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# Dehydroalanine-Based Diubiquitin Activity Probes

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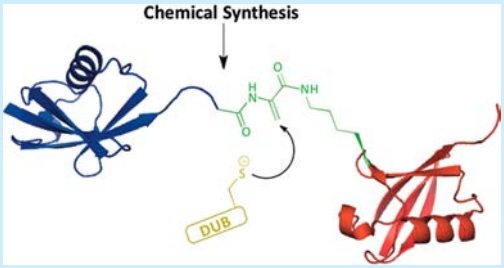
Supporting Information

**ABSTRACT:** A strategy for the synthesis of dehydroalanine based diubiquitin activity probes is described. The site-specific introduction of dehydroalanine was achieved from diubiquitin bearing Cys residue near the scissile bond between two ubiquitins linked through Lys48, Lys63 or in a head to tail fashion. The probes were characterized for their activities with various deubiquitinases, which open new opportunities in studying deubiquitinases in various settings.




Ubiquitination is a post-translational modification that involves the attachment of a ubiquitin (Ub) monomer or a polyubiquitin (polyUb) chain to a protein target affecting a wide range of cellular processes.[1] The process is counteracted by a family of enzymes, known as deubiquitinases (DUBs), which remove the Ub molecule from the substrate or cleave off the Ub chains.[2] DUBs are involved in a variety of regulatory processes, such as cell-cycle progression and tissue development; hence, several DUBs have been implicated in various neurological and neoplastic diseases,[3] making them potential therapeutic targets. DUBs can remove Ub or polyUb from proteins/peptides, process Ub precursors, and disassemble unanchored polyUb chains. Mechanistically, DUBs are divided into two groups, cysteine proteases (majority) and metalloproteases.[3] It is known that although some DUBs do not discriminate between chain linkages, several others do exhibit linkage specificity. The cleavage of the chain can occur from the ends (exo) or within the chains (endo).[2,3] In certain cases, DUBs are substrate specific, such that a specific sequence of the target is recognized, leading to a single-step chain removal.

There are approximately 80 DUBs encoded in the human genome; these have been identified by in silico and activity-based profiling.[3] In the latter approach, Ub-bearing C-terminal electrophile was used as an activity-based probe for the identification and study of members in the Ub system that utilize an active Cys residue.[4] Specifically, this approach was used to profile DUB activity in cancer cell lines, discover new DUB that has no sequence homology with any of the known DUBs, and recover subunits from the 19S cap of the proteasome.[4] In addition, this approach assisted the crystallization of several DUBs[5] and determining the potency and selectivity of DUBs inhibitors in cell culture models.[6,7]

Despite these advances, an obvious drawback of the above-described approaches is the use of only one Ub unit in the probes, which were generated by applying direct aminolysis on the Ub-thioester obtained via intein-based method. As a result, these probes have excluded the chain structure and dynamics of the sequence surrounding the isopeptide bond. Such structural features are, however, very important in the recognition of protein partners and play a vital role in the selective recognition of the Ub interacting proteins. For example, while the K48-linked di-Ub chain adopts predominantly a closed conformation, the K63-linked chain adopts an extended conformation without any contact between functionally important residues of the two monomers.[8,9] These differences are considered as the key factors of their different interactions with various Ub interacting proteins among them DUBs. Another important aspect that the mono-Ub based probes do not take into consideration is the different mode of interaction of DUBs with the chain, e.g., endo vs exo cleavage.[3,4] Hence, we reasoned that more sophisticated probes that incorporate the structural complexity and diversity of the Ub chains would be highly important to represent more precisely the selectivity of these chains toward DUBs and ligases.

Realizing the advantage of the new chemical methods that we have recently developed for the synthesis of Ub chains,[10] here we report our new strategy for the preparation of probes based on di-Ub chains.[11] Specifically, we report the synthesis and characterization of novel K48- or K63-linked di-Ub probes bearing dehyrdoalanine (DHA), as a "warhead" near the isopeptide bond. In addition, we have also prepared and characterized linear di-Ub chain bearing DHA functionality near the scissile amide bond. These probes were then analyzed for their behavior with various DUBs.

