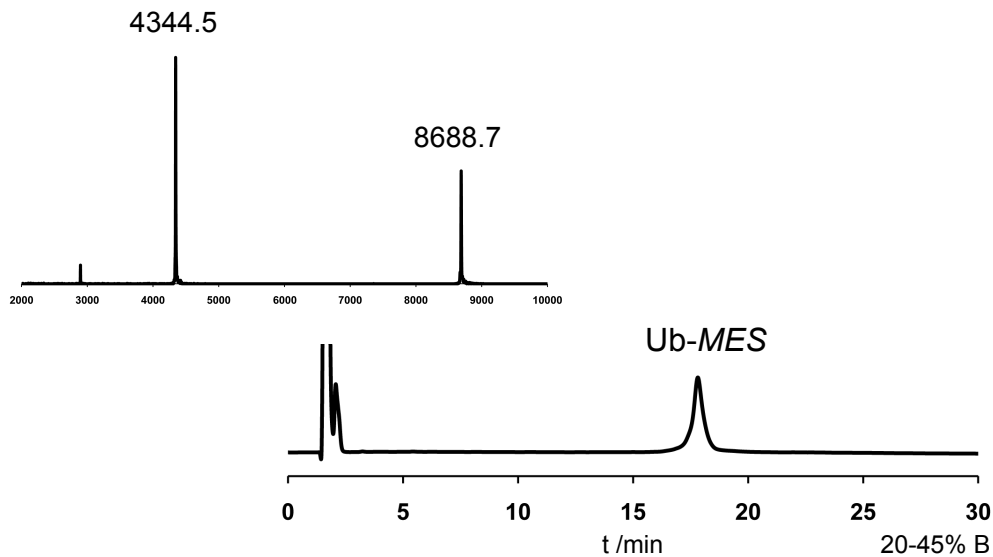
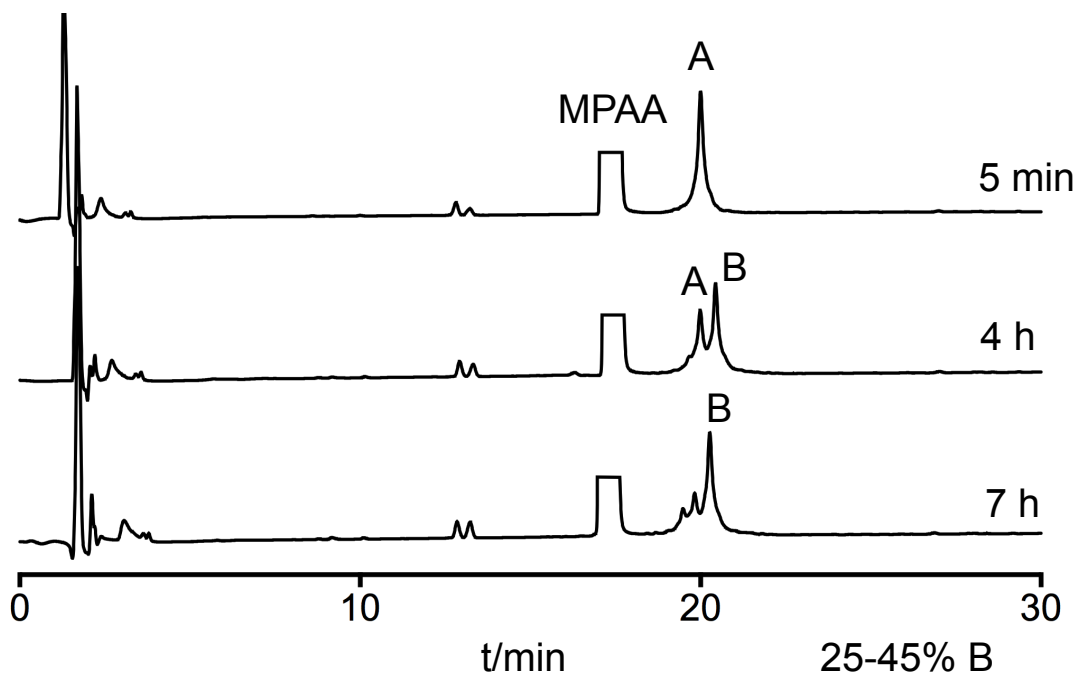


