A Bioorthogonal Click Chemistry Toolbox for Targeted Synthesis of Branched and Well-Defined Protein-Protein Conjugates

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A highly efficient technology for protein functionalization with commonly used bioorthogonal motifs for Diels-Alder cycloaddition with inverse electron demand (DA_{inv}). With the aim of precisely generating branched protein chimeras, we systematically assessed the reactivity, stability and side product formation of various bioorthogonal chemistries directly at the protein level. We demonstrate the efficiency and versatility of our conjugation platform using different functional proteins and the therapeutic antibody trastuzumab. This technology enables fast and routine access to tailored and hitherto inaccessible protein chimeras useful for a variety of scientific disciplines.

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A bioorthogonal click chemistry toolbox for targeted synthesis of branched and well-defined protein-protein conjugates

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Abstract

Bioorthogonal chemistry holds great potential to generate difficult-to-access protein-protein conjugate architectures. Current applications are hampered by challenging protein expression systems, slow conjugation chemistry, use of undesirable catalysts, or often do not result in quantitative product formation. Here we present a highly efficient technology for protein functionalization with commonly used bioorthogonal motifs for Diels-Alder cycloaddition with inverse electron demand (DA\textsubscript{inv}). With the aim of precisely generating branched protein chimeras, we systematically assessed the reactivity, stability and side product formation of various bioorthogonal chemistries directly at the protein level. We demonstrate the efficiency and versatility of our conjugation platform using different functional proteins and the therapeutic antibody trastuzumab. This technology enables fast and routine access to tailored and hitherto inaccessible protein chimeras useful for a variety of scientific disciplines. We expect our work to substantially enhance antibody applications such as immunodetection and protein toxin-based targeted cancer therapies.
Introduction

Bioorthogonal chemistry provides ample opportunities to be applied in the manufacturing of therapeutic biologics due to its biocompatible properties. Chemical conjugation strategies possess power to generate fusion types that are challenging to access using common protein expression or conjugation technologies. This is particularly the case for proteins that require special expression systems and thus limits the production as a fusion protein to a certain expression host. Examples for this are fusion proteins with toxic domains[1] or with non-proteinogenic features such as polysaccharides in vaccines[2]. Moreover, it is obvious that bioorthogonal chemical strategies can provide access to protein conjugate architectures that cannot be achieved by sole molecular biology methods (Scheme 1).[3]

To fully unleash the potential of bioorthogonal chemistry for the production of advanced biologicals, the method of conjugation needs to be highly efficient, quantitative and free of undesired side products to obtain homogeneous products - essential criteria to meet the high requirements of pharmaceutical manufacturing.[4] The demands are thus fundamentally different from applications for biomolecule labeling in complex mixtures, where incomplete labeling or minor side products are less of a problem. Among all bioorthogonal reactions, the Diels-Alder cycloaddition with inverse electron demand (DA_{inv}) between alkenes or alkynes (dienophiles) and N-heteroaromatic compounds (dienes) has become the most promising chemoselective bond-forming chemistry in terms of catalyst-free reaction conditions and reaction rates.[5] It is thus not surprising that DA_{inv} has already been adapted for the generation of antibody-drug conjugates (ADCs)[6] or investigated for diagnostic antibody radiolabeling[7] or related tumor pretargeting[8] approaches.

Advances in the past years gave rise to a variety of co-translational or posttranslational means to introduce DA_{inv} motifs site-specifically to proteins to prime them for subsequent site-specific derivatization.[9] A major challenge still remains in homogeneously labeling proteins with free choice of modification site and full independence of the expression system. In contrast to co-translational approaches, posttranslational labeling methods entail strict separation of installation of the bioorthogonal motif from protein translation and the expression host. This also minimizes exposure time to prevent the reactive scaffold from losing integrity[10] with the consequence of more efficient conjugation. Thus, a key consideration toward fast
and quantitative protein labeling is that the installed bioorthogonal motif is (I) as reactive as possible and (II) entirely stable until the chemoselective derivatization is performed (stability-reactivity-tradeoff). With the increasing number of bioorthogonal DA$_{\text{inv}}$ motifs reported in literature,$^{[5, 11]}$ the selection of those best suited for fast and highly efficient protein conjugation is not easy. A comprehensive comparison of the currently most used dienophile/diene pairs would be highly beneficial for experimentalists interested in bioconjugation, especially when the requirements for the production of biologics have to be met as benchmark characteristics.

Here we present the first extensive comparative study of various bioorthogonal chemistries for quantitative protein conjugation, aimed at identifying the ideal reaction partners for the production of defined and branched protein-protein conjugates (PPC) by DA$_{\text{inv}}$. Recently, we reported that lipoic acid protein ligase-based ligation of tetrazine substrates to proteins is compatible with quantitative and stoichiometric protein functionalization using dienophile probes.$^{[12]}$ Envisioning flexible conjugation workflows and compatibility with a variety of commercially available probes, we synthesized a large panel of the most popular dienophile and diene scaffolds as carboxylic acid derivatives and tested them as substrates for lipoic acid ligase (LplA). Several novel LplA$^{W37V}$ substrates for modular and selective protein modification were identified. Additionally, we developed an assay based on electrophoretic mobility changes of enhanced green fluorescent protein (EGFP) to set up a straightforward evaluation of each dienophile and diene motif in DA$_{\text{inv}}$-mediated $\textit{in vitro}$ protein functionalization. Among a novel methyltetrazine substrate, a stable bicyclononyne (BCN) substrate was found to be compatible with fast and quantitative protein derivatization. These substrates allow conducting site-specific and almost quantitative DA$_{\text{inv}}$-based protein-protein conjugation with second order rate constants up to $\sim 70 \text{ M}^{-1}\text{s}^{-1}$. We demonstrate the applicability and efficiency of our conjugation method with the full-length therapeutic antibody trastuzumab. All branched protein-protein fusions obtained were shown to be fully functional as proven in fluorescence, binding, and stability studies. The speed, robustness and efficiency of the method guarantees a simple workflow to generate protein-protein conjugates of choice within hours starting from recombinant proteins regardless of their expression host. With the technology presented here, we open up new avenues for advanced applications in therapeutics or diagnostics.
Results and Discussion

Synthesis and identification of well-accepted diene and dienophile substrates for site-specific ligation to peptide tags

To compare various bioorthogonal motifs for protein modification, a suitable assay platform is required that allows evaluation of modification efficiency and product integrity under conditions close to the desired application. While genetic code expansion is extremely powerful for the incorporation of dienes or dienophiles into proteins, specially evolved unnatural amino acid/tRNA-aminoacyl synthetase pairs are required for every scaffold to be tested. Adaptation for other expression hosts is challenging and limits its use and flexibility. Building on innovative work by the Ting group,[13] we chose the engineered lipoic acid protein ligase A LplA<sup>W37V</sup> from Escherichia coli that ligates unnatural lipoic acid analogues to the 13 amino acid recognition motif lipoate acceptor peptide (LAP). LAP can be attached terminally to as well as internally into recombinant proteins from any host organism.[13] Several substrates with bioorthogonal motifs for site-selective tag-based protein functionalization including azides,[14] norbornenes,[15] trans-cyclooctenes,[9b] methyltetrazines and triazines[12] have been described. We synthesized a panel of potential LplA<sup>W37V</sup> substrates based on all commonly used dienes and dienophiles for DA<sub>inv</sub>. This includes the axial 2<sup>E</sup> regioisomer of trans-cycloctenol (ax-TCO<sup>*a-d</sup>),[10b] endo-bicyclononylnyl-methanol (endo-BCN<sub>a-c</sub>),[16] mono- and dimethylcyclopropenyl-methanol (MMCy<sub>a-d</sub> and DMCy<sub>a-d</sub>)[17] derivatized with linear amino acids of varying length via a carbamate (Scheme 2, Table 1). For the terminal alkene candidates, commercially available enoic acids were selected. First, we used a HPLC-based assay to screen various putative substrate candidates for Mg<sup>2+</sup>-ATP-dependent ligation onto LAP within 15 min in phosphate buffer (pH 7.0) at 37 °C by both the wildtype-LplA and LplA<sup>W37V</sup>. From each substrate class, an optimal candidate for maximum ligation efficiency by the LplA<sup>W37V</sup> (Table S1) was identified. Carbamates with 5-aminopentanoic acid consistently gave the highest LplA<sup>W37V</sup> acceptance which is why we additionally prepared derivatives with cycloct-2-yn-1-ol (SCO<sub>S</sub>),[18] the equatorial 2<sup>E</sup> and 4<sup>E</sup> regioisomers of trans-cycloctenol (eq.-TCO<sup>*s</sup> and eq.-TCOs),[10b, 19] as well as the 2<sup>Z</sup> and 4<sup>Z</sup> isomers of cis-cyclooctenol (CCO<sup>*s</sup> and CCO<sub>S</sub>), and validated them in the HPLC assay. We also synthesized and tested the axial 4<sup>E</sup> TCO-based substrate first reported by the Ting group[9b] (denoted as ax.-TCOs) and the
norbornene substrate **Norbs** previously developed by our group\(^{[15]}\). Complementary to our formerly reported diene-based methyltetrazinylmethoxycarbonyl (**MeTzMeOcs**) and triazine amide (**Trzs**) substrates,\(^{[12]}\) we further synthesized methyltetrazinyl alkanoic acids (**MeTzAla-e**\(^{[17b]}\)) and identified the optimized substrate derivative **MeTzAl\(_b\)**. This gives a total set of 15 different bioorthogonal motifs as substrates for LplA\(^W37V\), covering the most commonly used diene and dienophile moieties in literature\(^{[11]}\) to conduct DA\(_\text{inv}\) in its entire reactivity range (\(k_2 = 10^{-2} \text{M}^{-1} \text{s}^{-1} – 10^5 \text{M}^{-1} \text{s}^{-1}\))\(^{[20]}\) (Scheme 2 and Table S1). Notably, the BCN and SCO moieties can further serve as 1,3-dipolarophiles for strain promoted alkyne-azide (SPAAC) or alkyne-nitrone (SPANC) cycloaddition, expanding the scope of the method to other bioorthogonal cycloaddition reactions.\(^{[16, 20b, 21]}\)

In the HPLC assay, all substrates resulted in uniform ligation products (Figures S2-S6 and S9-S13), except the TCO-based ones (Table S1 and Figures S7+S8). Closely examining the retention times and m/z of the **ax./eq.-TCO**\(_s\) ligation products in comparison with **CCO**\(_s\), trans-to-cis isomerization of the TCO-modified peptides was apparent (Table S1 and Figure S7B/D). Interestingly, **ax./eq.-TCO**\(_s\) stock solutions showed no sign of cis-isomerization prior to the LplA\(^W37V\) ligation reaction, indicating that the conditions in the peptide assay alone favor trans-to-cis isomerization (Figures S7C). TCO* substrates (**ax.-TCO**\(_b\) and **eq.-TCO**\(_s\)) with the previously reported more stable TCO* scaffold\(^{[10b]}\) were prone to isomerization as well, albeit much less pronounced as **ax./eq.-TCO**\(_s\). For **eq.-TCO**\(_s\), isomerization was barely detectable (Table S1 and Figure S8).

### Protein labeling, diene/dienophile performance and a tool to probe protein modification states

With the established substrate platform for peptide modification with all commonly used dienes and dienophiles, we moved on to evaluate the best performing LplA\(^W37V\) substrates for site-specific modification at the protein level.

During our work with a LAP tag internally inserted in a flexible loop of EGFP (EGFP\(^E172::\text{LAP}\))\(^{[22]}\) (Figure 1C), a change in electrophoretic mobility in semi-native SDS-PAGE (non-reducing and non-denaturing conditions) for both, substrate functionalized EGFP and the corresponding DA\(_\text{inv}\) cycloadducts (CA) was observed (Figure 1). While non-modified EGFP\(^E172::\text{LAP}\) migrates at an apparent molecular weight (MW) of ~35 kDa, all ligation products of our tested substrates undergo a slight shift to a lower apparent MW (~33 kDa). Comparable electrophoretic mobility changes due to
lipoylation or octanoylation for E2 and H proteins have been reported. Amino acid substitutions or posttranslational modifications can lead to an altered electrophoretic mobility and GFP’s intrinsic properties seem to enhance this phenomenon. Remarkably, semi-native SDS-PAGE analysis of the conjugation product, when dienophile-modified EGFP treated with tetrazole tetramethylrhodamine (TAMRA), results in an even more pronounced gel shift toward lower apparent MWs (~ 26 kDa) (Figure 1). This analysis thus allows a modification state discrimination by electrophoresis (MSDE) of EGFP to analyze the ligation and cycloaddition steps (Figure 1D).

Inspired by the ability to probe the protein modification states directly at the protein level, we started to investigate the ligation yields, DA inv conversion and side-reactions of all substrates (Figures 1, S14 and S15). Briefly, EGFP was quantitatively functionalized with each substrate (Figures S14 and S15). Subsequently, the dienophile-modified EGFP (10 µM) was treated with 5 eq. of the two tetrazine fluorophore conjugates MeTzBnNH-TAMRA or TzBnNH-TAMRA (Figure 1A, 1B, 1C, S14), while diene-modified EGFP was treated with 5 eq. of TCO-Prop-TAMRA or BCN-Pip-TAMRA (Figure S15). Reactions were performed at 37 °C in PBS for 15 min and quenched with a large excess of either ax.-TCO-OH or dimethyl tetrazine (MeTzMe), respectively. Each step of modification was analyzed by the previously described MSDE of EGFP. Additionally, separate reaction mixtures with LAP-tagged maltose-binding protein (MBP-LAP) were subjected to mass determination to further support the data from MSDE of EGFP. (Figure S16 and S19).

All types of TCOs have remarkably fast kinetics in DA inv that are particularly useful for intracellular labeling. However, in complex cellular settings, quantification of the extent of labeling is challenging. Previous works reported that TCO tends to isomerize to the almost non-reactive cis-isomer and TCO* shows elimination after DA inv-product formation. Thus, we wanted to evaluate the extent of these unwanted side-reactions during LplAW37V ligation and DA inv by using MSDE of EGFP. Although, ax.-TCOs-modified proteins undergo rapid conjugation with both tetrazine fluorophore probes, we observed this conjugation to be incomplete limited to a maximum of about 53 % (Figure 1B). This supports the notion that the previously observed trans-to-cis isomerization tendency in the peptide assay also takes place during protein labeling (Figure S7). Comparably, eq.-TCOs-modified EGFP showed higher isomerization propensity. Interestingly, proteins directly modified with CCOs were not fully unreactive
toward TzBnNH-TAMRA resulting in non-negligible CA formation (Figure 1B). For \textit{ax.-TCO}_b-modified EGFP treated with TzBnNH-TAMRA, an unreactive EGFP population substantiates isomerization of \textit{ax.-TCO}*, albeit less pronounced as for the TCO substrates (Figure 1B). Especially for MeTzBnH-TAMRA-treated proteins, a protein species with the apparent MW of non-modified EGFP\textsuperscript{E172::LAP} appeared (Figure 1B), which we attribute to the elimination of the carbamate function of the CA. This could be further verified by data from mass spectrometry (Figure S16). Less elimination was observed when using TzBnNH-TAMRA, which is in agreement with the previously reported effect of the tetrazine scaffold for elimination of the TCO*-tetrazine CA.\textsuperscript{[27]}

High CA yield, minimal elimination and low isomerization were achieved solely with the \textit{eq.-TCOs}/TzBnNH-TAMRA combination. However, we were unable to validate the reported exclusive orthogonality for MeTzBnNH-substituted probes under the chosen reaction conditions.\textsuperscript{[20b]} Under the conditions applied, both TCO and TCO* are unsuitable for quantitative conjugation in DA\textsubscript{inv} reaction.

