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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.
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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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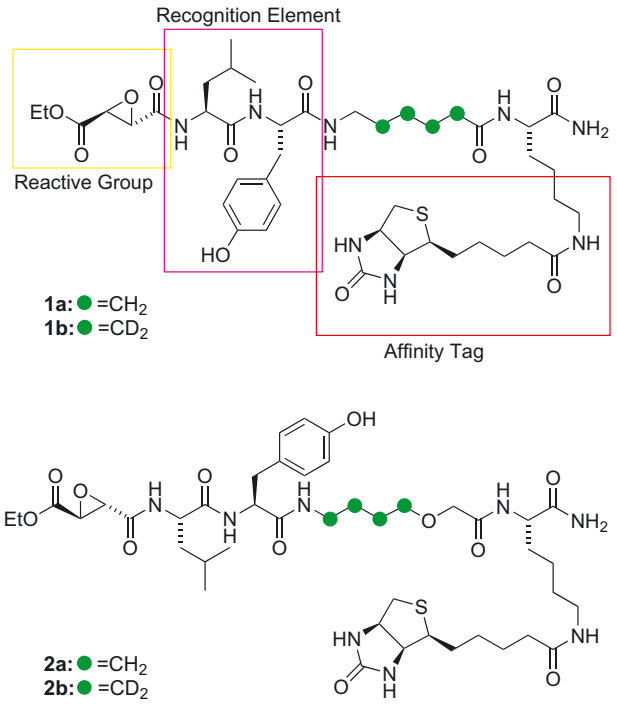
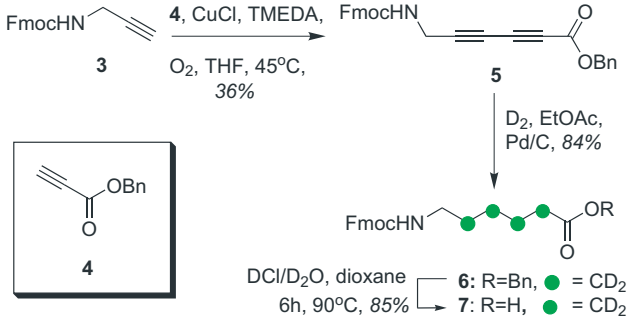


Figure 1. Target structures.

(light) of eight deuterium atoms in a biotin-containing cysteine-reactive probe. After labeling a denatured protein sample from two different sources with either the light or heavy ICAT probe, trypsinolysis of the combined fragments and enrichment in the biotinylated fragments, both the relative quantity and sequence identity of the proteins from which the biotinylated peptides originated are determined by automated multistage mass spectrometry.[15]

Here we describe the synthesis of a new azide-protected amino acid spacer, 7-azido-3-oxaheptanoic acid (N_3AohOH, **14a**) and two d_8-enriched amino acids, 2,2,-3,3,4,4,5,5-octadeutero-6-(Fmoc-amino)hexanoic acid (FmocAhxOH-d_8, **7**) and 4,4,5,5,6,6,7,7-octadeutero-7-azido-3-oxaheptanoic acid (N_3AohOH-d_8, **14b**). Their use in the synthesis of two sets of DCG-04-based isotopic encoded activity-based probes: compound **1b** (in combination with DCG-04 **1a**) and compounds **2a** and **2b** is demonstrated. We also show here that neither incorporation of an isotopic label (compounds **1b** and **2b**, Fig. 1) nor substitution of the aminohexanoic acid moiety by an 7-amino-3-oxa-heptanoic acid residue (compounds **2ab**) affects the broad spectrum affinity for cysteine proteases of the cathepsin family.

The synthesis of FmocAhx-d_8 **7** (Scheme 1) starts with a copper(I) catalyzed oxidative Glaser coupling[16] of Fmoc-protected propargylamine[17] (**3**) and benzyl propiolate[18] (**4**) to furnish diyne **5**. Upon reduction of diyne **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**,[19] no isotopic scrambling had taken place. Finally, the benzyl ester was hydrolyzed[20] using DCl in deuterium

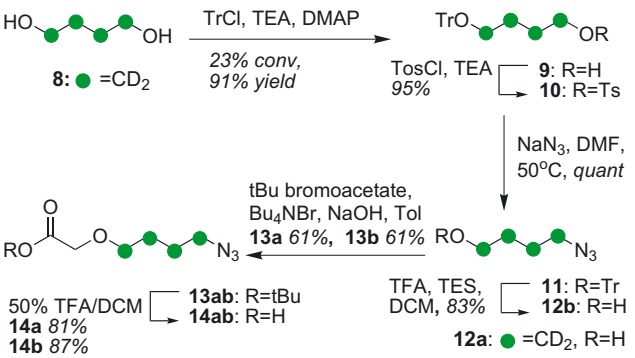


Scheme 1.

