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海外特別研究員最終報告書

独立行政法人 日本学術振興会 理事長 殿

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(氏名は必ず自署すること)

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。

なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地 (派遣先国名) 用務地: チューリッヒ (国名: スイス連邦)

2. 研究課題名 (和文) ※研究課題名は申請時のものと変わらないように記載すること。

SUMO タンパク質の化学合成とその新規標的タンパク質同定

3. 派遣期間: 平成 29 年 4 月 1 日 ~ 平成 31 年 2 月 23 日

4. 受入機関名及び部局名

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ETH Zuurich

5. 所期の目的の遂行状況及び成果…書式任意 **書式任意 (A4 判相当 3 ページ以上、英語で記入****も可)**

(研究・調査実施状況及びその成果の発表・関係学会への参加状況等)

(注)「6. 研究発表」以降については様式 10-別紙 1~4 に記入の上、併せて提出すること。

【Background and Aims】

Proteins are subjected to a huge variety of post-translational modifications, which regulate the function, localization, or activity. A prime example is the covalent attachment of ubiquitin or ubiquitin-like proteins such as SUMO (small ubiquitin-related modifier). Ubiquitin or SUMO conjugation contributes to various cellular processes through protein degradation or stabilization, regulation of transcription, and DNA repair. There is abundant evidence that defects of SUMO modification is highly associated with various diseases, such as cancers, diabetes, and neurodegenerative diseases. In addition, some viruses and bacteria interfere with the SUMOylation of host proteins during infection. Understanding of the role of SUMOylation in various cellular events can be used to identify biomarkers for diseases and possible targets for drug development. In this research, the author synthesizes SUMO proteins for irreversible conjugation, and identifies the proteins that are modified by SUMO. We will monitor changes in SUMO modification with different cell lines or under various stress and stimuli. This study will provide an updated landscape of SUMO-regulated cellular events, and contribute to drug development or medical treatment.

【Research plan】1. Chemical synthesis of SUMO3 probes with C-terminal mutation

The human genome encodes the three different functional SUMO isoforms (SUMO1, SUMO2, and SUMO3) that are conjugated to distinct but overlapping sets of target proteins. Conjugation of

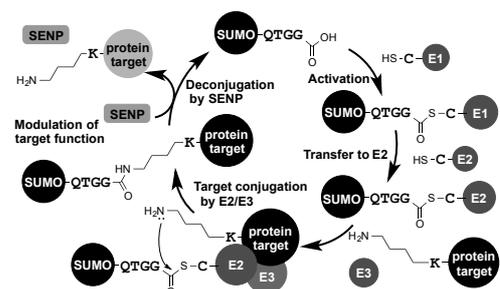


Figure 1. SUMO cycle

SUMO to its targets requires an E1-activating enzyme, an E2-conjugating enzyme, and E3 enzymes (Figure1).

Once conjugated to its target, SUMO can be deconjugated by SUMO-specific proteases (SENP) that tightly regulate the SUMOylation levels of proteins. To understand how SUMOylation can specifically control protein activity and hence cellular environment, it is necessary to identify the target of SUMOylation under various conditions. Much efforts has been dedicated to the identification of SUMO-conjugated proteins since its discovery. The most common approach used to isolate SUMOylated proteins is based on the expression of His-tagged versions of SUMO allowing the purification of SUMO-conjugated proteins by nickel chromatography. However, isolation of SUMOylated proteins has proven to be challenging. Indeed, for most SUMO substrates, only a small proportion of the total amount of protein is SUMO-attached. In addition, the high activity of SENP results in the rapid loss of SUMO conjugation in the absence of appropriate inhibitors. If SUMO that can be attached to the target proteins, but not removed by SENP is obtained, such SUMO can be a powerful tool to identify its targets. There are a few reports about a conjugatable, but not deconjugatable versions of SUMO containing the mutation within its C-terminal part (Figure 2). This is because that both E1 and E2 enzymes have large active site cavities and can accommodate a slightly kinked SUMO C terminus, however the narrow active site cleft of SENP does not tolerate it. To optimize the function of SUMO3 probe for irreversible conjugation, more C-terminal variants of SUMO are required.