We then evaluated the reactivity of our novel BCN- and SCO-LpIA\textsuperscript{W37V} substrates. Strained cyclic alkynes do not have an isomerization-susceptible configuration, are regarded to be stable and their cycloaddition products are not prone to elimination.\textsuperscript{[10b]} To our delight, \textit{endo-BCN}_b-functionalized proteins could be transformed nearly quantitatively to the CA form with both tetrazine-TAMRA conjugates (Figure 1B and S17, S19). SCO\textsubscript{S}-modified EGFP underwent almost full conversion to the CA with TzBnNH-TAMRA, but was only slightly reactive toward MeTzBnNH-TAMRA. This is in agreement with a previous work\textsuperscript{[20b]}, although complete orthogonality cannot be proven as reported.

While the strained cyclooctyne substrates already had demonstrated great potential for quantitative CAs, we were curious to investigate means to prevent the observed isomerization of the TCO. Isomerization of TCOs has mainly been attributed to the influence of thiols via a radical-based mechanism.\textsuperscript{[9b]} We selected the two radical scavengers Trolox\textsuperscript{[28]} and ascorbic acid as possible isomerization suppressors in the ligation mixture with \textit{eq.-TCOs} without effect (Figure S18A). Next, we suspected the cysteine residue (Cys85) located in the binding pocket of the substrate-bound form of the LpIA\textsuperscript{W37V} \textsuperscript{[29]} to be responsible for the observed TCO isomerization. The double mutant LpIA\textsuperscript{W37V/C85A} was prepared and displayed ligase activity for \textit{endo-BCN}_b and \textit{eq.-TCOs} but did not alleviate or abolish isomerization of TCO (Figure S18B).
This suggests that protein environments during ligation reaction are sufficient to trigger TCO isomerization and implies that other enzymes with close contact to corresponding TCO substrates for site-specific protein functionalization might be confronted with the same issue. Using BCN as a dienophile for DA$_{\text{inv}}$ based post-translational protein modification takes advantage of the reactivity-stability-tradeoff and outperforms TCO and TCO*^\text{\textregistered}. While maintaining a high reaction rate under the applied conditions BCN provides quantitative conjugation yields. With endo-BCN$_b$, we identified the most efficient substrate for LplA$_{W37V}$ to prime proteins for efficient DA$_{\text{inv}}$ conjugation. Ena-, Norb$_b$, MMCy$_b$- and DMCy$_b$-functionalized EGFPs were expectedly much less reactive in DA$_{\text{inv}}$, but their side-by-side comparison might be interesting for some readers (Figure S14). We could also confirm almost quantitative cycloaddition of tetrazine-modified EGFP for MeTzMeOc$_c$ with TCO and BCN probes and for the novel MeTzAl$_b$ with TCO under the chosen reaction conditions (Figure S15). Even the triazine substrate Trzs provided reasonable reactivity with both TCO and BCN probes.

**Targeted protein-protein conjugation using DA$_{\text{inv}}$**

Ideally, a method for protein conjugation can be applied directly to the recombinant protein expressed in the expression host of choice using enzymatic or chemical strategies. Sortase or split-intein strategies have been successfully used for mild and post-translational covalent bond formation between two or more native proteins to a continuous polypeptide chain.$^{[1, 31]}$ Especially for larger multidomain protein fusions it might be advantageous to precisely attach proteins at defined internal positions within a single polypeptide chain. The generation of these internal or branched protein fusions from individual native proteins purely by enzymatic means is a challenge. To our knowledge, only the approaches based on CnaB2 and D4 Ig-like domains (Spy-/Snoop- × Tag/Catcher) from the Howarth lab provide enzyme-catalyzed covalent internal protein connectivity in a building block principle.$^{[32]}$ Non-selective chemistry using multifunctional amine or thiol reactive crosslinkers has enabled internal protein-protein conjugation (PPC) much long before the advent of genetic fusions,$^{[33]}$ but is severely limited in site-specificity. This challenges conjugation control and leads to poorly defined conjugate populations$^{[34]}$ preventing usage for precise construction of branched protein architectures. PPC utilizing bioorthogonal reactions has attracted attention over the last years to due to its potential to overcome terminal attachment restrictions or non-selective reactions. These works mainly focus on SPAAC, oxime ligation or copper-catalyzed alkyne-azide cycloaddition (CuAAC) as
conjugation chemistries.\[^{3, 35}\] It is surprising that targeted PPC via DA\textsubscript{inv} has not been investigated yet, given that all potential tools in genetic code expansion are available for a couple of years.\[^{10a, 36}\] Apart from PPC between TCO- and tetrazine-modified proteins pre-modified via non-site-specific NHS chemistry,\[^{38}\] there is only one example for a targeted PPC over DA\textsubscript{inv} using tetracysteine tag-conjugatable tetrazine crosslinker and a genetically encoded TCO\.\[^{39}\]

Stimulated by the modularity of the stable LplA\textsuperscript{W37V} substrates and the quick and near-quantitative ligation reaction, we envisioned functionalizing proteins with a methylietrazine substrate (MeTzMeOcs or MeTzAl\textsubscript{b}) and an endo-BCN\textsubscript{b} dienophile substrate separately. Subsequently, the primed proteins can be combined for conjugation by the DA\textsubscript{inv} cycloaddition, benefitting from the fast reaction rate of tetrazines with strained dienophiles and the benign catalyst-free\[^{5}\] conditions (Figure 2). In addition to a minimal tag size, position independence for these recognition motifs is an integral prerequisite for obtaining the desired relative orientation in advanced conjugate architectures.\[^{22, 40}\] The LAP tag has been introduced internally into viral capsid proteins, G-protein coupled receptors and fluorescent proteins without disruption of the protein activity.\[^{22, 41}\] Introduction of LAP in permissive internal positions should therefore provide the basis to construct branched protein chimeras.

We thus chose the conjugation between the two model proteins MBP-LAP and an EGFP construct with an internal LAP tag (EGFP\textsuperscript{Q157::LAP}) as a proof of concept. For 1:2 protein ratios at 10 \(\mu\)M, 37 °C, PBS (pH 7.4) and 2-4 h incubation time, the PPC gave nearly quantitative yield related to the protein reaction partner which is not present in excess (fluorescent protein species at \(~85\) kDa, Figure 2A and Figure S21). Non-purified reaction mixtures of the protein cycloaddition reactions were further analyzed by intact protein mass determination (Figure 2C and S20). MBP-LAP(endo-BCN\textsubscript{b}), the protein in excess, gave the most prominent signal, followed by the protein-protein conjugate and no detectable tetrazine-modified EGFP construct, indicating full conversion to PPC product. The absence of homo-conjugates in the PPC reactions and the respective negative controls underpins the precision and fidelity of DA\textsubscript{inv} to obtain chimeric and covalent protein heterodimers. Notably, the \(N\)- and \(C\)-termini of both proteins remain in their native unmodified form providing additional branching options for even more complex protein chimeras.

To analyze the kinetics of this PPC method, we incubated endo-BCN\textsubscript{b}-functionalized EGFP\textsuperscript{E172::LAP} with varying concentrations of methylietrazine-functionalized mRuby3-
LAP and monitored the Förster resonance energy transfer (FRET) acceptor fluorescence of mRuby3 over time. Under pseudo-first order kinetics, remarkably high second order rate constants of \( \sim 70 \text{ M}^{-1}\text{s}^{-1} \) and \( \sim 50 \text{ M}^{-1}\text{s}^{-1} \) for the \textit{endo-BCN}b/\textit{MeTzMeOcs} and \textit{endo-BCN}b/\textit{MeTzAl}b combinations, respectively, were determined at 37 °C in PBS (Figure 3 and S22). In line with our expectations, FRET partners both equipped with \textit{endo-BCN}b did not show any FRET response (Figure 3).

**Functional protein-antibody conjugates**

Next, we wanted to demonstrate the power of our conjugation procedure for the generation of therapeutic protein chimeras. To underline the wide variety of applications, we genetically equipped the monoclonal antibody trastuzumab with the LAP motif on the C-termini of each heavy chain (trastuzumab-hc\textsuperscript{LAP}/hc\textsuperscript{WT}, further referred to as trastuzumab-(LAP)\textsubscript{2}). This immunoglobulin targets HER2-positive cancer cells and is applied as parental antibody in the FDA approved drug Herceptin. Very recently, LplA\textsuperscript{W37V}-mediated TCO ligation was used in combination with transglutaminase for one-pot dual labeling of trastuzumab. The data presented in the report indirectly point toward an incomplete conjugation of TCO modified trastuzumab-(LAP)\textsubscript{2} which is in agreement with our observation of an impaired stability of TCO for quantitative \textit{in vitro} bioconjugation.

We reasoned that antibody functionalization with \textit{endo-BCN}b might serve as a conjugation hub enabling both DA\textsubscript{inv}/SPAAC with small molecules (fluorophores or cytotoxic drugs) and even whole proteins. Ligation of \textit{endo-BCN}b to both hc-located LAP tags of intact trastuzumab was monitored by hydrophobic interaction chromatography (HIC, Figure 4A and S23) and quantitative conversion of trastuzumab to the doubly BCN-functionalized species within 3 min was observed, demonstrating the potential of the \textit{endo-BCN}b/LplA\textsuperscript{W37V} pair for rapid and clean antibody functionalization. This approach is significantly faster than transglutaminase, sortase or formylglycine generating enzymes, the most popular enzymes used for protein conjugation.

With fast, targeted and efficient posttranslational protein conjugation to antibodies being a hitherto unmet challenge, we sought to directly assess this concept with internally tetraine-functionalized EGFP. Protein conjugation to the hc of trastuzumab smoothly went to near completion at 40 \( \mu \text{M} \) of tetraine-modified EGFP\textsuperscript{Q157::LAP} and 10 \( \mu \text{M} \) of doubly BCN-functionalized trastuzumab-(LAP)\textsubscript{2} at 37 °C for 4 h in PBS
Encouraged by these promising results, the biological activity of doubly EGFP-modified trastuzumab (trastuzumab-(LAP\(\cdot\)EGFP)\(_2\), Figure 4C) was assessed via immunofluorescence staining of living eukaryotic cells using confocal microscopy (Figure 4D and S24A). As expected, the fluorescent signal of EGFP-conjugated trastuzumab accumulated at the outer cell membrane of the HER2-positive breast cancer cell line SK-BR-3, but not at the HER2-negative control cell line CHO-K1. No fluorescent signal was detected for the negative control conjugation (Figure 4D), supporting the lack of mutual cross reactivity between BCN-functionalized proteins in PPC reactions (Figure 2A and 4B). Incubation of both cell lines with a 1:1 mixture of trastuzumab-(LAP\(\cdot\)EGFP)\(_2\) and trastuzumab-(LAP\(\cdot\)TAMRA)\(_2\) resulted in colocalizing fluorescent signals of only on the HER2-positive cell line (Figure S24B).

To examine how the LAP tag and subsequent conjugation with a probe, either a small molecule probe (TAMRA) or a whole fluorescent protein (EGFP), influence biological properties of the antibody, subsequent binding studies were performed. A comparative binding study of trastuzumab-(LAP\(\cdot\)EGFP)\(_2\), trastuzumab-(LAP\(\cdot\)TAMRA)\(_2\) and trastuzumab-(LAP)\(_2\) on HER2-positive cells revealed potent binding of all constructs (Figure 4E and S25). To further substantiate these findings, we determined the binding constants on cells applying flow cytometry in vitro (Figure S26). Trastuzumab with the C-terminal LAP motif revealed single-digit nanomolar binding properties \((K_D = 5.6 \text{ nM})\) equal to unmodified wildtype trastuzumab \((K_D = 6.3 \text{ nM})\).\(^{[45]}\) Notably, trastuzumab equipped with EGFP exhibited equipotential binding \((K_D = 7.9 \text{ nM})\). Additionally, the further modified trastuzumab-(LAP\(\cdot\)TAMRA)\(_2\) construct revealed only a negligible loss of binding potential \((K_D = 12.9 \text{ nM})\). Encouraged by these results, we further investigated the impact of the LAP motif itself and the conjugation to EGFP on the stability of the antibody. Thermal shift assays revealed equal melting points for the trastuzumab-(LAP)\(_2\) compared to the unmodified wildtype antibody. Additionally, no loss in stability was found for trastuzumab-(LAP\(\cdot\)EGFP)\(_2\), as three melting points equal to the Fc- and the Fab-part of the unmodified trastuzumab and to solitaire EGFP were obtained (Figure S27 and Table S28).

**Antibody-drug conjugates (ADCs) by SPAAC click chemistry**

To investigate, whether our strategy of site-specific protein conjugation allows for the efficient generation of antibodies equipped with highly potent cytotoxins, we generated an ADC in a two-step procedure. Trastuzumab-(LAP)\(_2\) was modified with \textit{endo}-BCN\(_b\)
by LplA<sup>W37V</sup>-catalysis followed by conjugation of highly toxic monomethyl auristatin E (MMAE) by SPAAC applying N<sub>3</sub>-PEG<sub>3</sub>-vc-PABC-MMAE, resulting in trastuzumab-(LAP•MMAE)<sub>2</sub>. Next, we assessed the <i>in vitro</i> potency of the trastuzumab-(LAP•MMAE)<sub>2</sub>. For this, a cell proliferation assay on SK-BR-3 breast cancer cells and CHO-K1 cells was performed (Figure 5). Potent, subnanomolar (IC<sub>50</sub> = 0.31 nM) cell-killing properties were shown for trastuzumab-(LAP•MMAE)<sub>2</sub> on HER2-positive SK-BR-3 cells, while the construct was found innocuous to HER2-negative CHO-K1 cells. As expected, trastuzumab-(LAP)<sub>2</sub> lacking a toxin warhead was found non-toxic for both HER2-positive and negative cells.