Our strategy to generate Lys-linked di-Ub-based probes is illustrated in Scheme 1. Considering that several DUBs cleave the isopeptide bond between two Ub molecules, we reasoned that positioning an electrophile near the isopeptide bond would be an appropriate strategy to study the activity and selectivity of different Cys-based DUBs. For this, placing DHA functionality instead of Gly76, while keeping the isopeptide bond intact,

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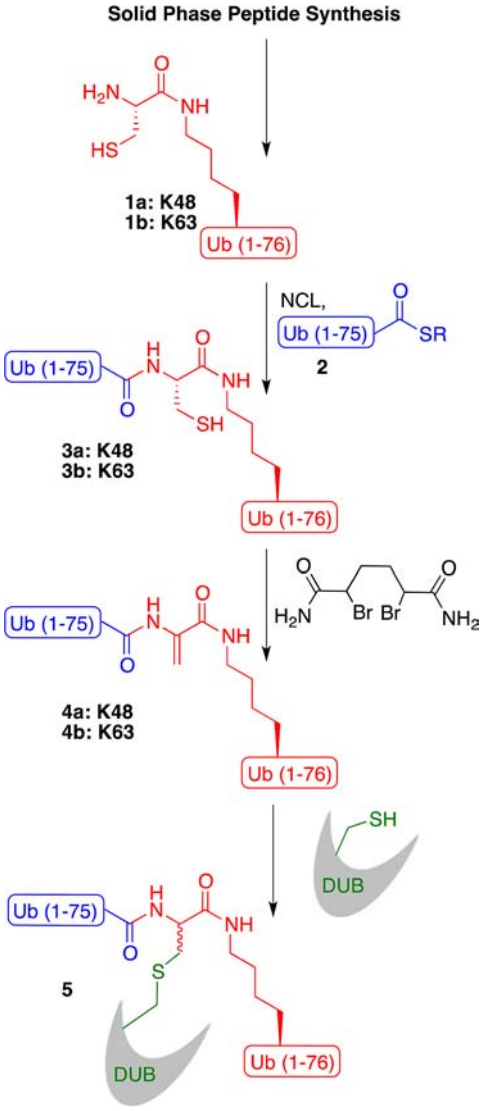
**Scheme 1. General Strategy for the Preparation of DHA-Based Di-Ub Probe for DUBs**




would generate a reactive electrophilic center for the catalytic cysteine found in DUBs. To achieve such a construct, Gly76 in di-Ub should be replaced with a DHA precursor that after the synthesis of di-Ub, can lead to the formation of the DHA functionality. We were also inspired by the work of Davis and co-workers on developing various reagents to convert the Cys residue in proteins to DHA moiety.[12] Hence, we decided to take advantage of placing the Cys residue at position 76 in Ub for a dual function; first to assist in the synthesis of the di-Ub chains and later to establish the DHA functionality.

The syntheses of K48- or K63-linked di-Ub bearing Gly76Cys replacement **3**, was carried out using SPPS and NCL.[13] During the synthesis of the proximal Ub, orthogonally protected Fmoc-Lys-(Dde)-OH was used in position 48 or 63. Subsequent to chain assembly, the $\varepsilon$-amine of the Lys residue was unmasked and coupled with Boc-Cys(Trt)-OH. After peptide cleavage and purification, fragment **1** was ligated by applying NCL[14] with the Ub(1−75)-thioester **2** to afford di-Ub **3** in 40% yield. Next, we turned our attention to the crucial step of converting the Cys in di-Ub **3** to DHA. Several reagents were recently reported for such a transformation, among them is the bisamide of the 1,4-dibromobutane core.[12,15] To convert the Cys76 in di-Ub to the DHA, di-Ub **3** was dissolved in 6 M Gn·HCl, pH 8, in presence of the bisamide reagent, and the reaction was followed by HPLC/ESI-MS.

Gratifyingly, after 3 h, the desired product **4** was observed as the major peak by HPLC analysis (Figure 1B) with a mass