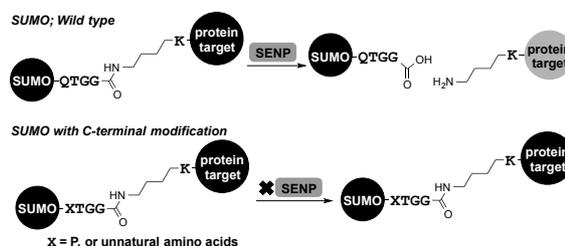
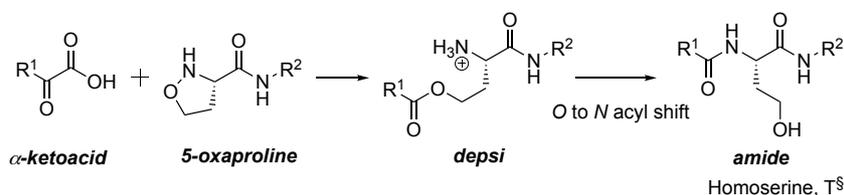


Figure 2. SUMO for irreversible conjugation

2. Design of synthetic SUMO3 probes and synthetic plan

Based on these knowledges, the author will introduce Q89P mutation into SUMO3, or noncanonical amino acid into Q89 positions to avoid the cleavage by SENP. Moreover biotin tag and Rhodamine will also be introduced at N-terminus to purify SUMO3 probe conjugated to its target and to see the localization. In addition to the biotin tag, fluorescent dye, and C-terminal mutation, cell penetrating peptides (CPPs) would be introduced in the second generation of SUMO3 probe for capturing the SUMOylation target in living cells. After we established synthetic SUMO3 probe sequence or contents, CPPs would be introduced to enable SUMO3 probe to get into the cells.

The chemical synthesis of SUMO probe can be achieved by multiple segment ligations using α -ketoacid-hydroxylamine (KAHA) ligation (Scheme 1). In KAHA ligation, a C-terminal peptide α -ketoacid reacts chemoselectively with a N-terminal peptide hydroxylamine to form an amide bond. This reaction is compatible with unprotected peptide side-chain functional groups, and works in DMF, DMSO or water. The KAHA ligation is very suitable for the assembly of peptide fragments and chemical syntheses of proteins. SUMO3 will be divided into three segments, and key bi-functional segments with an N-terminal hydroxylamine and C-terminal α -ketoacid can be accessed by Fmoc solid-phase peptide synthesis. Several C-terminal variants with the mutation and CPPs will be prepared to obtain the desired SUMO3 probe for irreversible conjugation and for getting into the living cells.



Scheme 1. KAHA ligation

3. Evaluation of the C-terminal modified SUMO3 probe

To evaluate the function of SUMO3 probe, we will perform in vitro SUMOylation reaction using RanGAP1, which is known to be the substrate of SUMO. Synthetic SUMO3 probe, E1, E2 enzymes, RanGAP1, and SENP will be incubated to generate SUMO3 probe-RanGAP1 conjugates. The enzymatic reaction products will be separated by SDS-PAGE followed by Western Blotting (WB) using anti-SUMO antibody. The synthetic SUMO which retains the conjugation with RanGAP1 will be the desired SUMO that can be

transferred to the target proteins but cannot be removed by SENP.

【Results and Discussion】

1. SUMO3 probe 1st 2nd generation

The first SUMO3 probe was synthesized by former PhD student in Bode group, Dr. Thomas Wucherpfenning (Figure 3). As we mentioned above, our SUMO3 probe harbors Biotin, as a tag, PEG linker and Rhodamin at N-terminus. Besides, we put Q89P mutation at C-terminal for irreversible conjugation. After the completion of synthesis, we had checked the activity of SUMO3 probe by in vitro SUMOylation assay followed by WB. This analysis showed the activity of SUMO3 probe to attached to RanGAP1 was enough, and we moved to the next step, in vitro SUMOylation assay with cell lysates and following proteomics analysis. The results of the proteomics analysis was not good as expected, there are very few number of the protein which is modified by SUMO3 probe.