**Conclusion**

In conclusion, we have developed a highly efficient method to synthesize well defined, homogeneous protein(-protein) conjugates utilizing enzyme-mediated protein modification and bioorthogonal DA<sub>inv</sub> chemistry. Various diene and dienophile substrates were synthesized and analyzed for ligation to proteins using LplA<sup>W37V</sup> as well as for their ability to yield homogeneous conjugates in subsequent DA<sub>inv</sub>. For this we have developed the MSDE of EGFP based on changes in electrophoretic mobility of the modified protein that allows for straightforward monitoring of each step of the procedure directly at the model protein EGFP-LAP. This assay facilitates error diagnosis during ligation reactions and provides information on the stability of the ligated substrates. To our knowledge, this is so far the first method to evaluate stability and reactivity of bioorthogonal motifs at the protein level. We identified novel tetrazine- and BCN substrates to be the best candidates for fast and quantitative <i>in vitro</i> conjugation of two functional proteins with rate constants of up to 70 M<sup>-1</sup> s<sup>-1</sup>. Our strategy yields quantitative homogeneous protein-protein conjugates without any detectable by-products or toxic remnants, within a single working day starting from purified protein. By efficiently labeling the therapeutic antibody trastuzumab with EGFP or the antimitotic agent MMAE, respectively, we have shown that the novel methodology offers great potential for advances in the production of ADCs and antibody protein toxin conjugates (APCs). Compared to standard methods, where protein tags are fused terminally, our approach provides great flexibility to conjugate a probe, drug or even a whole protein to various internal positions, resulting in branched protein architectures that are difficult to accomplish with other technologies. We assume that the versatility of this conjugation strategy bears potential for various
applications, e.g. for facile drug development\cite{44b}, generation of biomaterials\cite{46} or nanofabrication\cite{46-47}. In particular, with biologics being the fastest growing class of drugs we see the herein reported method for PPC as an attractive technology that can speed up development timelines and facilitate their manufacturing by addressing problems of reagent reproducibility, important aspects to enhance the biologics drug pipeline.

**Experimental Section**

For experimental details see supporting information.

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Scheme 1. Protein fusion and conjugate architectures accessible through expression (only left), enzymatic ligation strategies or site-specific bioorthogonal chemistry.
Scheme 2. (A) Two-step chemoenzymatic procedure with LpL\text{A}\text{W}^{37V} and click chemistry to generate protein-toxin and protein-protein conjugates. The carboxylic acid of the substrate is ligated enzymatically to a specific lysine resulting in an amide bond. (B) Substrate scope for LpL\text{A}\text{W}^{37V}-mediated site-specific protein functionalization and ensuing Diels-Alder cycloaddition with inverse electron demand (DA\text{inv}), strain-promoted alkyne-azide cycloaddition (SPAAC) or strain-promoted alkyne-nitrone cycloaddition (SPANC). References; a: Baalmann et al.,\textsuperscript{[12]} b: Liu et al.,\textsuperscript{[9b]} c: Best et al.,\textsuperscript{[15]}. 
Figure 1. Individual EGFP modification states during two-step labeling can be traced by semi-native SDS-PAGE (MSDE of EGFP). (A) Procedure of two-step EGFP modification and subsequent analysis. (B) In-gel fluorescence and CBB analysis of SDS gels assaying the reactivity of different dienophile LplA constructs directly at the protein level using EGFP$_{E172::LAP}$ highlights high modification yields utilizing combinations of endo-BCN$_b$ and SCO$_s$ with MeTzBnNH-TAMRA and TzBnNH-TAMRA and eq.-TCO*$_s$ together with TzBnNH-TAMRA (blue boxes). †: ~35 kDa. ‡: ~25 kDa. (C) Protein construct used. (D) Scheme illustrating the different shifts of the EGFP undergoing in the gel during modification steps. For the exhaustive side-by-side comparison of all DA$_{inv}$ substrates including the diene and less reactive dienophile substrates, see Figures. S14 and S15. Note: Yields have been corrected for an EGFP impurity of 6% at the height of the ligation products that was neither accessible for ligation nor cycloaddition reactions. Crystal structure PDB: 2Y0G.[30]
Figure 2. Protein-protein conjugation using model proteins MBP-LAP and EGFP<sub>Q157::LAP</sub>. Dienophile/diene combination of endo-BCN<sub>b</sub> with MeTzMeOcs or MeTzAlb are efficiently used for targeted conjugation of two functional entities by DA<sub>inv</sub>. (A) SDS-PAGE analysis highlights almost quantitative formation of a conjugation product between MBP-LAP(endo-BCN<sub>b</sub>) and EGFP<sub>Q157::LAP(MeTzMeOcs/MeTzAlb)</sub> (~ 70 kDa) within 2 h of incubation at 37 °C. (B) Schematic view of the model proteins equipped with the respective functional entity which primes for a branched protein fusion during DA<sub>inv</sub>. (C) Mass spectrometry analysis of conjugation reaction emphasizes the clean nature of the PPC reaction utilizing endo-BCN<sub>b</sub> and MeTzMeOcs. Crystal structure PDBs: 1ANF<sup>37</sup> and 2Y0G.
**Figure 3.** Reaction kinetics for targeted protein-protein conjugation between BCN-functionalized EGFP and methyltetrazine-functionalized mRuby3 monitored by FRET. Left graph shows mRuby3 FRET acceptor response resulting from formation of protein-protein conjugate under pseudo-first order conditions. Right graph shows determination of second order rate constant. Error bars in left graph: ±1 standard deviation; in right graph: ±1 highest error estimate. Crystal structure PDBs: 2Y0G and 3U0L[42].
**Figure 4.** Protein-antibody conjugation using DAinv with the substrate combination of endo-BCNb and MeTzMeOcs/MeTzAlb yields a functional protein conjugate with the full-length therapeutic antibody trastuzumab. (A) Rapid ligation of endo-BCNb to the LAP-tagged trastuzumab leads to doubly-functionalized trastuzumab within 3 min. BAR = BCN-to-antibody ratio. Also see Figure S23 for expanded ligation time. (B) SDS-PAGE analysis highlights nearly quantitative conjugation of methyltetrazine-modified EGFP to BCN-modified trastuzumab. hc = heavy chain, lc = light chain. (C) Scheme of EGFP conjugated to both hc of the antibody trastuzumab and resulting conjugate architecture. (D) Confocal microscopy visualizes localization of the trastuzumab-(LAP•EGFP)2 conjugate specifically to living human HER2-positive SK-BR-3 breast cancer cells with no binding for HER2-negative CHO-K1 cells. Scale bar = 20 µm. Conjugation control was done with trastuzumab-(LAP)2 and EGFP157:LAP both functionalized with endo-BCNb and processed equally as for the described conjugation reaction. (E) Flow cytometry analysis shows concentration-dependent binding of trastuzumab-(LAP•EGFP)2 and trastuzumab-(LAP•TAMRA)2 conjugates to HER2-positive SK-BR-3 cells. fl. = fluorescence.
Figure 5. Generation of the potent antibody-drug conjugate trastuzumab-(LAP•MMAE)$_2$ by *endo*-BCN$_b$ ligation followed by SPAAC with N$_3$-PEG$_3$-vc-PABC-MMAE. Top: Conjugate architecture. vc = valine-citrulline, PABC = *para*-aminobenzyl carbamate, MMAE = monomethyl auristatin E. Dotted lines represent cleavable bonds for drug release. Scissors indicate cleavage site for lysosomal proteases. Bottom: Cell proliferation assays with HER2-positive (SK-BR-3) and HER2-negative cells (CHO-K1) treated with trastuzumab-(LAP)$_2$ and trastuzumab-(LAP•MMAE)$_2$ (DAR2). Error bars in graphs: ±1 standard deviation.
Supporting Information

A bioorthogonal click chemistry toolbox for targeted synthesis of branched and well-defined protein-protein conjugates

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Table S1. Enzymatic ligation turnover of different substrate candidates and lipoic acid (LA) to LAP at 37°C for 15 min by wildtype LplA and LplA<sup>H37V</sup>. Conditions: 0.5 mM substrate candidate, 5 mM ATP, 5 mM Mg<sup>2+</sup>; 1 µM wildtype LplA or LplA<sup>H37V</sup>, 25 mM sodium phosphate buffer (pH 7.0), quenching: 250 mM EDTA. *t*<sub>n</sub> retention time, n.c. = no conversion, n.d. = not determined.

| substrate candidate | enzymatic conversion of LAP [%] | ligation product(s) |
|---------------------|---------------------------------|--------------------|
| entry               | LplA<sup>H37V</sup> | wildtype LplA | LplA<sup>H37V</sup> | wildtype LplA |
| LA                  | 96.0 ± 0.4                  | 96.3 ± 0.3        | 24.5                 |
| En<sub>n</sub>, n = 3| 13.2                        | 4.9 ± 0.7         | 8.1 ± 2.0            | 23.0                 |
| En<sub>n</sub>, n = 4| 18.5                        | 11.5 ± 2.0        | 40.7 ± 3.4           | 23.8                 |
| En<sub>n</sub>, n = 5| 22.8                        | 42.7 ± 0.9        | 44.6 ± 3.4           | 24.6                 |
| En<sub>n</sub>, n = 6| 28.9                        | 58.8 ± 1.8        | 52.4 ± 2.2           | 25.6                 |
| En<sub>n</sub>, n = 7| 29.7                        | 40.6 ± 1.9        | 28.2 ± 6.0           | 26.6                 |
| En<sub>n</sub>, n = 8| 32.7                        | 50.8 ± 6.1        | 6.1 ± 3.7            | 27.7                 |
| Nor<sub>b</sub> (± 2d<sup>i</sup>) | 20.6                        | 85.6 ± 6.3 | n.c. | 24.3                 |
| DMC<sub>y</sub>, n = 3| 18.4                        | 10.4 ± 0.2        | n.c. | 23.9                 |
| DMC<sub>y</sub>, n = 4| 20.1                        | 56.2 ± 0.9        | 1.0 ± 1.0            | 24.3                 |
| DMC<sub>y</sub>, n = 5| 22.3                        | 48.5 ± 6.3        | n.c. | 24.9                 |
| DMC<sub>y</sub>, n = 5| 24.5                        | 7.9 ± 6.3         | n.c. | 25.4                 |
| MMC<sub>y</sub>, n = 3| 15.0                        | 3.8 ± 2.7         | n.c. | 23.2                 |
| MMC<sub>y</sub>, n = 4| 17.0                        | 52.3 ± 1.1        | n.c. | 23.5                 |
| MMC<sub>y</sub>, n = 5| 19.3                        | 33.2 ± 4.9        | n.c. | 24.0                 |
| MMC<sub>y</sub>, n = 6| 21.8                        | 38.5 ± 3.3        | 6.9 ± 8.4            | 24.6                 |
| ax-TCO<sup>+</sup>, n = 3| 21.8                        | 5.2 ± 3.6         | n.c. | 25.3 | n.d. |
| ax-TCO<sup>+</sup>, n = 4| 23.4                        | 27.9 ± 0.6        | n.c. | 25.3 | 25.8 |
| ax-TCO<sup>+</sup>, n = 5| 25.3                        | 13.6 ± 0.1        | n.c. | 25.8 | 26.4 |
| ax-TCO<sup>+</sup>, n = 6| 27.4                        | 3.7 ± 0.1         | n.c. | 26.4 | 27.0 |
| eq-TCO<sup>+</sup>, n = 4| 24.1                        | 58.8 ± 5.2        | n.c. | 25.6 | 25.8 |
| CCO<sup>+</sup>, n = 4| 24.9                        | 61.8 ± 3.6        | n.c. | 25.8                 |
| ax-TCOs, n = 4 (± TCO2<sup>i</sup>) | 23.6                        | 61.3 ± 1.7        | n.c. | 25.4 | 25.5 |
| eq-TCOs, n = 4 | 22.9                   | 55.3 ± 0.9        | n.c. | 25.1 | 25.5 |
| CCOs, n = 4 | 24.2               | 72.8 ± 3.2        | n.c. | 25.5                 |
| SCOs, n = 4 | 21.8                   | 88.1 ± 4.0        | n.c. | 24.9                 |
| endo-BCN<sub>n</sub>, n = 3 | 20.5                   | 14.1 ± 6.6        | n.c. | 24.4                 |
| endo-BCN<sub>n</sub>, n = 4 | 22.0                   | 50.0 ± 0.9        | n.c. | 24.8                 |
| endo-BCN<sub>n</sub>, n = 5 | 23.8                   | 1.6 ± 2.0         | n.c. | 25.3                 |
| Trz<sub>3</sub> (± Trz<sup>i</sup>) | 3.3                   | 93.9 ± 0.1        | n.c. | 20.9                 |
| MeTzAl<sub>n</sub>, n = 5 | 12.3                   | 8.9 ± 1.4         | n.c. | 22.5                 |
| MeTzAl<sub>n</sub>, n = 6 | 16.4                   | 39.2 ± 1.4        | n.c. | 23.1                 |
| MeTzAl<sub>n</sub>, n = 7 | 19.8                   | 33.1 ± 2.7        | n.c. | 23.7                 |
| MeTzAl<sub>n</sub>, n = 8 | 23.0                   | 20.7 ± 2.6        | n.c. | 24.5                 |
| MeTzAl<sub>n</sub>, n = 9 | 25.8                   | 7.3 ± 2.4         | n.c. | 25.4                 |
| MeTzMeOcs, n = 4 (± MeTz3<sup>i</sup>) | 9.7                   | 37.1 ± 2.7        | n.c. | 22.0                 |
Figure S2. Ligation of (R)-lipoic acid to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM LA, 5 mM ATP, 5 mM Mg²⁺, 1 µM LplA³⁷V, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). (C) High resolution mass spectrum of the ligation product LAP(LA).

Figure S3. Ligation of 8-nonenoic acid (En) as terminal alkene substrate to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM En, 5 mM ATP, 5 mM Mg²⁺, 1 µM LplA³⁷V, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). The asterisk indicates impurity of the peptide batch. (C) High resolution mass spectrum of the ligation product LAP(En).
**Figure S4.** Ligation of the norborne substrate Norbs to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM Norbs, 5 mM ATP, 5 mM Mg^{2+}, 1 µM LplA^W37V, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). The asterisk indicates impurity of the peptide batch. (C) High resolution mass spectrum of the ligation product LAP(Norbs).

**Figure S5.** Ligation of the dimethylcyclopropene substrate DMCyb to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM DMCyb, 5 mM ATP, 5 mM Mg^{2+}, 1 µM LplA^W37V, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). (C) High resolution mass spectrum of the ligation product LAP(DMCyb).
Figure S6. Ligation of the monomethylcyclopropene substrate MMCyb to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM MMCyb, 5 mM ATP, 5 mM Mg²⁺, 1 µM LpL₉₇V, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). (C) High resolution mass spectrum of the ligation product LAP(MMCyb).
Figure S7. Ligation of the trans-cyclooctene substrates \textit{ax}./\textit{eq}.-\text{TCO}_5\text{S} and cis-cyclooctene substrate \text{CCO}_5 to LAP and investigation of trans-to-cis isomerization.

