling experiments with cell lysates of the mouse macrophage cell line J774. Cell lysates were incubated with DCG-04 (1a) as a control and with the new probes 1b, 2a, and 2b for 60 min at 37°C. The resulting mixtures were separated by SDS-PAGE. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, followed by chemiluminescence induced by horseradish peroxidase–streptavidin conjugate (Fig. 2). Probes 2ab label the cysteine proteases CTS B, L, S, and Z in a cell lysate with the same efficiency as DCG-04, which has been shown previously to effectively target these proteolytic enzymes. This suggests that both sets of isotopic coded spacers (13C, 15N) and activity-based probes targeting other proteins. 

In summary, we have presented the efficient synthesis of two pairs of isotopic coded spacers and we have shown that their incorporation into a known cysteine protease inhibitor does not alter the inhibitory profile of the label. This opens the way to quantitative functional proteomics studies on a functional subset of the proteome, namely the cathepsin family of cysteine proteases.

Importantly, this concept may be extended toward other isotopic coded spacers (13C, 15N) and activity-based probes targeting other proteins. Current research efforts are aimed in that direction.

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References and notes


19. The reduction of 6-(Fmoc-amino)-hexa-2,4-diyn-1-ol, 6-(Fmoc-amino)-hexa-2,4-diyn-1-tetrahydropyranyl ether and 6-(Fmoc-amino)-hexa-2,4-diynoic acid methyl ester, prepared via Glaser oxidative couplings of Fmoc-protected propargylamine with propargyl alcohol, 2-(prop-2-yn-1-yloxy)tetrahydro-2H-pyran and methyl propiolate, respectively, under the same conditions resulted in substantial amounts of hexa-, hepta-, and nonadeuterated products.