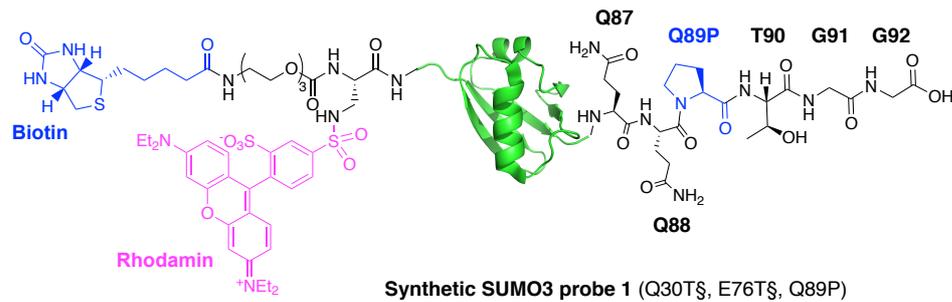


Figure 3. The first SUMO3 probe

For proteomics analysis, the SUMO3 probe – protein conjugates were enriched by Avidin agarose from in vitro SUMOylation mixture. Next, the enriched SUMO3 probe – protein conjugates were digested by trypsin to give peptide fragments. Finally these peptide fragments were analyzed by MS. However, it turned out that the peptide fragments harboring the branched peptides from the C-terminal of SUMO3 probe, which could be the proof of the modification by SUMO3 probe, were hard to detect by MS analysis (Figure 4). During the digestion process, the trypsin cut the peptide chain at lysine and arginine, which possess positively charged side chain. Looking at the SUMO3 peptide sequence, the trypsin cleavage site closest to the C-terminal is Arg60, which would give 32 amino acids branch on the peptides from the modified protein. Such a peptide fragments are quite large for MS analysis and gave lower signal compared to the smaller peptide fragments, which could give higher signal. Therefore, we could have got few number of the proteins modified by SUMO3 probe 1st generation. To resolve this issue, we had decided to put the additional mutation at C-terminal, T90K, which would introduce the positive charge and thus trypsin cleavage site very close to C-terminal. This mutation would give only two amino acids branch on the peptide fragments from the modified protein, and this could make peptide fragment harboring SUMO3 probe modification much smaller. We set out the synthesis of SUMO3 probe 2nd generation with T90K mutation.