(A) Reaction scheme. Ligation of trans-cyclooctene substrates leads to the formation of the CCO-LAP ligation product LAP(\text{CCO}_5). \text{CCO}_5 is also accepted by the LplA^{W37V}. (B) Exemplary chromatograms of reactions under the following conditions: 0.5 mM cyclooctene substrate (\textit{ax}.-\text{TCO}_5, \textit{eq}.-\text{TCO}_5 or \text{CCO}_5), 5 mM ATP, 5 mM Mg\text{\textsuperscript{2+}}, 1 \mu M LplA^{W37V}, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). The asterisk indicates an impurity of the peptide batch. (C) Chromatograms of the substrate stock solutions \textit{ax}.-\text{TCO}_5, \textit{eq}.-\text{TCO}_5 and \text{CCO}_5 show no \text{CCO}_5 being present prior to ligation. (D) High resolution mass spectra of all ligation products with LAP.
Figure S8. Ligation of the trans-cyclooctene* substrates ax.-TCO*$_b$, eq.-TCO*$_s$ and cis-cyclooctene substrate CCO*$_s$ to LAP and investigation of trans-to-cis isomerization. (A) Reaction scheme. Ligation of trans-cyclooctene* substrates leads to the formation of the CCO*$_s$-LAP ligation product LAP(CCO*$_s$). CCO*$_s$ is also accepted by the LpIA*W37V. (B) Exemplary chromatograms of reactions under the following conditions: 0.5 mM cyclooctene substrate (ax.-TCO*$_b$, eq.-TCO*$_s$, or CCO*$_s$), 5 mM ATP, 5 mM Mg$^{2+}$, 1 µM LpIA*W37V, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). The asterisk indicates an impurity of the peptide batch. (C) Chromatograms of the substrate stock solutions ax.-TCO*$_b$, eq.-TCO*$_s$, and CCO*$_s$. (D) High resolution mass spectra of all ligation products with LAP.
Figure S9. Ligation of the strained cyclooctyne substrate SCO₃ to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM SCO₃, 5 mM ATP, 5 mM Mg²⁺, 1 µM Lplₐ⁸⁷⁷, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). (C) High resolution mass spectrum of the ligation product LAP(SCO₃).

Figure S10. Ligation of the bicyclononyne substrate endo-BCN₆ to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM endo-BCN₆, 5 mM ATP, 5 mM Mg²⁺, 1 µM Lplₐ⁸⁷⁷, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). (C) High resolution mass spectrum of the ligation product LAP(endo-BCN₆).
Figure S11. Ligation of the 1,2,4-triazine substrate Trz₅S to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM Trz₅S, 5 mM ATP, 5 mM Mg²⁺, 1 µM LplA¹⁵³⁷⁷, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). (C) High resolution mass spectrum of the ligation product LAP(Trz₅S).

Figure S12. Ligation of the methyltetrazinylmethoxycarbonyl substrate MeTzMeOc₅ to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM MeTzMeOc₅, 5 mM ATP, 5 mM Mg²⁺, 1 µM LplA¹⁵³⁷⁷, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). The asterisk indicates an impurity of the peptide batch. (C) High resolution mass spectrum of the ligation product LAP(MeTzMeOc₅).
Figure S13. Ligation of the methyltetrazynalkanoic acid substrate MeTzAlb to LAP. (A) Reaction scheme. (B) Exemplary chromatogram of ligation reaction under following conditions: 0.5 mM MeTzAlb, 5 mM ATP, 5 mM Mg^{2+}, 1 µM LpaW37V, 25 mM sodium phosphate buffer (pH 7.0), 37 °C, 15 min (quenching: 250 mM EDTA). (C) High resolution mass spectrum of the ligation product LAP(MeTzAlb).
Figure S14. Comparison of two-step labeling of EGFP with several dienophile substrates (step I) and two tetrazine-TAMRA conjugates (step II) by change in electrophoretic mobility (MSDE-assay). (A) Used protein construct, conditions and workflow. (B) Scheme depicts how to analyze the SDS gels. (C) Structures of used tetrazine-TAMRA conjugates. (D) Structure of ax-TCO-OH. (E) Substrate structures. (F) Ligation of substrates (step I) and cycloadduct formation during DA_{inv} (step II) is monitored by change in electrophoretic mobility during SDS-PAGE (CBB staining and in-gel fluorescence overlay of EGFP [green] and TAMRA [orange] fluorescence) and can be quantified by densitometry (below gels). Best-performing substrate/probe combinations are highlighted in blue. 10 % acrylamide gels. CBB = Coomassie Brilliant Blue, M = size marker, †: ~ 35 kDa, ‡: ~ 25 kDa.
Figure S15. Comparison of two-step labeling of EGFP with several diene substrates (step I) and two dienophile-TAMRA conjugates (step II) by change in electrophoretic mobility (MSDE-assay). (A) Used protein construct, conditions and workflow. (B) Scheme depicts how to analyze the SDS gels. (C) Structures of used dienophile-TAMRA conjugates. (D) Structure of dimethyl tetrazine MeTzMe. (E) Substrate structures. (F) Ligation of substrates (step I) and cycloadduct formation during DAinv (step II) is monitored by change in electrophoretic mobility during SDS-PAGE (CBB staining and in-gel fluorescence overlay of EGFP [green] and TAMRA [orange] fluorescence) and can be quantified by densitometry (below gels). Best-performing substrate/probe combinations are highlighted in magenta. 10 % acrylamide gels. CBB = Coomassie Brilliant Blue, M = size marker, † : ~ 35 kDa, ‡ : ~ 25 kDa.
Figure S16. Mass spectrometry analysis of TCO-functionalization and subsequent DA inv with MeTzBnNH-TAMRA at protein level. (A) Scheme of MBP-LAP used, structure and calculated average mass of different species in the two-step labeling procedure. (B + C) Deconvoluted ESI mass spectra of non-modified MBP-LAP (top), MBP-LAP after being quantitatively functionalized with ax.-TCO or ax.-TCO* (middle) and after treatment of functionalized protein with MeTzBnNH-TAMRA (bottom). Proteins were washed several times with PBS before DA inv conditions: 10 µM functionalized MBP-LAP, 50 µM MeTzBnNH-TAMRA, PBS, 37 °C, 15 min (no quenching). Mass determination was performed without prior separation or enrichment of certain species. All peaks were in nearly perfect agreement with the calculated average masses. The asterisk points out to an impurity in the original protein sample that cannot be modified. Note: CCO or CCO* bound to proteins is indicated by a non-reactive protein population as a result of isomerization. MBP = maltose-binding protein, CA = cycloadduct.
Figure S17. The change in electrophoretic mobility of EGFP<sub>E172::LAP</sub> allows for tracking the progress of substrate ligation directly at the protein level. (A) Left: SDS-PAGE analysis of the ligation of the LplA<sub>W37V</sub> substrate endo-BCN<sub>0</sub> to EGFP<sub>E172::LAP</sub> carried out for the given time. EGFP<sub>E172::LAP</sub> is shown to migrate to a smaller molecular weight upon modification with endo-BCN<sub>0</sub> than in its unmodified state. The change in electrophoretic mobility during SDS-PAGE is monitored by CBB staining and in-gel fluorescence of EGFP [green] fluorescence and can be quantified by densitometry (above gels). Right: Ligation of EGFP<sub>E172::LAP</sub> was quantified to be complete after 15 min of incubation using densitometry analysis. (0) min indicates migration behavior of unmodified EGFP<sub>E172::LAP</sub> in the absence of LplA<sub>W37V</sub> and the substrate endo-BCN<sub>0</sub>. (B) SDS-PAGE analysis of the ligation of the LplA<sub>W37V</sub> substrate endo-BCN<sub>0</sub> to MBP-LAP did not result in any change in electrophoretic mobility of the BCN-modified protein. (0) min indicates migration behavior of unmodified MBP-LAP in the absence of LplA<sub>W37V</sub> and the substrate endo-BCN<sub>0</sub>. 10 % acrylamide gels. CBB = Coomassie Brilliant Blue, MBP = maltose-binding protein, M = size marker, †: ~55 kDa, ‡: ~40 kDa, ¶: ~35 kDa.

Figure S18. Radical scavengers and an engineered cysteine mutant version of the LplA<sub>W37V</sub> do not prevent eq.-TCO<sub>3</sub> from isomerization during the ligation process with EGFP<sub>E172::LAP</sub>. Ligation of substrates (step I) and cycloadduct formation during DA<sub>inv</sub> (step II) is monitored by a change in electrophoretic mobility during SDS-PAGE (CBB staining and in-gel fluorescence overlay of EGFP [green] and TAMRA [orange] fluorescence) and can be quantified by densitometry (below gels). (A) SDS-PAGE analysis shows that radical scavengers trolox and ascorbic acid (500 µM each) used during two-step ligation procedure do not prevent trans-to-cis isomerization of eq.-TCO<sub>3</sub>. (B) The replacement of a cysteine with an alanine in the binding pocket of substrate-bound LplA<sub>W37V</sub> using the engineered LplA<sub>W37V/C85A</sub> has no influence in preventing trans-to-cis isomerization of eq.-TCO<sub>3</sub> during two-step ligation. 10 % acrylamide gels. CBB = Coomassie Brilliant Blue, M = size marker, †: ~35 kDa, ‡: ~25 kDa.
Figure S19. Mass spectrometry analysis of MBP functionalization with monomethylcyclopropene (MMCy) or bicyclononyne (endo-BCN) and subsequent DA with MeTzBnNH-TAMRA at protein level. (A) Scheme of used protein, as well as structure and calculated average mass of different species in the two-step labeling procedure. (B) Deconvoluted ESI mass spectra of non-modified MBP-LAP (top) and MBP-LAP after being functionalized quantitatively with MMCy (bottom). (C) Deconvoluted ESI mass spectra of non-modified MBP-LAP (top), MBP-LAP after being quantitatively functionalized with endo-BCN (middle) and after treatment of functionalized protein with MeTzBnNH-TAMRA (bottom). Proteins were washed several times with PBS before DA. DA conditions: 10 µM functionalized MBP-LAP, 50 µM MeTzBnNH-TAMRA, PBS, 37 °C, 15 min (no quenching). Mass determination was performed without prior separation or enrichment of certain species. All peaks were in nearly perfect agreement with the calculated average masses. The asterisk points out to an impurity in the original protein sample that cannot be modified by LplA<sup>W37V</sup>. 
Figure S20. Mass-spectrometric evaluation of protein-protein conjugation between MBP and EGFP. Scheme of proteins (A) and substrate structures (B) used for mass spectrometry analysis. (C) Connectivity of resulting protein-protein conjugate. (D) Deconvoluted ESI mass spectra of functionalized protein reaction partners (olive green and blue trace) and PPC reaction mixture (orange trace). A magnified section of the orange trace shown on the right confirms the formation of the protein-protein cycloadduct. Mass determination was performed without prior separation or enrichment of certain species. All peaks were in nearly perfect consistency with the calculated masses. Conditions: 20 µM MBP-LAP(endo-BCN₅), 10 µM EGFPQ₁₅₇::LAP(MeTzAlb), PBS (pH 7.4), 37 °C, 2 h (no quenching). MBP = maltose-binding protein, PPC = protein-protein conjugation.

Figure S21. Time course analysis of protein-protein conjugation between EGFPQ₁₅₇::LAP and MBP-LAP. (A) SDS-PAGE analysis with CBB staining and in-gel fluorescence of EGFP [green]. Proteins were modified quantitatively with endo-BCN₅ or MeTzMeOcs prior to conjugation at 37 °C in PBS. The conjugation reaction was stopped at the given time points by the addition of 1 mM of MeTzMe. (B) Quantification of CBB-stained protein-protein conjugate (see A) in dependence of reaction time by densitometry. An exemplary fit for second order kinetic product formation is indicated in grey. CBB = Coomassie Brilliant Blue, MBP = maltose-binding protein.
Figure S22. Kinetics of protein-protein conjugation between BCN-functionalized EGFP and methyltetrazine-functionalized mRuby3. Scheme of proteins (A) and substrate structures (B) used for kinetic measurements. (C) Determination of second order rate constants $k_2$ between 0.4 µM of EGFP$^{[172]:LAP}$ (FRET donor) and 2-8 µM of methyltetrazine-functionalized mRuby3-LAP (FRET acceptor) under pseudo-first order conditions in PBS at 37 °C. Donor excitation: (488 ± 5) nm. Acceptor detection: (579 ± 5) nm. Acceptor fluorescence ($F_A$) was processed as described by Hoffmann et al.$^{[4]}$. Upper graphs show combined, intercept-corrected mRuby3 acceptor fluorescence traces per condition and representative exponential fits. For determination of $k_2$, the individual acceptor traces were fitted assuming an inverse monoeponential function. The obtained $k_{2\text{obs}}$ were plotted against the methyltetrazine-functionalized mRuby3-LAP concentration and $k_2$ was obtained as the slope of the linear fit (lower graphs). As expected, no response in acceptor fluorescence was observed when endo-BCN-functionalized mRuby3-LAP was used (right side, negative control). Error bars in upper graphs = standard deviation from two replicates. Error bars in lower graphs = highest error estimate from two individual $k_{2\text{obs}}$ and their standard errors. Inset shows scheme of protein connectivity.
Figure S23. Kinetic measurements of LplA<sup>W37V</sup>-mediated trastuzumab-(LAP)<sub>2</sub> modification with endo-BCN<sub>B</sub> within 0-120 min of incubation at 37 °C by hydrophobic interaction chromatography (HIC). A shift of unmodified trastuzumab-(LAP)<sub>2</sub> (BAR0) toward a retention time of ~17 min indicates a modification of trastuzumab-(LAP)<sub>2</sub> to full extent (BAR2). BAR = BCN-to- Antibody Ratio. Note: No species corresponding to single-modified trastuzumab-(LAP)<sub>2</sub> (BAR1) was detected due to the rapid and complete modification of the antibody within three minutes of incubation. For chromatograms with this intermediate species, see Fig. 4A in the main paper.
Figure S24. Confocal microscopy visualizes localization of the trastuzumab conjugates specifically to living HER2-positive SK-BR-3 cells with no binding for HER2-negative CHO-K1 cells. (A) Immunostaining with 100 nM of trastuzumab-(LAP-EGFP)$_2$. Conjugation control was done with trastuzumab and EGFP$^{Ox7}$-LAP both functionalized with endo-BCN, and processed equally as for the described conjugation reaction. (B) Co-staining with 100 nM of of trastuzumab-(LAP-EGFP)$_2$ and 100 nM of trastuzumab-(LAP-TAMRA)$_2$. Scale bar = 20 μm.
**Figure S25.** Flow cytometry analysis of cellular binding of trastuzumab-(LAP)$_2$ stained with anti-human IgG Fc antibody-phycoerythrin (anti-IgG-Fc•PE) on HER2-positive cells (SK-BR-3). Concentration dependent binding of trastuzumab-(LAP)$_2$ is shown for two concentrations (6 + 250 nM). Excitation: 520 nm.

