Unnatural amino acids for in vivo protein labeling

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The incorporation of unnatural or also called non-canonical amino acids (ncAAs) into proteins via the cellular biosynthetic machinery is frequently achieved by Amber stop codon suppression. This technique is often termed a genetic code expansion. In the past, we used the tRNA and the pyrrolysine synthetase (pCMV $tRNA^{Pyl}$/PylRS) from archaeobacter *Methanosarcina mazei* as it is recognizing lysine-based amino acids with an amide or a carbamate substitution at the side chain amino group. To accommodate larger molecular entities, we used a synthetase altered at two residues (Y306A, Y384F, termed $tRNA^{Pyl}$/$PylRS^{AF}$). We successfully incorporated cyclooctyne groups for performing 3+2 cycloaddition reactions in vitro and in intact cells (Plass et al., 2011). In order to increase reaction speed we then switched to 4+2 cycloaddition reactions of the Diels-Alder type that ideally employ a strained *trans*-cyclooctene moiety and a tetrazine (Plass et al., 2012). We then used the difference in the reaction rates of cyclooctenes with H- and methyl-tetrazines to introduce two distinct labels in an intact cell in a kinetically controlled fashion (Nikic et al., 2014).

Here we present highly improved synthetic access to the axially and the equatorially linked trans-cyclooct-2-ene isomers. We further show that the axially connected isomer is significantly more stable. We then determined the reaction rate constants of the Diels-Alder reaction with H-tetrazines by stopped-flow experiments. We demonstrate the effect of the improved reactivity on the labeling of the insulin receptor on the surface of intact cells. In addition, results on potential occurrences of ß-elimination reactions of the click reaction products and loss of label are quantitatively analyzed.

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