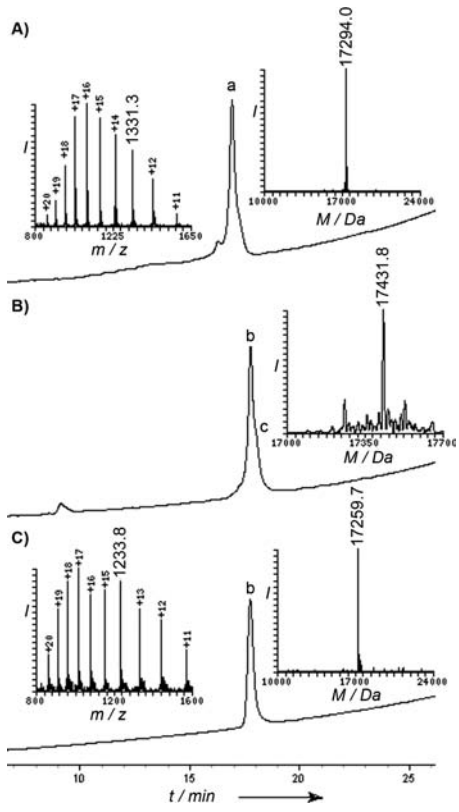



**Figure 1.** Representative synthesis of K48-linked di-Ub probe: (A) reaction at time zero showing the purified ligation product **3a** (peak a) with the observed mass of 17294 Da (calcd *m/z*. 17292.7 Da). (b) HPLC/ESI-MS analysis of the reaction crude after 3 h; peak b corresponds to the desired product **4a** with the observed deconvoluted mass of 17259.7 Da (calcd *m/z* 17259.8 Da); peak c corresponds to the alkylated cyclized intermediate with the observed mass of 17431.8 Da (calcd *m/z* 17433.9 Da) in addition to other side reactions with unidentified masses. (c) Purified K48-linked di-Ub probe.

decrease of 34 Da from the starting material and is consistent with the loss of $H_2S$. We also observed a shoulder to the major peak, which corresponds to the alkylated cyclized intermediate (+138 Da).[15] Longer time of reaction did not drive this step furhter and other side reactions started to appear; hence, the reaction was quenched and purified by semipreparative column giving the desired product in 31% isolated yield.

While the above strategy can be applied to any of the Lys-linked di-Ub chains, in the case of the linear di-Ub the Cys should be placed at the N-terminal of the proximal Ub. Hence, the N-terminal Met1, which is involved in the peptide bond connecting the two-Ub units, was replaced with a Cys residue to enable first NCL followed by DHA formation (Scheme 2). The ligation between Cys-Ub1 **6** and Ub2-thioester **7** was completed within 1.5 h by applying standard NCL conditions (Supporting Information). The ligation product **8** was converted to linear DHA probe **9** as described above (Supporting Information, Figure S6). A portion of the ligation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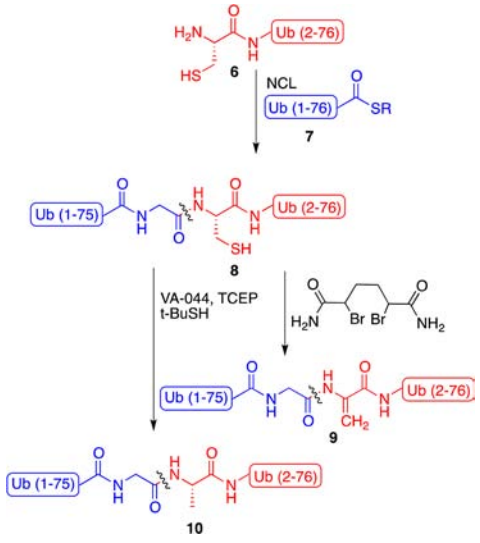
**Scheme 2. Chemical Synthesis of DHA Probe Based on Linear Di-Ub**




product **8** was desulfurized[16] using metal-free condtions[17] to give linear Di-Ub **10** (Supporting Information, Figure S5).

Having the K63-, K48-linked and linear di-Ub probes in hand, we then tested several DUBs for their behaviors with these probes. We first tested probes **4a** and **4b** with the following DUBs:[2] (1) USP2 and IsoT, from the Ub specific protease (USP) family, known to have high promiscuity for Ub chains; (2) OTUB1 and OTUB2 from the ovarian tumor (OTU) family which are known to cleave the K63- and K48-linked chain to different extents; (3) OTULIN and cylindromatosis-associated DUB (CYLD), which do not cleave the K48-linked di-Ub. However CYLD cleaves K63-linked di-Ub, while OTULIN cleaves the linear chain.

The above-described enzymes were first tested for their abilities to hydrolyze the chemically prepared native K63- and K48-linked di-Ub to verify the integrity of these chains and for a comparison with our probes. As expected, after 30 min incubation USP2, ISOT, OTUB1, and OTUB2 hydrolyzed these chains to different levels. OTULIN and CYLD did not hydrolyze K48-linked chain (Supporting Information, Figures S7 and S8); however, CYLD was able to cleave the K63-linked chains. We then treated separately each of these DUBs with probe **4a** for 30 min, and the reactions were analyzed by Western blotting. Our analyses revealed that the designed probe is able to label the known DUBs that are cleaving the native K48-linked di-Ub, i.e., IsoT, USP2, OTUB1, and OTUB2, as indicated by the appearance of new bands that reflect the mass of the specific DUB and the di-Ub probe (Figure 2A). On the other hand, those DUBs that cannot cleave this chain remained unreacted. Our analyses also revealed that the labeling level with the reactive DUBs varies, which could be as a result of the DUB specificity and the orientation of the DHA moiety in the active site. In the case of probe **4b** with USP2, we observed 24% labeling after 1 h, while with IsoT both probes reached up to 60% labeling (Supporting Information, Figure S10). Notably, in the case of USP2, a slight cleavage of the probe was observed. This is consistent with our previous data on the modification of di-Ub chain at the Gly76, where Gly76Ala replacment leads to much higher stability against DUBs, however still slight cleavage was observed.[13]