**Figure S26.** Dissociation constants ($K_D$) of trastuzumab conjugates on cells. $K_D$ of antibody conjugates trastuzumab-(LAP•EGFP)$_2$, trastuzumab-(LAP•TAMRA)$_2$ and unconjugated trastuzumab-(LAP)$_2$ were determined on trypsinized HER2-positive cells (SK-BR-3) by flow cytometry. For analysis, normalized RMF was plotted against the trastuzumab conjugate concentration and $K_D$ of each compound was determined using Graph Pad Prism. RMF = Relative Mean Fluorescence.
Figure S27. Thermal shift assay to derive the melting temperature ($T_m$) of the trastuzumab-(LAP-EGFP)$_2$ conjugate. Top: Thermal stability of wildtype trastuzumab (wine-red) compared to trastuzumab-(LAP)$_2$ (green) Bottom: Thermal stability of trastuzumab-(LAP)$_2$ (green) compared to trastuzumab-(LAP-EGFP)$_2$ (pink) and EGFP$^{Q157::LAP}$ (light green). PBS (blue) was used as a control.

Table S28. Obtained melting points of wildtype trastuzumab, trastuzumab-(LAP)$_2$, trastuzumab-(LAP-EGFP)$_2$ and EGFP$^{Q157::LAP}$.

| entry               | melting point 1 [$^\circ$C] | melting point 2 [$^\circ$C] | melting point 3 [$^\circ$C] |
|---------------------|-----------------------------|-----------------------------|-----------------------------|
| wildtype trastuzumab| 70.1                        | 78.4                        | -                           |
| trastuzumab-(LAP)$_2$ | 70.5                        | 78.0                        | -                           |
| trastuzumab-(LAP-EGFP)$_2$ | 70.7                      | 77.4                        | 84.9                        |
| EGFP$^{Q157::LAP}$     | 84.6                        | -                           | -                           |
Experimental procedures

Disposables were obtained from Greiner Bio-One GmbH, Corning Inc., Sarstedt AG & Co., Kimtech, Merck KGaA, Eppendorf AG, Bemis Company Inc., Brand GmbH + Co. KG and STARLAB GmbH. Unless stated otherwise, enzymes, kits and reagents were used according to the manufacturers’ protocols. Goat anti-human IgG Fc PE eBioscience was obtained from Thermo Fisher Scientific.

The term water here exclusively stands for purified water from the Milli-Q device (Merck KGaA). All pH values for buffers were determined with a pH meter (EL20, Mettler-Toledo International Inc.) at room temperature (RT). Ratios or proportions are volumetric if not indicated otherwise.

Sequencing of DNA was performed by Microsynth AG using the Economy Run Service with the specified requirements. Oligos and double stranded DNA were obtained from Integrated DNA Technologies Inc.

Mass spectrometry

Protein masses were analyzed with high resolution electrospray ionization (HR-ESI) on a Bruker micrOTOF-Q II (Bruker Corp.). Low m/z between 50 and 1200 were calibrated using internal sodium formate clusters and the enhanced quadratic mode. Calibration for higher m/z was performed with the ESI-L Low Concentration Tuning Mix (Agilent Technologies Inc.). Runs were evaluated using DataAnalysis ESI-compass (Bruker Corp.).

Mass spectrometry analysis for intact protein mass determination was performed using a maXis UHR-TOF (Bruker Corp.) at the ZMBH Core Facility for mass spectrometry and proteomics (Core Facility for Mass Spectrometry & Proteomics [CFMP], Zentrum für Molekulare Biologie der Universität Heidelberg [ZMBH]). Mass spectra of intact proteins were calculated using Max Entropy Deconvolution of the ESI-Spectra with the software DataAnalysis ESI-compass.

Nuclear magnetic resonance spectroscopy (NMR)

Spectra were recorded with the NMR spectrometer Varian Mercury 300 MHz or Varian VNMRS 500 MHz at 25° C. Analysis of spectra was carried out with MestReNova (Mestrelab Research SL) and referenced to the NMR solvent peak according to Fulmer et al.[5]. Carbon spectra were mainly recorded with AP\textsuperscript{T} NMR experiments.

Reverse phase high pressure liquid chromatography (RP-HPLC)

Analytical separations were carried out on a 1100 Series HPLC system with photodiode array detection (Agilent Technologies Inc.). Analytical runs were performed using a Luna 2.5 μ C18(2) 100 Å column (100 x 3 mm, Phenomenex Inc.) and a flowrate of 0.5 mL/min. Gradient for analytical separations: linear gradient of 10-90% MeCN in water supplemented with 0.1 % trifluoroacetic acid within 45 min. Peaks were collected, lyophilized and subjected to mass spectrometric analysis when closer analysis was necessary. For preparative runs, the column Luna 5 μ C18(2) 100 Å (250 x 15 mm, Phenomenex Inc.) with a flowrate of 5 mL/min was used.

Hydrophobic interaction chromatography (HIC)

Analytical hydrophobic interaction chromatography (HIC) was performed on an Agilent Infinity 1260 system with a VWD detector equipped with a Tosoh Bioscience TSKgel Butyl-NPR (2.5 μ, 4.6 mm x 3.5 cm) column applying a linear gradient from 0 to 100% B in 30 min (Eluent A: 1.5 M ammonium sulfate and 25 mM TRIS-HCl pH 7.5. Eluent B: 25 mM TRIS-HCl pH 7.5).

In-gel fluorescence analysis

In-gel fluorescence was measured with the Typhoon FLA 9500 Fluorescence scanner (GE Healthcare Life Sciences). Unstained, washed SDS gels were scanned via excitation with appropriate lasers and emission filter combinations (see below) and different photomultiplier voltages. Scans with signals out of the linear-dynamic range were discarded.

| fluorophore    | laser excitation | emission filter       |
|----------------|------------------|-----------------------|
| EGFP/Fluorescein| 488 nm           | LPB (515 LP), long pass, ≥ 515 nm |
| TAMRA          | 532 nm           | LPG (575 LP), long pass, ≥ 575 nm |

Densitometry

The Gel Analysis tool within ImageJ was used for densitometry analysis of SDS gels. Pictures of gel data files were aligned, and signal tracks were calculated from the CBB-stain of the gel. Peaks were integrated manually and normalized to obtain the signal intensities of the individual bands.
Molecular cloning and plasmids

The expression plasmid pMBP-LAP was prepared as described in Baalmann et al.[8] The construction of plasmids for the expression of His6-LplA<sup>WT</sup> and His6-LplA<sup>W37V</sup> (pLplA<sup>W37V</sup>) is described by Hauke et al.[7]

**pLplA<sup>W37V/C85A</sup>**

The pLplA<sup>W37V/C85A</sup> expression plasmid was generated by site-directed mutagenesis PCR amplification with the primer pair 5′-CGG CAA TAC CGG GTT TAC TCT TAT GCC TGG C-3′ and 5′-AGA TCG TGG AAC GCG G-3′. Amplification of the pLplA<sup>W37V</sup> plasmid with the above-mentioned oligonucleotides was carried out at an annealing temperature of 63 °C and 3 % of dimethyl sulfoxide (DMSO) using the Phusion DNA polymerase (Thermo Fisher Scientific). The PCR reaction mixture was treated with DpnI (New England BioLabs) and was purified over a preparative agarose gel (QIAquick Gel Extraction Kit, Qiagen). The obtained DNA was treated with T4 Polynucleotide Kinase (Thermo Fisher Scientific) and subsequently ligated using T4 DNA ligase (Thermo Fisher Scientific). After ligation, the obtained plasmid was transformed into E. coli DH5α. The identity of the sequence in the plasmid was analyzed by Sanger sequencing (T7 fw promoter primer + T7 rv terminator primer).

**pEFGP**

p1GF (gift from Scott Gradia, pET His6 GFP TEV LIC cloning vector, Addgene plasmid # 29663) was amplified with the primer pair 5′-ATG GTC TCA CAA GTA ACG GAT CCG CGG C-3′ and 5′-TTG GTC TCA CTA GTT AAG GAC CTC CAT G-3′ using the Phusion DNA polymerase at 58 °C with 3 % DMSO. The amplified was purified using a preparative agarose gel and digested with DpnI. After PCR Purification (GeneJET PCR Purification Kit, Thermo Fisher Scientific), a BsaI digest (New England BioLabs) was performed followed by another PCR purification and ligation using T4 DNA ligase. After transformation in E. coli DH5α, the identity of the sequence was analyzed by Sanger sequencing (T7 fw promoter primer + T7 rv terminator primer).

**pEFGF<sup>E172::LAP</sup>** and pEFGF<sup>E157::LAP</sup>

His6-EFGP expression plasmids with internal LAP tags, pEFGF<sup>E172::LAP</sup> and pEFGF<sup>E157::LAP</sup> were generated by PCR amplification of pEFG using the primer pair 5′-ATG GTC TCA CAA GTA CCG CGG CAG CGT GCA GCT CG-3′ and 5′-TAG GTC TCT AAC CCT CGA CTT GGT GGA GTA TCT G-3′ for insertion of the LAP tag following Gln157; and with the primer pair 5′-AGG TCG TGG AAC GAA GGT ACG AGT AAC ATT GCT AAC ATT G-3′ and 5′-AGG TCT CAA ACC CTG TCT TGG GAT GAT ATA GCT ATG G-3′ for LAP insertion after Gln157. Amplification was carried out at an annealing temperature of 62 °C and 3 % DMSO using the Phusion DNA polymerase. After amplification, the template DNA was digested using DpnI. The PCR product was purified by a preparative agarase gel. The PCR-purified vector backbone and dsDNA with the sequence for LAP (5′-ATG GTC TCA CTT AAG ACC AAA GTC AGG AGC GAG GCT AGA AGC GAC GTA GTC TCT ATT GAG G-3′ and 5′-AGG TCT CAA ACC CTG TCT TGG GAT GAT ATA GCT ATG G-3′) were digested with Bsal and both fragments were PCR purified. Ligation of was performed using T4 DNA ligase. After transformation in E. coli DH5α, the identity of the sequence was analyzed by Sanger sequencing (T7 fw promoter primer + T7 rv terminator primer).

**pmRuby3-LAP**

pET28c was amplified with the primer pair 5′-ACG TGC TCA TAA GAT CGG CGG CAG GAT G-3′ and 5′-CAG TGC TCT TGA CGC CGG TGC GAT GAT G-3′ using the Phusion DNA polymerase with 62 °C annealing temperature and 3 % DMSO. The amplified was purified over a preparative agarase gel, digested with DpnI and PCR purified. A double stranded DNA construct coding for mRuby3 (ds(mRuby3-LAP-PA), see appendix, optimized for E. coli K12, http://www.idtdna.com/CodonOpt) and the linearized pET28c were digested with Bsal, PCR purified, ligated with T4 DNA ligase and transformed into E. coli DH5α. The identity of the sequence was analyzed by Sanger sequencing (T7 fw promoter primer + T7 rv terminator primer).

**Trastuzumab cloning, expression and purification**

Trastuzumab-(LAP)<sub>2</sub> was produced by transient transfection of Exp293F with two respective pTT5 plasmids applying polyethyleneimine (PEI). The LAP recognition motif was added to the C-terminus of the heavy chain using standard PCR procedures. 20 µg of each plasmid were mixed with 120 µg of PEI in serum-free Exp293 expression medium and added to 30 mL of Exp293F cells, in a density of 2.5×10<sup>6</sup> cells/mL, in a dropwise fashion under continuous shaking. After 24 h, cells were fed with 0.5 % (w/v) tryptone. After 120 h, the cell supernatant was purified by protein A affinity chromatography using HiTrap Protein A HP columns (GE Healthcare). A 1:1.5 dilution of antibody production supernatant in running buffer (20 mM sodium phosphate, pH 7.0) was applied to the prior to this equilibrated column. Elution was carried out using 100 mM citrate buffer (pH 3). The eluted sample was immediately neutralized with 1 M TRIS-HCl (pH 9). The eluate was dialyzed against PBS and concentrated using Amicon Ultra centrifugation filters (Merck KGaA).

**Protein expression and purification from E. coli**

Expression of MBP-LAP, wildtype LpA and LpA<sup>W37V</sup> is described in our publication by Baalmann et al.[6]. The plasmid encoding the protein of interest was transformed into the respective competent E. coli BL21(DE3) expression strain (New England BioLabs or Thermo Fisher Scientific Inc.). Bacterial cells were grown at 37 °C in terrific broth[10] supplemented with the respective antibiotic in a baffled flask to an A<sub>600</sub> of 0.6 and protein expression was induced with 0.5 mM of IPTG (isopropyl β-D-1-thiogalactopyranoside) and carried out for 4-5 h (37 °C induction temperature) or overnight (RT induction temperature) (see below). After harvest of cells by centrifugation at 4000 rpm at 4 °C, cell pellets were resuspended in 20 mL of lysis buffer (protease inhibitors [Complete EDTA-free Protease Inhibitor Cocktail, Roche] and 0.05 % Triton-X-100, 0.5 M NaCl, 30 mM imidazole, 20 mM sodium phosphate, pH 7.4). After
sonification and centrifugation (37,500 rcf), the supernatant was sterile-filtered (0.45 µm) and loaded onto a HisTrap HP Ni-NTA affinity column (1 mL, GE Healthcare). The column-bound protein was eluted using an imidazole gradient (30 → 100 mM imidazole, 0.5 M NaCl, 20 mM sodium phosphate, pH 7.4) on a FPLC-protein purification system (BioLogic DuoFlow, Bio-Rad) at 4 °C and the fractions were analyzed for purity by SDS-PAGE. Eluted protein fractions were pooled and concentrated using Amicon Ultra 15 mL centrifugal filter devices (10 kDa cutoff, Merck KGaA) and washed several times with PBS. Proteins were aliquoted, snap-frozen in liquid N2 and stored at -80 °C.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and MSDE-assay

SDS gels were casted with a 40 % (w/v) acrylamide/bis-acrylamide solution (Rotiphores Gel 40 [19:1], Carl Roth GmbH + Co. KG). Protein samples were supplemented with 4 × reducing (Laemmli formulation) or 4 × non-reducing SDS-sample buffer (Laemmli formulation omitting 2-mercaptoethanol)[9], heated at 95 °C for 10 min (in the case of denaturing conditions) and subsequently loaded onto the SDS-gel with PageRuler Prestained Protein Ladder (Thermo Fisher Scientific Inc.) as protein standard. SDS-PAGE was carried out with constant voltage between 100 and 170 V and analyzed by in-gel fluorescence and/or Coomassie Brilliant Blue staining (“Blue Silver” formulation)[10].

LplA acceptance assay on peptide level

LplA acceptance was measured as previously described[9] for a reaction time of 15 min. For peak characterization, peaks were collected, lyophilized and subjected to HR-ESI mass spectrometry.