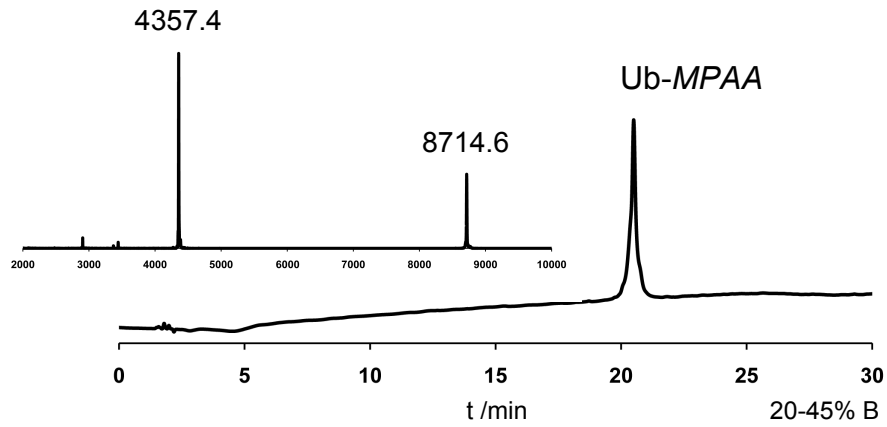
Supplementary Figure 1. Model ligation of NEMO₂₉₈₋₃₀₈hdmb with LAPAG-MPAL. Time-course monitoring of ligation reaction by analytical HPLC, RP-C18, (2 μ L aliquots of the reaction mixture diluted in 10 μ L H₂O were injected) and MALDI-TOF MS of purified peptide C (ligation product with hdmb auxiliary), $m/z = 1873.6$ [M+H]⁺, calc. = 1876.9, * = MPAA. Analytical HPLC of crude product after hdmb removal from purified peptide C and MALDI-TOF MS, $m/z = 1679.3$ [M+H]⁺, calc. = 1679.8, D = ligation product without hdmb.



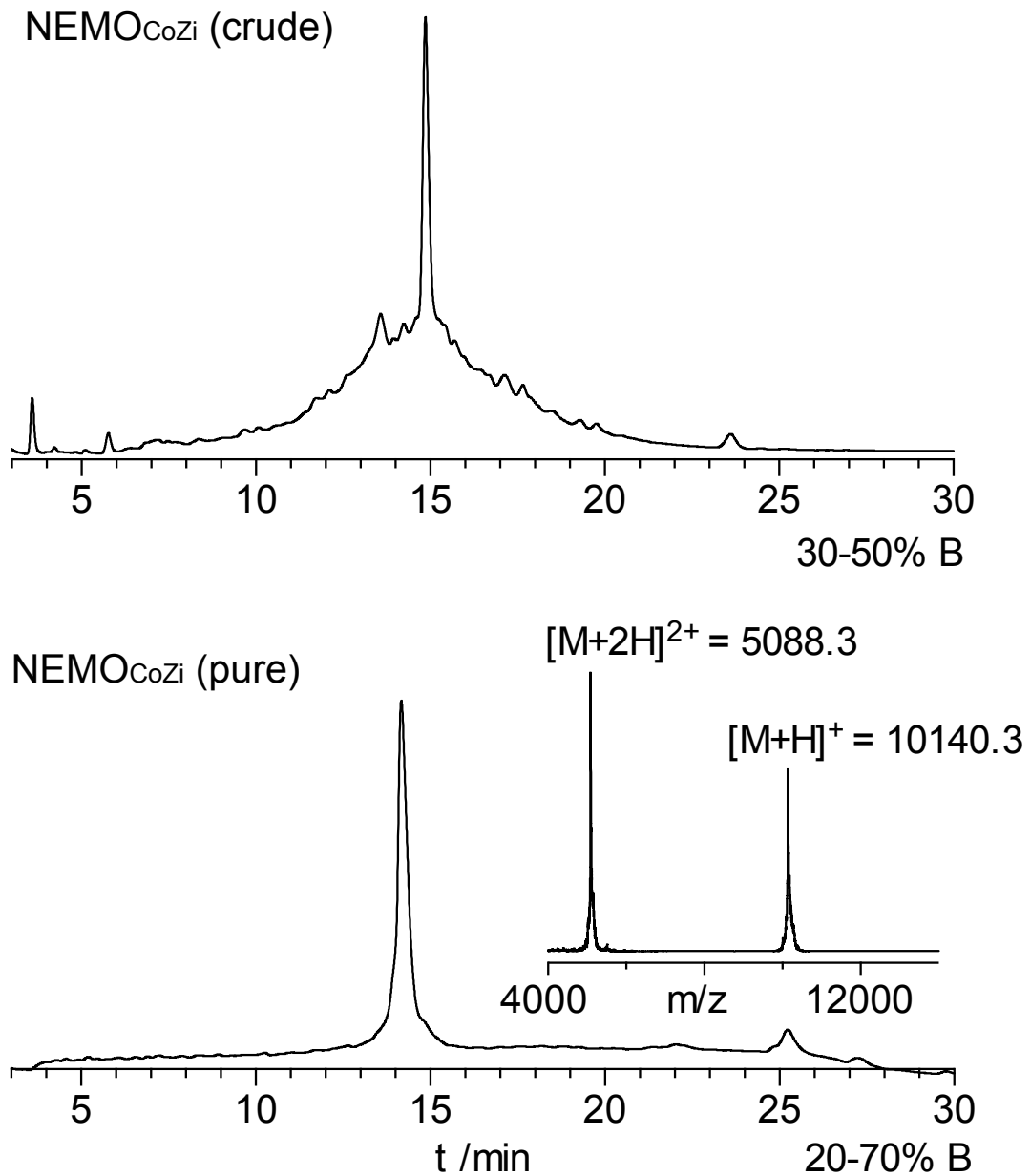
Supplementary Figure 2. Synthesis of Ub-MES thioester by N,S acyl transfer and thioester exchange from Ub- α MeCys. Analytical HPLC (RP-C18) and MALDI-TOF MS of Ub-MES, $m/z = 8689$ $[M+H]^+$, calc. Av. = 8689.