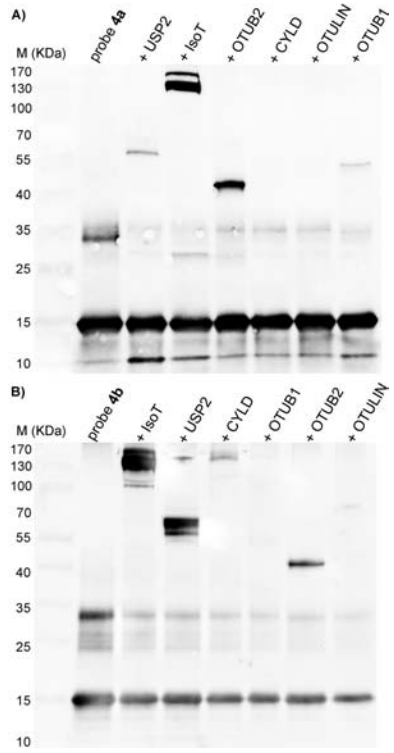



**Figure 2.** Western blot analyses of the (A) K48- and (B) K63-linked di-Ub probes with different DUBs. Each probe was incubated separately with the specific DUBs for 30 min and analyzed by SDS-PAGE and Western blot using anti-Ub antibody. Bands at higher MW indicating the labeling and reflecting the mass of the specific DUB in addition to di-Ub. The band appears at 35 KDa is an unidentified product and shows only in the Western blot analyses.

Examining the K63-linked di-Ub chain probe under similar conditions with the above listed DUBs validated nicely the known behavior of this chain with these DUBs. As seen from Figure 2B, IsoT and USP2, OTUB2, and CYLD reacted with the probe while OTULIN and OTUB1 did not show any reactivity confirming the specificity of this probe. Notably, this probe exhibited complete stability with the various DUBs where no isopeptide bond cleavage was observed.

Next, we examined the behavior of the linear di-Ub probe with two DUBs that are known to cleave this chain, OTULIN and USP2. Interestingly, both DUBs cleaved this probe completely rather than reacting with the DHA unit (Supporting Information, Figures S9). In the Lys-linked di-Ub chains, the DHA unit was placed instead of Gly76 of the distal Ub, which is the obvious logical position both in terms of synthesis and close vicinity to the cleavable isopeptide bond (Scheme 1). However, in the linear di-Ub case there are two possibilities to place the DHA unit relative to scissile amide bond. In the current study, the DHA unit was placed prior to the scissile amide bond (P1′), replacing Met1 with the DHA, which apparently was not accessible for the nucleophilic attack by the reactive Cys of the specific DUB.

In summary, we have presented a novel strategy to prepare di-Ub activity probes for various DUBs. The strategy should be amenable to all di-Ub chains as well to ubiquitinated proteins that are accessible by chemical and semisynthesis of protein[18] as well as applying the genetically site-specific introduction of $N^{\varepsilon}$-L-cysteineyl-L-Lys in recombinant proteins.[19] The synthesis of other Lys-linked di-Ub probes, the redesign of the linear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chain probe, examining how changing the position of the DHA unit within the Lys-linked di-Ub chains could influence the efficiency of the labeling and their applications in studying DUBs profiling and inhibition in cellular environment, is currently underway.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental procedures and data for synthesis and analysis of probes. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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(11) While this manuscript was under preparation and under review, two papers were published reporting the preparation of di-Ub probes: (1) Zhuang and co-workers reported activity-based di-Ub probes (K48 and K63) by introducing Michael acceptor in the linker between the two Ubs. The authors mutated the desired Lys residue with Cys in the proximal Ub, which was reacted with the C- terminal of the other Ub bearing $\alpha$-bromo-unsaturated ketone functionality. For more details see: Li, G.; Liang, Q.; Gong, P.; Tencer, A. H.; Zhuang, Z. *Chem. Commun.* **2014**, *50*, 216. (2) Kessler and co-workers reported di-Ub probes based on all eight different linkages in which the proximal Ub was linked by Cu(I)-catalyzed 1,4-triazole formation to the C terminus of the distal Ub bearing a reactive electrophile. For more details, see: McGouran, J. F.; Gaertner, S. R.; Altun, M.; Kramer, H. B.; Kessler, B. M. *Chem. Biol.* DOI: 10.1016/j.chembiol.2013.10.012.
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