Quantitative in vitro ligation of substrates to proteins

Ligation reactions were carried out in a volume ranging from 100-2000 µL with the following components: 5 or 10 µM of dual or single LAP-tagged target protein, respectively, from PBS stock, 5 mM Mg(OAc)₂, 5 mM ATP, 0.25 - 1 mM substrate (from DMSO stock), 1 µM LplA₁₃₇₇₉/C₈₅₆ (from PBS stock, pH 7.4) in 25 mM sodium phosphate buffer (pH 7.0). Quantitative ligation took place for 10 µM of any of our LAP-tagged proteins with 0.25 mM (cycloctyne substrates), 1 mM (tetrazine substrates) or 0.5 mM (all other substrates) of substrate at 37 °C within 1 h, although complete ligation reaction is usually faster for most substrates (for example trastuzumab-BCN functionalization which is essentially complete within 5 min). Here, it should be noted that ligation of internally LAP-tagged EGFP constructs proceeds slightly slower compared to the terminally LAP-tagged proteins MBP or trastuzumab (see corresponding figures and main text) most probably due to increased conformational strain of LAP. Protein solutions were incubated at 37 °C for 1 h (or the specified time for time course measurements). For time course ligation monitoring, a master mix was used. Samples for the individual time point measurements were taken out of the master mix and were added to EDTA samples (final concentration 250 mM) to stop the reaction at distinct time points. For subsequent DAinv or SPAAC substrate removal, the solutions were washed 4 (small molecule functionalization, ~ 1.5 h total washing time) or 8 times (protein-protein conjugation, ~ 2.5 h total washing time) with PBS and concentrated in Amicon Ultra-Centrifugal filter units (Merck KGaA) with the appropriate molecular cutoff at 4 °C as described previously.[9] To challenge the isomerization properties of the TCO substrate, radical scavengers were added to all ligation mixtures and PBS used for wash steps. Protein concentration was determined with the Bradford assay (for aromatic substrates and protein-protein conjugation) or spectrophotometrically using the Nanodrop ND-100 (Thermo Fisher Scientific) and the calculated molar extinction coefficients (ProtParam tool, http://web.expasy.org/protparam/).

Cycloaddition using DAinv

DAinv was conducted at a target protein concentration of 10 µM with the respective probe from a DMSO stock at 37 °C for the desired amount of time under shaking (total DMSO content < 5 %). For the change in electrophoretic mobility and DAinv time course measurements, reactions were stopped by addition of 50 eq. of MeTzMe (for dienophile-probe conjugates) or ax-TCO-OH (for tetrazine-probe conjugates).
Protein-protein conjugation (PPC)

Proteins were modified quantitatively with the appropriate substrate, washed extensively and subsequently mixed in the corresponding ratios and incubated at 37 °C under shaking for the desired time. For time course analysis, MeTzMe (1 mM) was added to stop the reactions. Samples were analyzed by SDS-PAGE. After gel electrophoresis, gels were analyzed by in-gel fluorescence and CBB staining. For mass spectrometry analysis of the PPC, a separate reaction mix containing 10 µM of EGFP157-LAP and 20 µM of MBP-LAP(endo-BCNα) was used.

Kinetic measurements of PPC

Purified EGFP172::LAP and mRuby3-LAP were functionalized quantitatively with endo-BCNα, MetTzMeOc or MetTzAl (standard ligation protocol). EGFP172::LAP(endo-BCNα) was diluted in PBS to a final concentration of 400 nM. Several dilutions of mRuby3-LAP (2, 4, 6 or 8 µM), functionalized with endo-BCNα, MetTzMeOc or MetTzAl, were prepared in PBS. Prior to measurement, all protein solutions were equilibrated at 37 °C for 15 min. Reactions were carried out in duplicate per condition at 37 °C with a working volume of 200 µL in a black microwell plate with flat bottom (BRAND GMBH + CO KG) and orbital shaking for 3 s between every cycle. Conjugation was started by the addition of 100 µL of mRuby3-LAP solution to 100 µL of EGFP solution per well. Excitation was performed at (488 ± 5) nm and fluorescence was recorded at (579 ± 5) nm in a Tecan safire2 microtiter plate reader (Tecan Trading AG). Additionally, donor only, acceptor only and blank spectra were recorded. Acceptor fluorescence (Fα) was processed as described by Hoffmann et al.4 For determination of kobs, the individual acceptor traces were fitted assuming an inverse monophasic function \[ F_α(t) = A \times (1 - \exp(-k_{obs} \times t)) \]. The obtained kobs values including the corresponding standard errors \(\sigma_{k_{obs}}\) from the fit were plotted against the methyltetrazine-functionalized mRuby3-LAP concentration. kobs including the standard error were received as the slope of the linear fit \[ k_{obs}(qcceptor) = k_0 \times \sigma_{qcceptor} \]. Highest error estimate: \[\text{abs}(0.5 \times (k_{obs, a} - k_{obs, b})) + 0.5 \times (\sigma_{k_{obs,a}} + \sigma_{k_{obs,b}})\]. Data processing was done with OriginPro 2018 (OriginLab Corporation).

Kinetics of LplA-mediated antibody modification

Kinetics measurements of LplA-mediated antibody modification with BCN-substrate were performed on an Agilent Infinity 1260 system equipped with a TSKgel Butyl-NPR (2.5 µM, 4.6 mm×3.5 cm, Tosoh Bioscience) applying a linear gradient from 0 to 100 % of eluent B in 30 min (Eluent A: 1.5 M ammonium sulfate in 25 mM TRIS-HCl [pH 7.5], Eluent B: 25 mM TRIS-HCl [pH 7.5]). Reactions were stopped prior to measurements by the addition of EDTA yielding a final concentration of 250 mM. Measurements were performed from 0 to 5 min in 1 min steps, from 5 to 20 min in 5 min steps and up to a final time of 120 min.

Cell culture

Cells were incubated under standard conditions at 37 °C in a humidified incubator with 5 % CO2. SK-BR-3 breast cancer cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) culture medium under addition of 10 % fetal bovine serum (FBS). CHO-K1 cells were grown in DMEM/F-12 (Thermo Fisher Scientific) with 10 % FBS added. Non-adherent Exp293F cells (Thermo Fisher Scientific) were grown in serum-free Exp293 expression medium (Thermo Fisher Scientific) on an orbital shaker at 110 rpm.

Cellular binding by flow cytometry

Human breast cancer SK-BR-3 cells were trypsinized from the culture flask and spun down. Cells (2 x 10⁶) were washed three times with PBS containing 1 % (w/v) of bovine serum albumin (BSA) followed by incubation with trastuzumab-(LAP)2, trastuzumab-(LAP-EGFP)2 or trastuzumab-(LAP-TAMRA)2 (50 µL, 50 nM in PBS + 1 % [w/v] BSA) for 30 min on ice. Subsequently, cells were washed three times with PBS containing 1 % (w/v) of BSA. Then trastuzumab-(LAP)2 was incubated with fluorescently labeled secondary antibody goat anti-human IgG Fc, PE-conjugated eBioscience (100 µL, 1/100 dilution) for 20 min on ice. In the case of fluorescent trastuzumab-(LAP-EGFP)2 or trastuzumab-(LAP-TAMRA)2, no secondary antibody was applied. Subsequently, cells were washed three times with PBS + 1 % of BSA. Cells were analyzed by flow cytometry using a BD Influx cell-sorting device (Becton, Dickinson and Company). Cells only incubated with the fluorescently labeled secondary antibody goat anti-human IgG Fc, PE-conjugated eBioscience were used as isotype control. Data analysis and visualization was carried out with FlowJo v10 (Becton, Dickinson and Company).

Determination of K0 by flow cytometry

Human breast cancer SK-BR-3 cells were trypsinized from the culture flask and spun down. Cells were counted and 2 x 10⁵ cells per well were seeded in a 96-well round-bottom plate. Cells were washed three times with PBS containing 1 % (w/v) of BSA followed by incubation with trastuzumab-(LAP)2, trastuzumab-(LAP-EGFP)2 or trastuzumab-(LAP-TAMRA)2, in varying concentrations, for 30 min on ice. Trastuzumab-(LAP)2 was subsequently incubated with the fluorescently labeled secondary antibody goat anti-human IgG Fc, PE-conjugated eBioscience (100 µL, 1/100 dilution) for 20 min on ice. In the case of fluorescent trastuzumab-(LAP-EGFP)2 or trastuzumab-(LAP-TAMRA)2, no secondary antibody was applied. Cells were washed three times with PBS + 1 % (w/v) of BSA and diluted in 400 µL and analyzed by flow cytometry using a BD Influx cell-sorting device (Becton, Dickinson and Company). Normalized mean fluorescence was plotted against antibody concentration. A single point measurement was executed.
Confocal microscopy

One day prior to the experiment, SK-BR-3 cells and CHO-K1 cells were plated at $4 \times 10^4$ cells/well into a 8-chamber glass bottom slide (Nunc Lab-Tek II Chamber Slide System, Thermo Fisher Scientific) with a culture area of 0.4 cm$^2$/well (working volume per well: 200 µL). Glass slides were etched with an aqueous 0.1 M HF solution prior to seeding. SK-BR-3 cells were cultured in DMEM + 10 % of FBS and penicillin/streptomycin, CHO-K1 cells were cultured in DMEM/F-12 medium supplemented with 10 % of FBS and penicillin/streptomycin. On the following day, the media was removed; cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and subsequently incubated with 100 nM of trastuzumab-(LAP)-EGFP$_2$, trastuzumab-(LAP)-TAMRA$_2$ or the conjugation control (trastuzumab-(LAP)$_2$ and EGFP$_{172}$-LAP) both modified with endo-BCN$_6$ in the respective culture medium in a humified cell incubator for 1 h. Following incubation of the probes, cells were incubated with Hoechst stain 33342 (Thermo Fisher Scientific) at 8 µM in DMEM for 5 min. Cells were washed 4 times with DPBS and incubated in 200 µL of Leibovitz’s L-15 medium without phenol red (Thermo Fisher Scientific) at 37 °C during imaging. Confocal laser scanning microscopy was performed at the Nikon A1R confocal mounted on an inverted Nikon Ti2 microscope with a 60× objective (Nikon N Apo 60× NA 1.4 λs Oil). The Hoechst channel was set to excite at 405 nm, the GFP channel was set to excite at 488 nm and the TRITC channel was set to excite at 558 nm. Bright field images were acquired at 488 nm.

Thermal shift assay

Thermal shift assays were performed on a Prometheus NT.48 nano DSF detection system (NanoTemper Technologies GmbH) with 0.5 °C/30 s from 20 to 100 °C or from 20 to 90 °C, respectively. Values were collected from melting curves using the corresponding analysis software. The 350/330 nm ratio was plotted against the temperature. Reactions for the comparison of trastuzumab-(LAP)-EGFP$_2$, trastuzumab-(LAP)$_2$ and EGFP$_{Q157}$-LAP were performed in PBS at a concentration of 0.25 mg/mL. Reactions for the comparison of trastuzumab-(LAP)$_2$ with wildtype trastuzumab were performed at a concentration of 0.5 mg/mL.

Cytotoxicity assay

Trastuzumab-(LAP)$_2$ was treated with endo-BCN$_6$ (50 eq), Lpl$^{W37V}$ (0.1 eq.), 5 mM ATP, 5 mM Mg(OAc)$_2$ in 25 mM of sodium phosphate buffer (pH 7.0) for 15 min at 37°C. Trastuzumab-(LAP)(endo-BCN$_6$)$_2$ was immobilized on a Protein A HP SpinTrap (GE Healthcare) and purified by repeated washing with PBS from excess substrate. SPAAC reaction with N$_2$PEG$_3$-vc-PABC-MMAE (50 eq.) (MedChemExpress) was performed in Protein A bound state at 37 °C o/n. Excess N$_2$PEG$_3$-vc-PABC-MMAE was removed by extensive washing with PBS and the generated antibody-drug conjugate (ADC) was eluted with elution buffer (80 mM citric acid, 18 mM trisodium citrate, pH 3). Eluate was subsequently neutralized with 1 M TRIS-HCl, pH 9, dialyzed against PBS and concentrated using an Amicon Ultra centrifugation filter (Merck Millipore).

HER2-positive breast cancer SK-BR-3 cells and HER2-negative CHO-K1 cells were seeded in a 96-well plate in a density of $6.5 \times 10^4$ cells/well with penicillin/streptomycin. After 24 h incubation in a humidified cell incubator, cells were treated with trastuzumab-(LAP)$_2$ and trastuzumab-(LAP-MMAE)$_2$ in the respective concentrations and incubated for 80 h in a humidified cell incubator. Cell viability was measured using CellTiter96 AQ$_{soula}$ One Solution Cell Proliferation Assay (Promega) following the instructions of the supplier using a Tecan Infinite F200 Pro reader (Tecan Trading AG) for spectrophotometric analysis.
Chemicals and synthesis procedures

All chemicals and reagents were purchased from commercial sources like Sigma-Aldrich Corp., abcr GmbH, Thermo Scientific Inc., ChemPUR Feinchemikalien und Forschungsbedarf GmbH, Merck KGaA, Iris Biotech GmbH, Fluorochem Ltd. and VWR International and used without any further purification unless otherwise stated. Deuterated solvents were obtained from Sigma-Aldrich Corp. (now Merck KGaA) or Euriso-Top GmbH.

(RS)-lipoic acid and enolic acids En$_3$E$_n$ were acquired from commercial sources. Norb$_2$ was synthesized as described previously.$^{[1, 3]}$ Synthesis and characterization of MeTzMeOcs, Trzs, 12a, 12b, endo-BCN-OH, 13, MeTzBnNH$_2$+HCl, MeTzMe, 5-TAMRA, TCO-Prop-TAMRA and BCN-Pip-TAMRA is described in our publication by Bälmann et al.$^{[5]}$ Synthesis and characterization of compounds CCO$_2$-OH, ax-TCO$_2$-OH, eq.-TCO$_2$-OH, 9a and ax-TCO$_2$ is described in our publication by Wieczorek et al.$^{[11]}$ TzBnNH-TAMRA (known as Tetrazine-5-TAMRA) was purchased from Jena Bioscience GmbH. SCO-OH was purchased from Sirius Fine Chemicals SiChem GmbH. N$_5$-PEG$_3$-vc-PABC-MMAE was purchased from MedChemExpress. Note: see accompanying figures and text for further details.

Unless stated otherwise, all reactions were conducted at room temperature (RT). When handling anhydrous solvents or reagents, all reactions were conducted under argon atmosphere in baked out Schlenk flasks or equivalently treated glassware. For adjusting the pH during aqueous workups, pH paper (pH-Indikatorpapier pH 1 - 14 Universalindikator, Merck KGaA) was used.

Progress of chemical reactions was monitored analytically either with normal phase silica gel thin layer chromatography plates (0.20 mm POLIGRAM SIL G/UV254, Macherey-Nagel GmbH & Co. KG) or with reverse phase silica gel thin layer chromatography plates (DC Kieselgel 60 RP-18 F$_{254}$S, Merck KGaA). Visualization was done via fluorescence quenching under UV light. If applicable, thin layer chromatography plates were stained with ninhydrin staining solution (1.5 g ninhydrin staining solution (1.5 g ninhydrin in 100 mL of n-butanol and 3 mL of AcOH), potassium permanganate staining solution (1.5 g of KMnO$_4$, 10 g of K$_2$CO$_3$ and 25 mL of 10% [w/v] NaOH in 200 mL of water) or bromocresol green staining solution (0.04 g of bromocresol green in 100 mL of EtOH, titrated with an aqueous 0.1 M NaOH solution until blue).