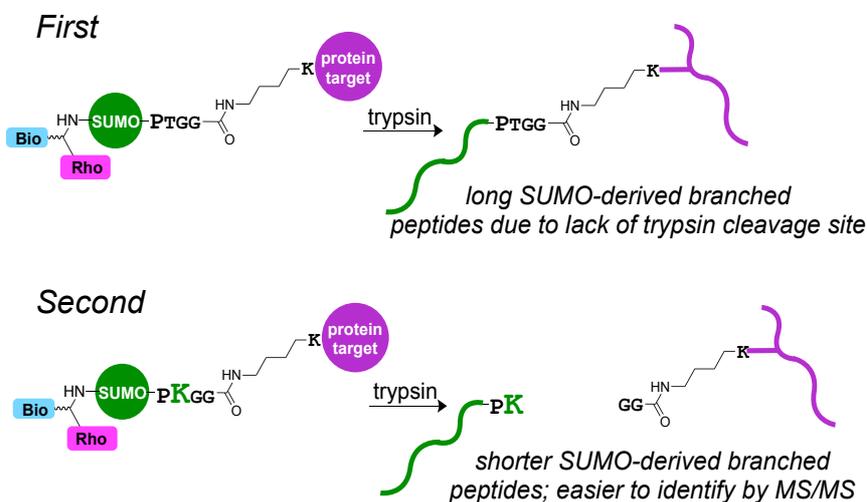
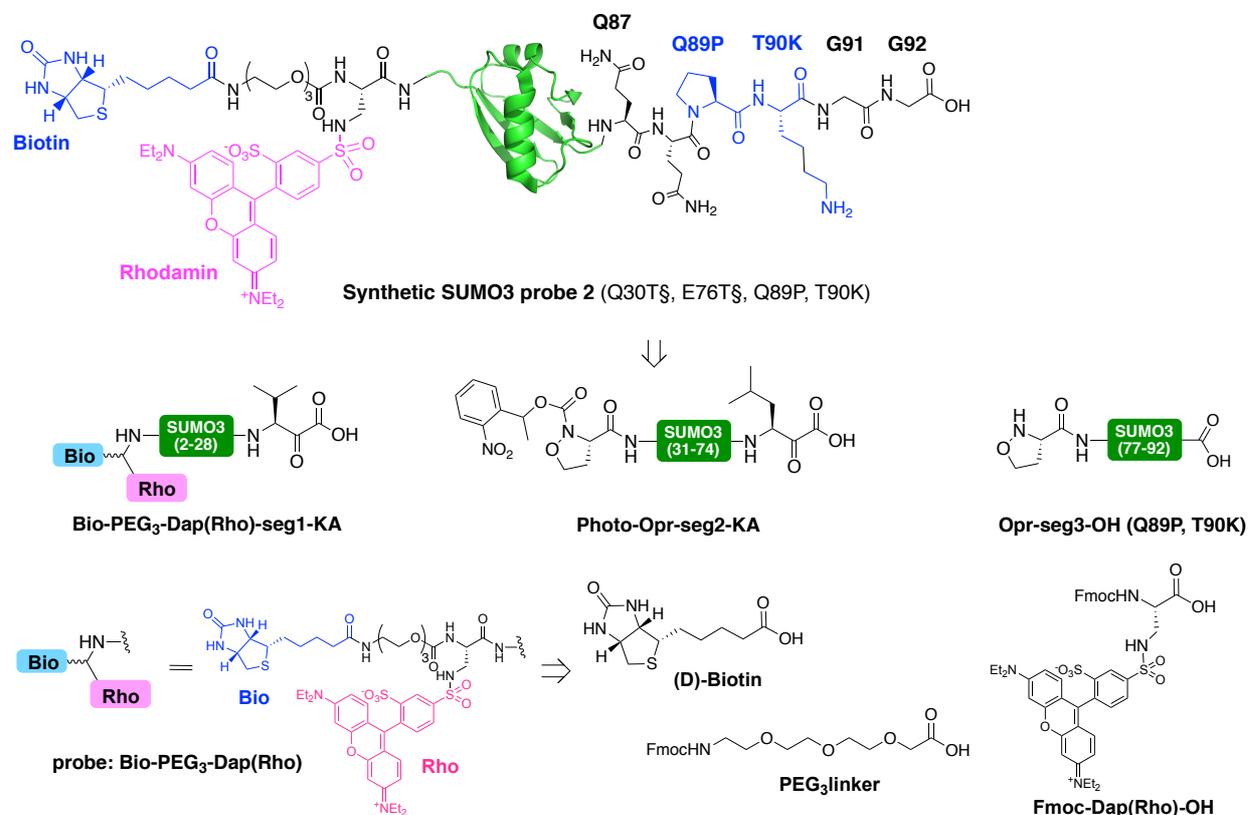