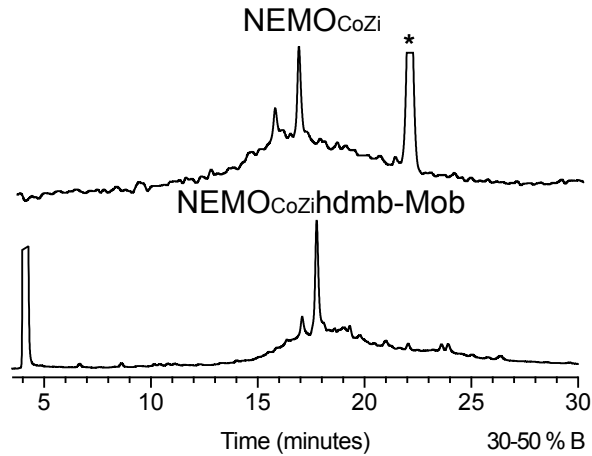
Supplementary Figure 3. Time course of Ub-MPAA thioester synthesis by N,S acyl transfer and thioester exchange from Ub- α MeCys monitored by analytical HPLC (RP-C18) injecting 10 μ L aliquots of the reaction mixture. A = Ub- α MeCys, B = Ub-MPAA thioester.



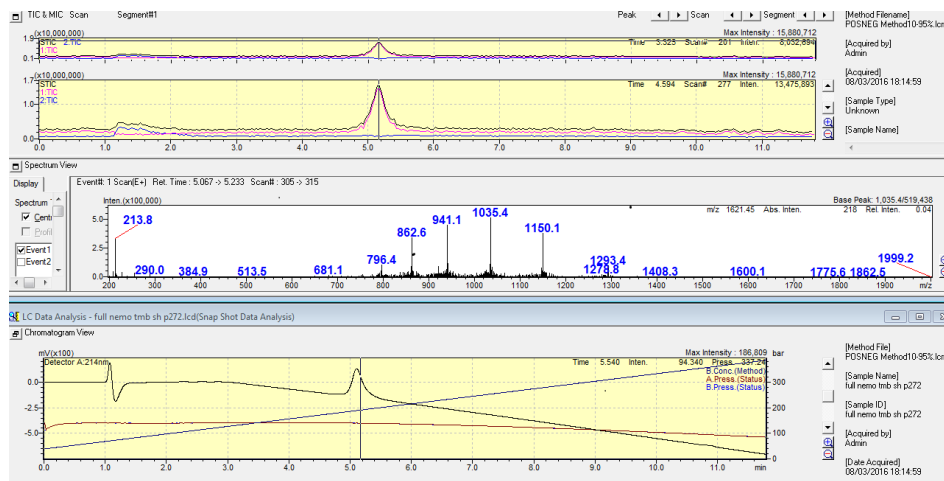
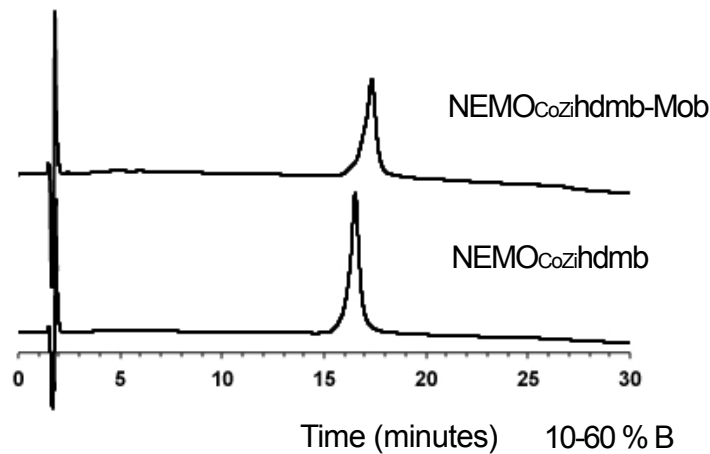
Supplementary Figure 4. Analytical HPLC (RP-C18) and MALDI-TOF MS of purified Ub-MPAA, $m/z = 8715$ $[M+H]^+$, calc. Av. = 8715.



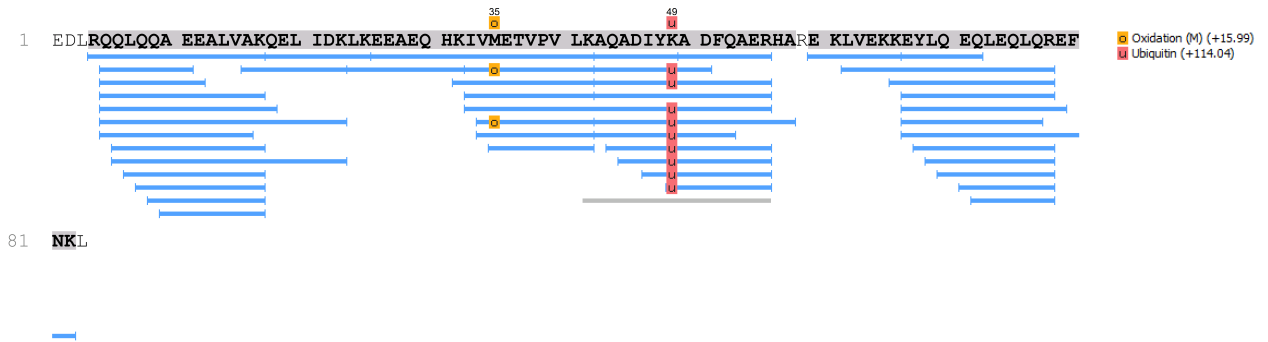