General synthesis procedure for 4-nitrophenyl carbonates

The synthesis is a modified literature procedure.$^{[12]}$

To a solution of the respective alcohol (100 mg, 0.792 mmol, 1.00 eq.) in anhydrous dichloromethane (DCM) (0.05 - 0.1 M solution), a solution of 4-nitrophenylchloroformate (1.10 eq.) in anhydrous DCM (2 mL) was added. Anhydrous pyridine (Py, 2.5 eq.) was added dropwise during which a colorless precipitate formed. After 1.5 h, conversion of the starting material was complete, and the reaction was quenched by the addition of a saturated aqueous NH$_4$Cl solution (20 mL/eq.). The layers were separated, and the aqueous layer was extracted three times with DCM. The combined organic layers were washed with a saturated aqueous NaCl solution, dried over MgSO$_4$ and filtered. After removal of the volatile constituents in vacuo, the crude product was purified by normal phase silica gel flash chromatography (FC, CH$_2$Cl$_2$ → CH$_2$Cl$_2$:EtOAc 97:3; CH$_2$Cl$_2$ = cyclohexane).

General carbamate synthesis procedure 1

For every substrate, the corresponding 4-nitrophenyl carbonate was dissolved in anhydrous dimethylformamide (DMF, 50-100 mM) and the respective powdered linear amino acid was added to the reaction mixture. N,N-diisopropylethylamine (DIPEA; 2.0-3.0 eq.) was added dropwise and the reaction mixture was stirred overnight. The reaction mixtures were diluted with an appropriate volume of water (100 mL/mmol 4-nitrophenyl carbonate), washed with DCM and the organic layer was discarded. The aqueous layer was adjusted to pH 2-3 with an aqueous AcOH solution (5%). The aqueous layer was extracted three times with DCM. The combined organic layers were washed with a saturated aqueous NaCl solution, dried over MgSO$_4$ and filtered. After removal of the volatile constituents in vacuo, the crude product was purified over FC (DCM → DCM + 1 % AcOH → DCM:EtOAc + 1 % AcOH). After coevaporation with Toluene (PhMe) to remove residual AcOH, the desired products were obtained.

General carbamate synthesis procedure 2

To the alcohol (1-2 mmol, 1 eq.) in anhydrous DCM (10 mL/eq.) was added anhydrous Py (6 eq.) and cooled to 0 °C. A solution of 4-nitrophenylchloroformate (2.20 eq.) in anhydrous DCM (1 mL/mmol starting material) was added until the starting material was consumed (20 min). After the addition of water (15 mL/eq.), separation into two layers took place and the organic layer was washed three times with a saturated aqueous NaHCO$_3$ solution and once with water. The organic layer was dried over MgSO$_4$, filtered and concentrated in vacuo. The crude product was dissolved with DMF (5 mL/eq.). The respective powdered linear amino acid (1.5 - 3.0 eq.) and DIPEA (2.25-3.00 eq.) were added and the reaction mixtures were stirred overnight. Water was added, the aqueous layer was washed with DCM and the organic layer was discarded. The aqueous layer was adjusted to pH 2-3 with an aqueous AcOH solution (5%). The aqueous layer was extracted three times with DCM. The combined organic layers were washed with a saturated aqueous NaCl solution, dried over MgSO$_4$ and filtered. After removal of the volatile constituents in vacuo, the crude product was purified over FC (DCM → DCM + 1 % AcOH → DCM:EtOAc + 1 % AcOH).
Cyclopropene substrate candidates (synthesis overview)

Scheme S29. Synthesis of cyclopropene substrate candidates for LplA<sup>29</sup> : a: Rh(0)OAc<sub>2</sub>, DCM, 0 °C → RT, o/n, 89 %; b: Rh(0)OAc<sub>2</sub>, DCM, 0 °C → RT, overnight (o.n), 66 %; c: 1) disobutylaluminium hydride (DIBAL-H), Et<sub>2</sub>O/THF, 0 °C, o/n; 2) H<sub>2</sub>O, 35 %; d: 1) 4-nitrophenyl chloroformate, Py, DCM, 0 °C, 20 min, DMCY<sub>d</sub>: 4-aminobutanoic acid; for DMCY<sub>d</sub>: 5-aminopenicanoic acid; for DMCY<sub>c</sub>: 6-aminohexanoic acid; for DMCY<sub>b</sub>: 7-aminohexanoic acid, DIPEA, DMF, RT, o/n, 3) AcOH, 21 % (DMCY<sub>c</sub>), 19 % (DMCY<sub>d</sub>), 28 % (DMCY<sub>b</sub>) or 21 % (DMCY<sub>a</sub>); e: 1) DIBAL-H, 0 °C, 27.5 h, 2) H<sub>2</sub>O, 54 %; f: 1) TBAF, 4Å molecular sieve, THF, RT, 1.5 M, 27.5 h, 2) H<sub>2</sub>O, 77 %; g: 1) 4-aminobutanoic acid; for DMCY<sub>d</sub>: 5-aminopenicanoic acid; for DMCY<sub>b</sub>: 6-aminohexanoic acid; for DMCY<sub>c</sub>: 7-aminohexanoic acid, DIPEA, DMF, RT, o/n, 2) AcOH, 54 % (DMCY<sub>d</sub>), 75 % (DMCY<sub>c</sub>), 91 % (DMCY<sub>b</sub>) or 59 % (DMCY<sub>a</sub>).

Ethyl 2,3-dimethylcycloprop-2-ene-1-carboxylate (1)

At 0 °C, rhodium(II) acetate (127 mg, 0.287 mmol, 1.0 eq.) was suspended in 2-butyne (3.10 g, 57.3 mmol, 1.00 eq.) under argon atmosphere. A solution of ethyl diazoacetate in anhydrous DCM (1.6 M, 3.27 g, 28.7 mmol, 1.0 eq.) was added dropwise over 9 h at 0 °C, the reaction mixture was allowed to reach RT and stirred overnight. The reaction mixture was filtered over normal phase silica gel. The crude product solution was carefully concentrated under reduced pressure (700 mbar) and purified by FC (n-pentane:Et<sub>2</sub>O 30:1 → 10:1) to afford the target compound as colorless liquid in 66 % yield (2.65 g, 18.9 mmol).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 4.11 (q, J = 7.1 Hz, 2H), 2.03 (s, 6H), 1.99 (s, 1H), 1.24 (t, J = 7.1 Hz, 3H), 0.18 (s, 9H). 1<sup>3</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 176.9, 102.4, 60.0, 23.1, 14.6, 9.7. HR-ESI, calcd for [C<sub>8</sub>H<sub>12</sub>O<sub>2</sub>Na<sup>+</sup>]: 163.0730, found: 163.0739.

Ethyl 2-methyl-3-(trimethylsilyl)cycloprop-2-ene-1-carboxylate (2)

At 0 °C, rhodium(II) acetate (79 mg, 0.18 mmol, 1.0 eq.) was suspended in 1-(trimethylsilyl)prop-1-yn (4.00 g, 35.6 mmol, 2.00 eq.) under argon atmosphere. A solution of ethyl diazoacetate in anhydrous DCM (1.78 M, 2.03 g, 17.8 mmol, 1.0 eq.) was added dropwise over 10 h at 0 °C, the reaction mixture was allowed to reach RT and stirred overnight. The reaction mixture was filtered over normal phase silica gel. The crude product solution was carefully concentrated under reduced pressure (700 mbar) and purified by FC (n-pentane:Et<sub>2</sub>O 40:1 → 15:1) to afford the target compound as colorless liquid in 89 % yield (3.15 g, 15.9 mmol).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 4.10 (qd, J = 7.1, 2.5 Hz, 2H), 2.18 (s, 3H), 1.97 (s, 1H), 1.23 (t, J = 7.1 Hz, 3H), 0.18 (s, 9H). 1<sup>3</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 177.0, 122.6, 60.0, 23.1, 14.6, 11.8, -16. HR-ESI, calcd. for [C<sub>10</sub>H<sub>18</sub>O<sub>2</sub>Si+Na<sup>+</sup>]: 221.0968, found: 221.0968. R<sub>e</sub>(n-pentane:Et<sub>2</sub>O 9:1) = 0.26.

(2,3-Dimethylcycloprop-2-ene-1-yl)methanol (3)

A solution of DIBAL-H in anhydrous tetrahydrofuran (THF, 1 M, 23.6 mL, 23.6 mmol, 1.25 eq.) was diluted with anhydrous Et<sub>2</sub>O (25 mL) and cooled to 0 °C. To this solution, a solution of 2 in anhydrous Et<sub>2</sub>O (0.76 M, 2.65 g, 18.9 mmol, 1.00 eq.) was added. The reaction mixture was allowed to reach RT and stirred overnight. The reaction mixture was cooled to 0 °C and a saturated aqueous potassium sodium tartrate solution was added under vigorous stirring (60 mL). The mixture was stirred overnight during which the gel-like structure dissolved, and layer separation took place. The aqueous layer was extracted three times with Et<sub>2</sub>O (3 × 50 mL) and the combined organic layers were washed with a saturated aqueous NaCl solution (50 mL). After drying over Na<sub>2</sub>SO<sub>4</sub> and filtration, the crude product was concentrated carefully under reduced pressure and purified over FC (n-pentane:Et<sub>2</sub>O 2:1 → 1:1). The desired compound was obtained as a colorless liquid in 35 % yield (650 mg, 6.62 mmol).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 3.52 (d, J = 4.4 Hz, 2H), 2.02 (s, 6H), 1.52 (t, J = 4.4 Hz, 1H).
**Dimethylcyclopropene substrate candidates (DMCy$_a$-DMCy$_d$)**

General carbamate synthesis procedure 2 with 4. Following amino acids were used: DMCy$_a$: 4-aminobutanoic acid; DMCy$_b$: 5-aminopentanoic acid; DMCy$_c$: 6-aminohexanoic acid; DMCy$_d$: 7-aminooctanoic acid; 1.5 eq. each. Prior to drying over Na$_2$SO$_4$, the combined organic layers were washed four times with an aqueous LiCl solution (5% w/w). For elution of the target compounds during FC, following solvent mixtures were used: DCM:EtOAc 4:1 + 1% AcOH for DMCy$_a$-DMCy$_d$ or DCM:EtOAc 10:1 + 1% AcOH for DMCy$_c$-DMCy$_d$. After purification, products were coevaporated with PhMe (3 × 1 mL) to remove residual AcOH.

4-(((2,3-Dimethylcycloprop-2-ene-1-yl)ethoxy)carbonyl)amino)butanoic acid (DMCy$_a$):
colorless viscous liquid, 21% (25 mg, 0.11 mmol)

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 9.34 (s, 1H), 4.89 (s, 1H), 4.09 – 3.78 (m, 2H), 3.30 – 3.14 (m, 2H), 2.39 (t, $J$ = 7.2 Hz, 2H), 1.98 (s, 6H), 1.90 – 1.77 (m, 2H), 1.55 – 1.41 (m, 1H). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 178.3, 157.4, 109.9, 72.7, 40.2, 31.4, 25.3, 19.5, 10.5. HR-ESI, calcd. for [C$_{13}$H$_{20}$NO$_4$+Na]$^+$: 250.1050, found: 250.1057.

5-(((2,3-Dimethylcycloprop-2-ene-1-yl)ethoxy)carbonyl)amino)pentanoic acid (DMCy$_b$):
colorless solid, 19% (25 mg, 0.10 mmol)

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.49 (s, 1H), 4.90 – 4.73 (m, 1H), 3.91 (d, $J$ = 5.1 Hz, 2H), 3.23 – 3.11 (m, 2H), 2.36 (t, $J$ = 7.2 Hz, 2H), 2.08 – 1.87 (m, 6H), 1.71 – 1.61 (m, 2H), 1.60 – 1.51 (m, 2H), 1.51 – 1.44 (m, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 178.7, 157.3, 110.0, 72.6, 40.6, 33.7, 29.6, 22.0, 19.6, 10.5. HR-ESI, calcd. for [C$_{13}$H$_{19}$NO$_4$+Na]$^+$: 264.1206, found: 264.1203.

6-(((2,3-Dimethylcycloprop-2-ene-1-yl)ethoxy)carbonyl)amino)hexanoic acid (DMCy$_c$):
colorless solid, 28% (37 mg, 0.14 mmol)

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.77 (s, 1H), 4.83 – 4.64 (m, 1H), 3.99 – 3.87 (m, 2H), 3.21 – 3.04 (m, 2H), 2.33 (t, $J$ = 7.4 Hz, 2H), 2.05 – 1.90 (m, 6H), 1.71 – 1.58 (m, 2H), 1.58 – 1.43 (m, 3H), 1.41 – 1.32 (m, 2H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 179.0, 157.2, 109.9, 72.5, 40.8, 34.1, 29.8, 26.3, 24.5, 19.6, 10.5. HR-ESI, calcd. for [C$_{13}$H$_{21}$NO$_4$+Na]$^+$: 278.1363, found: 278.1367.

7-(((2,3-Dimethylcycloprop-2-ene-1-yl)ethoxy)carbonyl)heptanoic acid (DMCy$_d$):
colorless solid, 21% (29 mg, 0.11 mmol)

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.83 (s, 1H), 4.77 – 4.61 (m, 1H), 4.03 – 3.86 (m, 2H), 3.24 – 3.07 (m, 2H), 2.33 (t, $J$ = 7.4 Hz, 2H), 2.05 – 1.93 (m, 6H), 1.68 – 1.59 (m, 2H), 1.54 – 1.45 (m, 3H), 1.39 – 1.30 (m, $J$ = 5.5, 4.4 Hz, 4H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 179.1, 157.2, 110.0, 72.5, 41.0, 34.1, 30.0, 28.8, 26.5, 24.8, 19.6, 10.5. HR-ESI, calcd. for [C$_{13}$H$_{23}$NO$_4$+Na]$^+$: 292.1519, found: 292.1521.

(2-Methyl-3-(trimethylsilyl)cyclopent-2-ene-1-yl)ethanol (4)

2 (800 mg, 4.03 mmol, 1.00 eq.) was dissolved in anhydrous THF (5 mL) under argon atmosphere and cooled to 0 °C. A solution of Dibal-H in THF (1 M, 10 mL, 10 mmol, 2.5 eq.) was added dropwise (0.4 mL/h) and stirred at 0 °C for further 2.5 h. Water was added (2 mL) and the reaction mixture was added to a rapidly stirring half-saturated aqueous potassium sodium tartrate solution (200 mL) and overlaid with Et$_2$O (20 mL). After several hours of stirring layer separation took place. The ether layer was separated, and the aqueous layer was extracted three times with Et$_2$O (3 × 20 mL). The combined organic layers were washed with a saturated aqueous NaCl solution, dried over MgSO$_4$, filtered and concentrated carefully under reduced pressure. The residue was purified over FC (DCM) and the target compound was isolated as a colorless viscous liquid in 54% yield (340 mg, 2.18 mmol).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 3.48 (d, $J$ = 4.6 Hz, 2H), 2.21 (s, 3H), 1.56 (t, $J$ = 4.6 Hz, 1H), 0.17 (s, 9H). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 135.6, 111.4, 69.5, 22.3, 13.6, -1.0. $R_f$(CH$_2$CHOA 1:1) = 0.59.