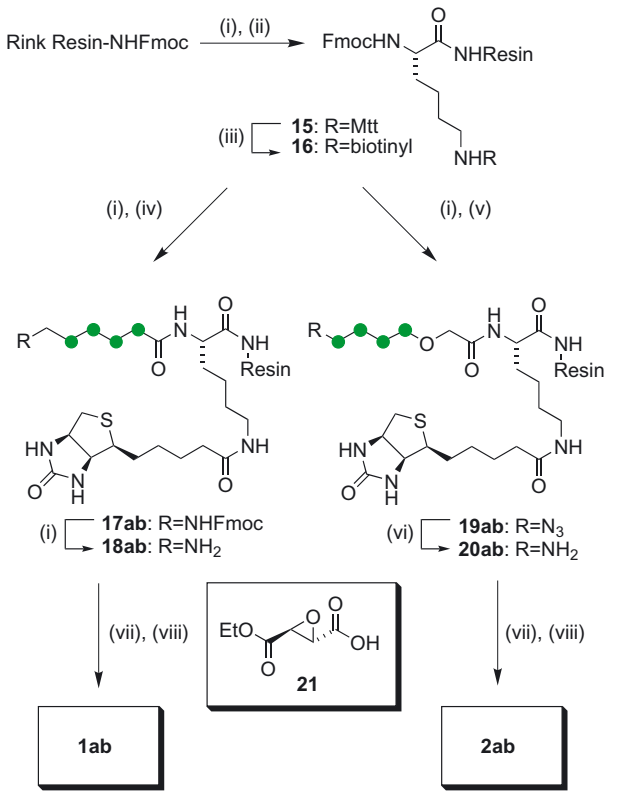
oxide and dioxane to give isotopically coded Fmoc-AhxOH-d_8 (**7**).

The syntheses of N_3Aoh-d_0 (**14a**) and N_3Aoh-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_2Cl_2) afforded azidoalcohol **12b**. Both known 4-azido-1-butanol[21] **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_2Cl_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.[22] Standard solid phase peptide synthesis (SPPS) with the sequential addition of a spacer (**7**, **14a**, and **14b**, respectively), FmocTyr(OtBu)OH, FmocLeuOH, and ethyl (2*S*,3*S*)oxirane-2,3-dicarboxylate,[23] acidic cleavage from the resin and purification by repeated precipitation afforded target compounds **1b**, and **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.



Scheme 2.



Scheme 3. Reagents and conditions: (i) 20% piperidine in NMP; (ii) FmocLys(Mtt)OH, HCTU, DiPEA, NMP; (iii) 1% TFA/CH_2Cl_2, then biotin, HCTU, DiPEA, NMP; (iv) **7a** or **7b**, HCTU, DiPEA, NMP; (v) **14a** or **14b**, HCTU, DiPEA, NMP; (vi) Me_3P, 20% H_2O, dioxane; (vii) Repeated cycles of SPPS: Fmoc cleavage: 20% piperidine in NMP; amino acid condensation: Fmoc-protected amino acid, HCTU, DiPEA, NMP; Fmoc-protected building blocks were used in the following order: FmocTyr(OtBu)OH, FmocLeuOH, **21**; (viii) TFA/H_2O 95/5.

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.[10] This suggests that both sets of isotopic coded activity-based probes **1ab** and **2ab** are viable quantitative functional proteomics tools for the cathepsin family of cysteine proteases.

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.

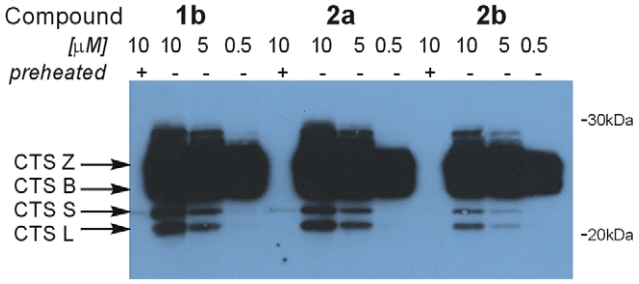


Figure 2. Derivatization of the DCG-04 molecule does not alter targeting of active proteases species in J774 cells. Crude extracts prepared from J774 cells were labeled for 1 h at 37 °C with different concentrations of compounds **1b**, **2a**, and **2b**. As a control, extracts were preheated for 5 min at 100 °C prior to labeling (lanes 1, 5, and 9). Proteins were then separated by SDS-PAGE on 12.5% gels and labeled polypeptides visualized by streptavidin blotting. Polypeptide species corresponding to CTS Z, B, S, and L are indicated based on previous studies.[10]

Importantly, this concept may be extended toward other isotopic coded spacers (13C, 15N) and activity-based probes targeting other proteins.[2,3] Current research efforts are aimed in that direction.

Acknowledgements

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