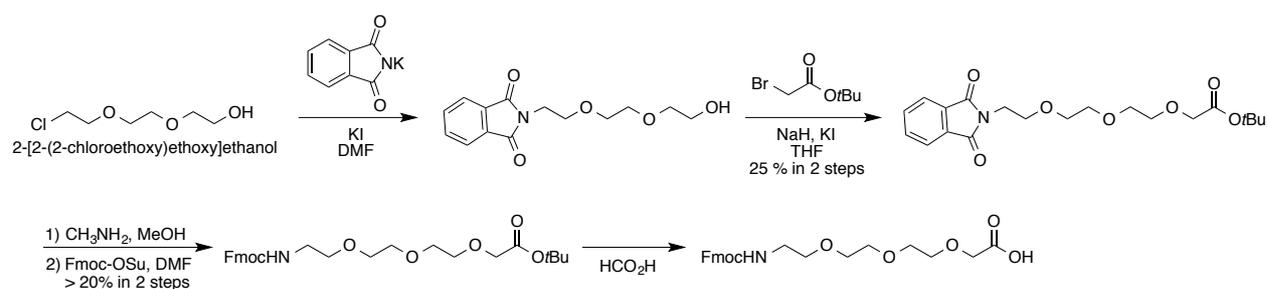
Figure 4. Branched peptide fragments with SUMO probe C-terminal after trypsin cleavage

In general, SUMO3 probe was prepared by the sequential ligation of three peptide segments (Scheme 2). Segment 1 starts from Ser2 and ends with Val29 ketoacid (KA). Segment 1 contains biotin, PEG linker and Rhodamin at N-terminus (seg1-KA). Segment 2 starts with Oxaproline (Opr) with photolabile protecting group followed by Phe31 and terminates at Leu75KA (Photo-Opr-seg2-KA). Segment 3 starts with Opr followed by Met77 and finishes at SUMO3 C-terminal, Gly92 (Opr-seg3-OH).