Supplementary Figure 5. Analytical HPLC (RP-C18) of crude and purified NEMO_{CoZi} (after removal of Alloc). *Biotin*-EDLRQQLQQAEELVAKQELIDKLKEEAEQHKIVMETVPV LKAQADIYKADFQAERHAREKLVEKKEYLQEQLEQLQREFNKL-NH₂. Insert shows MALDI-TOF MS, m/z = 10140 [M+H]⁺, calc. Av. = 10144.



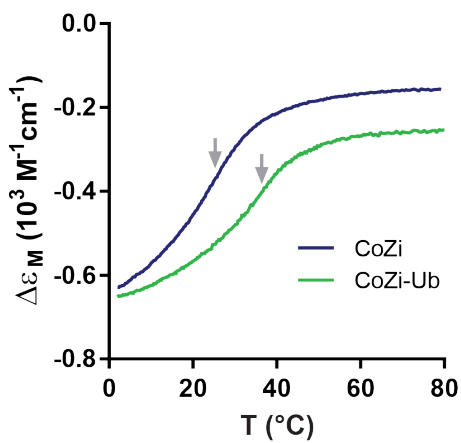
SupplementaryFigure 6. On-resin installation of the ligation auxiliary hdmb (**3**) into $\text{NEMO}_{\text{CoZi}}$. Analytical HPLC (RP-C18) of crude $\text{NEMO}_{\text{CoZi}}$ and $\text{NEMO}_{\text{CoZi}}\text{hdmb-Mob}$. * = scavenger