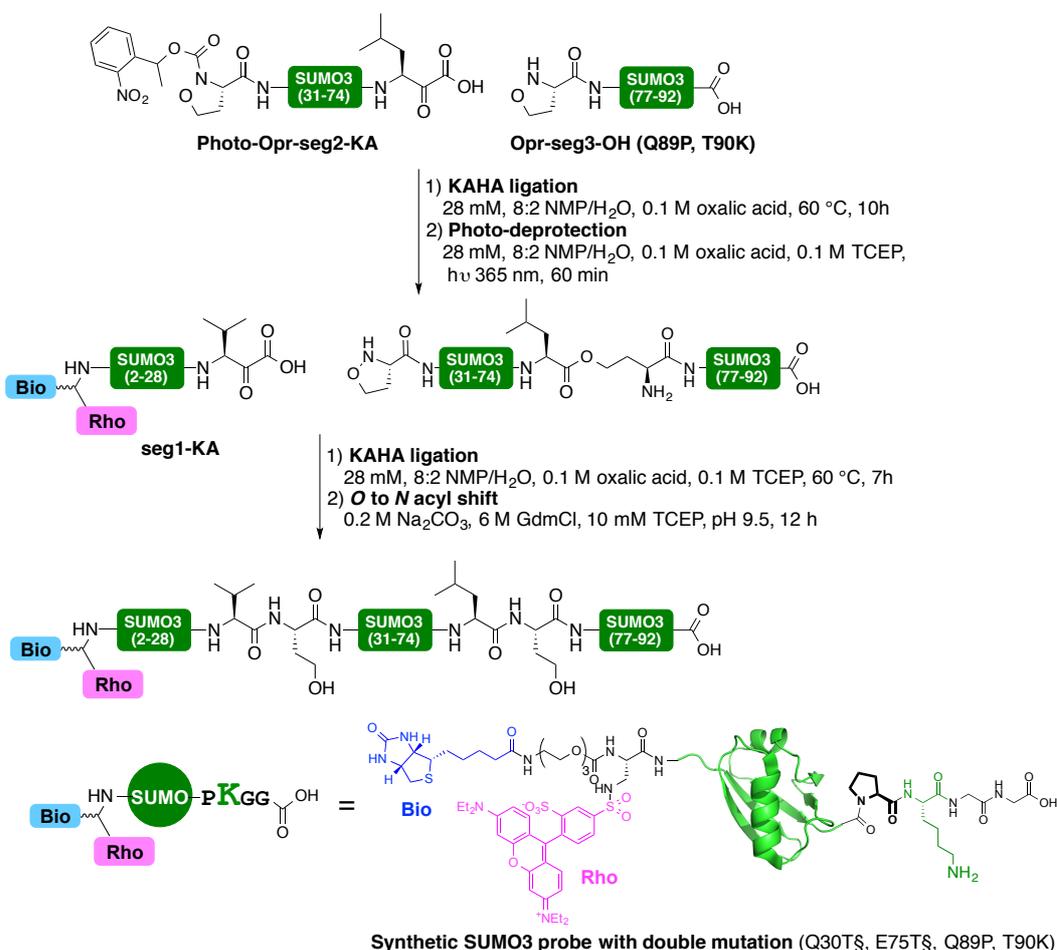
Scheme 2. Synthetic plan of SUMO3 probe

Each segments were prepared by Fmoc solid phase peptide synthesis (SPPS), and each N-terminal was coupled with either Opr, Photolabile protected Opr, or Rhodamin, PEG linker, Biotin. To introduce Rhodamin into segment 1, Fmoc-Diaminopropane (Rhodamin) –OH [Fmoc-Dap(Rho)-OH] were prepared by the coupling of Fmoc-Dap-OH and Rhodamin B sulphonyl chloride. PEG linker was prepared from 2-[2-(2-chloroethoxy)ethoxy]ethanol in five steps as described in the scheme (Scheme 3). After the SPPS of segment 1, first Fmoc-Dap (Rho)-OH was coupled followed by the Fmoc deprotection, and then coupling of PEG linker followed by Fmoc deprotection as well. At the end, biotin was coupled and complete segment 1 was cleaved from the resin. Crude peptides were purified by preparative HPLC, lyophilized and gave pure peptide segments as a white powder.



With three peptides segments in hand, we carried out the sequential ligation (Scheme 4). First, segment 2,

Photo-Opr-seg2-KA, and segment 3, Opr-seg3-OH, were ligated at 60 °C, 28 mM in ligation buffer for 10 hours. After the completion of the ligation, reaction mixture was photoirradiated at 365 nm to afford photo-deprotected Opr at N-terminal of the ligation product. After the photo-deprotection, segment 1 was added into the reaction mixture and 2nd KAHA ligation was proceeded, followed by the *O* to *N* acyl shift. After the purification of the crude ligation mixture by prep HPLC, the full length of SUMO3 probe was lyophilized to give pink powder.



Scheme 4. Synthesis of SUMO3 probe 2nd generation

Next, we checked the activity of SUMO3 probe 2nd generation by in vitro SUMOylation assay followed by WB, and tried in vitro SUMOylation assay with cell lysates and following proteomics analysis as well. However, the activity of SUMO3 probe 2nd generation was not high enough and the results of the proteomics analysis did not improve. We assumed this is due to the T90K mutation, as such a big change very close to C-terminal of SUMO3 probe could result in the poor activity of SUMO3 probe itself, even it could give the small peptide branch and could make proteomics analysis easier. Based on this result, we decided to change the mutation site for positive charge. As we still need trypsin cleavage site close to C-terminal, we chose the Q87R mutation as a new trypsin cleavage site, and moved to the SUMO3 probe 3rd generation.

2. SUMO3 probe 3rd generation and 4th generation

To synthesis SUMO3 probe 3rd generation, the new segment 3 with Q87R mutation was prepared by SPPS (Figure 5). Afterwards, the three segments were ligated in the same fashion as 2nd generation. After finishing the synthesis, the activity of the SUMO3 probe 3rd generation was checked by in vitro SUMOylation assay followed by WB. As the activity of SUMO3 probe 3rd generation got better compared with that of 2nd generation, we tried in vitro SUMOylation assay with cell lysates and following proteomics analysis. The results of the proteomics analysis improved, and number of the proteins modified by SUMO3 probe increased. However, the number of proteins we got was still not good enough. At that moment, we realized that there was still significant issue we have to fix. The issue was SUMO dimer. It was known that SUMO itself could be the substrate for SUMOylation, and in some cases SUMO chains could be formed. Under the condition of the in vitro SUMOylation assay, there were

always some amount of SUMO3 dimers. As the concentration of the SUMO3 dimer could be higher than any other SUMO3 probe – protein conjugation, they gave strong signal during the MS analysis which diminished the signals from other SUMO3 probe – protein conjugates.

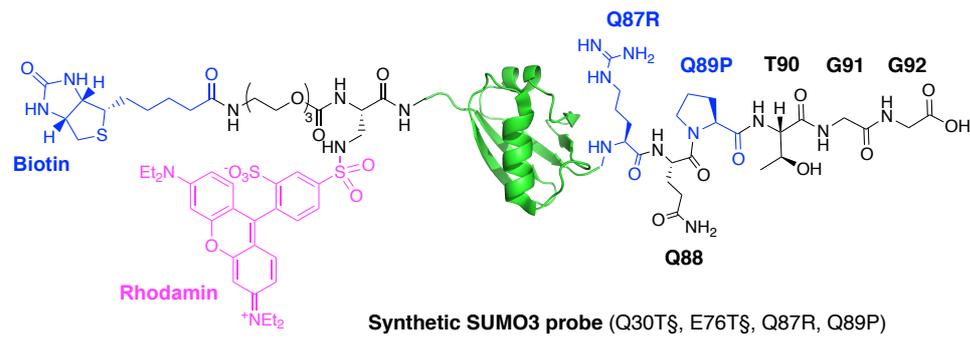


Figure 5. SUMO3 probe 3rd generation

Taken this result, we decided to add additional mutations. The new mutations are K5R, K7R, and K11R. Lysine 5, 7, and 11 are three possible SUMOylation site of SUMO, therefore these three mutations could prevent the formation of SUMO3 probe dimer. The design of SUMO3 probe 4th is showed below (Figure 6).

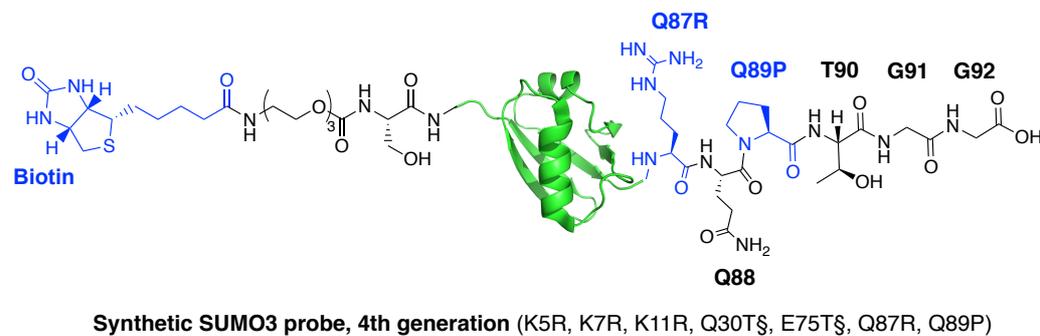


Figure 6. SUMO3 probe 4th generation

The activity of SUMO3 probe 4th generation was evaluated by in vitro SUMOylation assays. In this assay, cell lysates were prepared, and incubated with SUMO3 probes and other essential components of SUMOylation. After in vitro SUMOylation, the reaction mixture was purified by avidin agarose, and purified proteins were analyzed by proteomics analysis. After proteomics analysis, the list of protein with SUMO modification was achieved, however, the number of the proteins in the list was not sufficient. Although the signal from the SUMO dimer got lower, the list of the protein was not great as expected. To improve the results after proteomics, the final SUMO3 probe was synthesized, and purification procedure was investigated to improve the result. However, each results were not sufficient for publication.