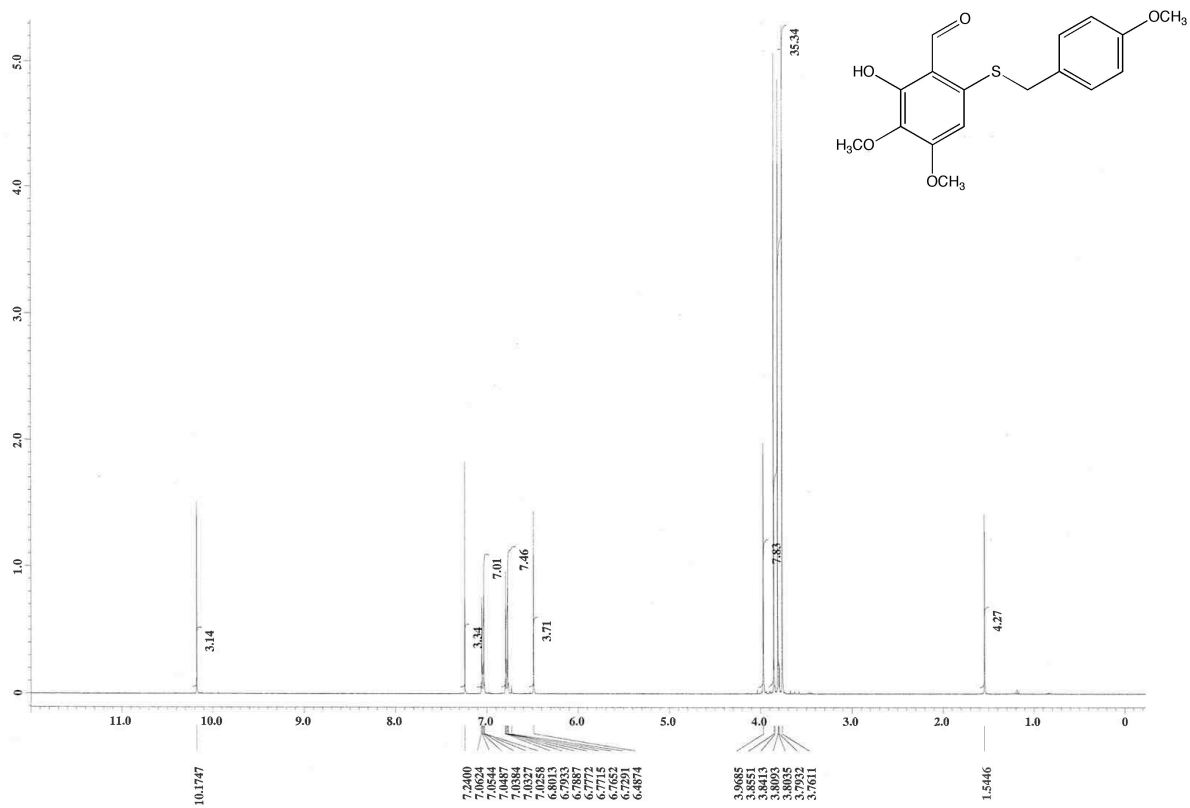
Supplementary Figure 7. Removal of Mob protecting group in solution. Analytical HPLC (RP-C18) of pure $\text{NEMO}_{\text{CoZi}}\text{hdmb-Mob}$ and $\text{NEMO}_{\text{CoZi}}\text{hdmb}$ and LCMS of $\text{NEMO}_{\text{CoZi}}\text{hdm}$ $m/z = 10342$ $[\text{M}+\text{H}]^+$, calc. Av. = 10342.



Supplementary Figure 8. Sequence coverage of NEMO_{CoZi}-Ub derived from LC-MSMS indicating ubiquitylation site (red). Oxidation of Met³⁵ was also detected (orange).



Supplementary Figure 9. Comparison of thermal melting curves of synthetic NEMO_{CoZi}-Ub (8 μM) and NEMO_{CoZi} (16 μM), measured by circular dichroism at 222 nm. The melting temperatures (grey arrows) of NEMO_{CoZi}-Ub and NEMO_{CoZi} are 36.4 °C and 24.9 °C, respectively.



Supplementary Figure 10. ¹H NMR of hdm b aldehyde 3.