(2-Methylcyclopent-2-ene-1-yl)methyl(4-nitrophenyl)carbonate (5)

The synthesis procedure was performed according to a modified literature procedure.[13] 4 (500 mg, 3.20 mmol, 1.00 eq.) was added to a suspension of activated powdered molecular sieve (4 Å) in anhydrous THF (25 mL). A solution of tetrabutylammonium fluoride (TBAF) in THF (1 M, 3.52 mmol, 1.10 eq.) was added and stirred until the starting material was consumed completely (ca. 1.5 h). $R_f$(CH$_2$CHOA 1:1) = 0.35. The reaction mixture was cooled to 0 °C, diluted with anhydrous Py (13 mL) and a solution of 4-nitrophenyl chloroformate in anhydrous THF was added (1.73 M, 1.74 g, 8.63 mmol, 2.7 eq.). After being stirred at 0 °C for 3 h, the reaction mixture was filtered, and the residue was washed with DCM (50 mL). The filtrate was washed with a saturated aqueous NH$_4$Cl solution (40 mL) and the aqueous layer was extracted two times with DCM (2 × 50 mL). The combined organic layers were dried over Na$_2$SO$_4$, filtered and the volatile constituents were removed under reduced pressure. After FC (CH$_2$CHOA 20:1), the title compound was obtained as a colorless liquid in 53% yield (420 mg, 1.69 mmol) that solidified at -20 °C.
**1H NMR** (300 MHz, CDCl$_3$) δ 8.37 – 8.20 (m, 2H), 7.50 – 7.32 (m, 2H), 6.67 – 6.55 (m, 1H), 4.25 – 4.10 (m, 2H), 2.20 – 2.15 (m, 3H), 1.81 – 1.74 (m, 1H). **13C NMR** (75 MHz, CDCl$_3$) δ 155.9, 152.8, 145.4, 125.4, 122.0, 120.3, 101.8, 77.5, 16.8, 11.8. HR-ESI, calcd. for [C$_{12}$H$_7$NO$_5$+Na$^+$]: 272.0529, found: 272.0533.

### Monomethylcyclopropene substrate candidates (MMCy$_a$-MMCy$_d$)

General carbamate synthesis procedure 1 (paragraph Fehler! Verweisquelle konnte nicht gefunden werden.) with 5. Following amino acids were used: MMCy$_a$: 4-aminobutanoic acid; MMCy$_b$: 5-aminopentanoic acid; MMCy$_c$: 6-aminohexanoic acid; MMCy$_d$: 7-aminoheptanoic acid; 1.5 eq. each).

For elution of the target compounds during FC, following solvent mixtures were used: DCM:EtOAc 4:1 for MMCy$_a$-MMCy$_b$ or 10:1 for MMCy$_c$-MMCy$_d$.

#### 4-(((2-Methylcycloprop-2-en-1-yl)methoxy)carbonyl)amino)butanoic acid (MMCy$_a$):

- colorless viscous liquid, 54 % (23.1 mg, 0.108 mmol)

**1H NMR** (300 MHz, CDCl$_3$) δ 9.97 (s, 1H), 6.71 – 6.31 (m, 1H), 5.05 – 4.70 (m, 1H), 4.09 – 3.79 (m, 2H), 3.29 – 3.16 (m, 2H), 2.40 (t, J = 7.3 Hz, 2H), 2.19 – 2.04 (m, 3H), 1.90 – 1.78 (m, 2H), 1.72 – 1.52 (m, 1H). **13C NMR** (75 MHz, CDCl$_3$) δ 178.3, 157.3, 120.8, 102.3, 72.6, 40.3, 31.3, 25.3, 17.3, 11.8. HR-ESI, calcd. for [C$_{10}$H$_{15}$NO$_4$+Na$^+$]: 236.0893, found: 236.0908.

#### 5-(((2-Methylcycloprop-2-en-1-yl)methoxy)carbonyl)amino)pentanoic acid (MMCy$_b$):

- colorless viscous liquid, 75 % (11 mg, 0.048 mmol)

**1H NMR** (500 MHz, CDCl$_3$) δ 6.56 (s, 1H), 4.77 (s, 1H), 3.92 (t, J = 4.1 Hz, 2H), 3.24 – 3.13 (m, 2H), 2.38 (t, J = 7.3 Hz, 2H), 2.20 – 2.04 (m, 3H), 1.73 – 1.62 (m, 3H), 1.59 – 1.53 (m, 2H). **13C NMR** (126 MHz, CDCl$_3$) δ 178.6, 157.2, 120.8, 102.3, 72.4, 40.6, 33.6, 29.6, 21.9, 17.4, 11.8. HR-ESI, calcd. for [C$_{11}$H$_{17}$NO$_4$+Na$^+$]: 250.1050, found: 250.1053.

#### 6-(((2-Methylcycloprop-2-en-1-yl)methoxy)carbonyl)amino)hexanoic acid (MMCy$_c$):

- colorless viscous liquid, 91 % (14 mg, 0.058 mmol)

**1H NMR** (300 MHz, CDCl$_3$) δ 9.60 (s, 1H), 6.72 – 6.44 (m, 1H), 4.87 – 4.58 (m, 1H), 3.97 – 3.83 (m, 2H), 3.28 – 3.08 (m, 2H), 2.35 (t, J = 7.4 Hz, 2H), 2.19 – 2.03 (m, 3H), 1.72 – 1.59 (m, 3H), 1.58 – 1.46 (m, 2H), 1.46 – 1.33 (m, 2H). **13C NMR** (75 MHz, CDCl$_3$) δ 178.9, 157.1, 120.9, 102.3, 72.4, 40.9, 34.0, 29.8, 26.3, 24.5, 17.4, 11.8. HR-ESI, calcd. for [C$_{12}$H$_{19}$NO$_4$+Na$^+$]: 264.1206, found: 264.1218.

#### 7-(((2-Methylcycloprop-2-en-1-yl)methoxy)carbonyl)amino)heptanoic acid (MMCy$_d$):

- colorless viscous liquid, 59 % (25 mg, 0.098 mmol)

**1H NMR** (300 MHz, CDCl$_3$) δ 10.62 (s, 1H), 6.67 – 6.44 (m, 1H), 4.86 – 4.63 (m, 1H), 4.23 – 3.75 (m, 2H), 3.22 – 3.06 (m, 2H), 2.33 (t, J = 7.4 Hz, 2H), 2.18 – 2.10 (m, 3H), 1.70 – 1.56 (m, 3H), 1.55 – 1.43 (m, 2H), 1.41 – 1.27 (m, 4H). **13C NMR** (75 MHz, CDCl$_3$) δ 179.5, 157.1, 120.8, 102.3, 72.4, 41.0, 34.2, 29.9, 28.8, 26.5, 24.7, 17.3, 11.8. HR-ESI, calcd. for [C$_{13}$H$_{21}$NO$_4$+Na$^+$]: 278.1363, found: 278.1373.
Cyclooctene substrates (synthesis overview)

\[
\begin{array}{c}
\text{a} \\
\text{b} \\
\text{c} \\
\text{d} \\
\text{e} \\
\text{f} \\
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\end{array}
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Scheme S30. Synthesis of trans- and cis-cyclooctene substrates for LpiA<sup>W37V</sup>. a: mCPBA, DCM, 0 °C → RT, 4 h, 44 %; b: LiAlH<sub>4</sub>, 0 °C → RT, o/n, 88 %; c: hv, PhCOOCH<sub>3</sub>, AgNO<sub>3</sub> on normal phase silica gel, n-hexane:EtOAc 99:1, 12 h, 77 %; d: 4-nitrophenyl chloroformate, Py, RT, DCM, 39 % (for ax-TCO-OH) and 56 % (for eq-TCO-OH); e: 1) 5-aminopentanoic acid, NEt<sub>3</sub>, DMF, RT, o/n, 2) AcOH, 27 %; f: 4-nitrophenyl chloroformate, Py, DCM, RT, 1.5 h, 78 %; g: 1) 5-aminopentanoic acid, DIPEA, DMF, RT, o/n, 2) AcOH, 71 %.

(Z)-9-oxabicyclo[6.1.0]non-4-ene (6)

Following a synthesis procedure from literature,<sup>14</sup> to 1,5-cyclooctadiene (8.8 g, 81 mmol, 1.00 eq.) in anhydrous DCM (100 mL) stirring under argon atmosphere at 0 °C was added a solution of meta-chloroperbenzoic acid (mCPBA, 17.1 g, 99.1 mmol, 1.22 eq.) in anhydrous DCM (150 mL) dropwise. The solution was allowed to reach RT and kept stirring for 4 h. The reaction mixture was filtered, and the organic layer was washed with an aqueous NaOH solution (1 M, 50 mL), with an aqueous saturated NaHCO<sub>3</sub> solution (50 mL) and finally washed with a saturated NaCl solution (50 mL). The organic layer was concentrated in vacuo and purified over normal phase flash chromatography (10:1 CH₂Cl₂/EtOAc) to afford the desired product as an off-colored viscous liquid in 44 % yield (4.484 g, 35.53 mmol).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 5.57 (ddd, <i>J</i> = 5.2, 2.5, 1.0 Hz, 2H), 3.11 – 2.92 (m, 2H), 2.56 – 2.32 (m, 2H), 2.21 – 1.93 (m, 6H).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 129.0, 56.9, 28.2, 23.8.

EI-MS, calcd for [C<sub>11</sub>H<sub>14</sub>O]+•: 126.1, found: 126.0.

(Z)-cyclooct-4-en-1-ol (CCO-OH)

Following a literature procedure,<sup>14</sup> to a solution of 6 (6.00 g, 48.3 mmol, 1.00 eq.) stirring in anhydrous THF (30 mL) at 0 °C was added a LiAlH<sub>4</sub> solution (1 M in anhydrous THF, 24.2 mL, 24.2 mmol, 0.501 eq.) dropwise. The reaction mixture was stirred for 1 h at 0 °C, allowed to warm up to RT and stirred overnight. The reaction was quenched by the addition of water (5 mL). The precipitate was removed by filtration and washed with Et<sub>2</sub>O (40 mL). The filtrate combined with the ether wash was dried over MgSO<sub>4</sub>, filtered, concentrated in vacuo and the residue was purified over FC (3:1 CH₂Cl₂/EtOAc) to afford the desired product as a colorless viscous liquid in 68 % yield (5.440 g, 42.7 mmol).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 5.77 – 5.65 (m, 1H), 5.65 – 5.52 (m, 1H), 3.89 – 3.74 (m, 1H), 2.36 – 1.45 (m, 11H).<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 130.3, 129.7, 72.9, 37.9, 36.4, 25.8, 25.0, 22.9. El-MS, calcd for [C<sub>9</sub>H<sub>12</sub>O]<sup>+</sup>: 126.1, found: 126.0.
**axial-(4E)-cyclooct-4-en-1-ol (ax.-TCO-OH) and equatorial-(4E)-cyclooct-4-en-1-ol (eq.-TCO-OH)**

The literature procedure for light-induced cis-to-trans isomerization by Royzen et al.[15] was followed with 5.00 g (39.5 mmol, 1.00 eq.) of racemic CCO-OH as starting material. After normal phase flash chromatography, two diastereomeric fractions were isolated as colorless viscous liquids with a characteristic smell in 75 % total yield (fraction with pure ax. enantiomers: 27 % yield, 1.35 g, 10.7 mmol; fraction with pure eq. enantiomers, 25 % yield, 1.25 g, 9.90 mmol; mix fraction with ax. and eq. enantiomers in molar ratio 81:19: 23 % yield, 1.13 g, 8.95 mmol).

**axial-(4E)-cyclooct-4-en-1-ol (ax.-TCO-OH):**

1H NMR (300 MHz, CDCl$_3$) δ 5.63 – 5.49 (m, 1H), 5.44 – 5.29 (m, 1H), 3.49 – 3.37 (m, 1H), 2.40 – 2.17 (m, 3H), 2.01 – 1.84 (m, 4H), 1.70 – 1.42 (m, 4H).

**equatorial-(4E)-cyclooct-4-en-1-ol (eq.-TCO-OH):**

1H NMR (300 MHz, CDCl$_3$) δ 5.66 – 5.45 (m, 2H), 4.07 – 3.99 (m, 1H), 2.44 – 2.30 (m, 1H), 2.28 – 2.16 (m, 2H), 2.17 – 2.04 (m, 2H), 1.97 – 1.54 (m, 5H).

**axial-(4E)-cyclooct-4-en-1-yl (4-nitrophenyl)carbonate (7a) and equatorial-(4E)-cyclooct-4-en-1-yl (4-nitrophenyl)carbonate (7b)**

Synthesis procedures were performed according to the Liu et al.[2]

**axial-(4E)-cyclooct-4-en-1-yl (4-nitrophenyl)carbonate (7a):** colorless solid, 39 % (652 mg, 2.24 mmol)

1H NMR (300 MHz, CD$_2$D$_6$) δ 7.84 – 7.51 (m, 2H), 6.94 – 6.65 (m, 2H), 5.39 – 5.07 (m, 2H), 4.47 – 4.28 (m, 1H), 2.24 – 1.93 (m, 4H), 1.91 – 1.72 (m, 2H), 1.72 – 1.53 (m, 3H), 1.41 – 1.30 (m, 1H).

**equatorial-(4E)-cyclooct-4-en-1-yl (4-nitrophenyl)carbonate (7b):** colorless solid, 56 % (948 mg, 3.25 mmol)

1H NMR (500 MHz, CD$_2$D$_6$) δ 7.79 – 7.53 (m, 2H), 6.91 – 6.57 (m, 2H), 5.83 – 5.70 (m, 1H), 5.40 – 5.28 (m, 1H), 4.86 – 4.77 (m, 1H), 2.46 – 2.32 (m, 1H), 2.24 – 2.07 (m, 3H), 1.99 – 1.88 (m, 1H), 1.77 – 1.66 (m, 1H), 1.67 – 1.54 (m, 2H), 1.30 – 1.19 (m, 1H), 0.96 – 0.84 (m, 1H).

**axial-(4E)-5-(((cyclooct-4-en-1-yl)-oxy)carbonyl)amino)pentanoic acid (ax.-TCOs) and equatorial-(4E)-5-(((cyclooct-4-en-1-yl-oxy)carbonyl)amino)pentanoic acid (eq.-TCOs)**

Following literature procedures,[3] to 7a or 7b (500 mg, 1.726 mmol, 1.000 eq.) stirring in anhydrous DMF (8.5 mL) was added NE$_3$(0.7 mL, 5 mmol, 3 eq.) and 5-aminopentanoic acid (303 mg, 2.58 mmol, 1.49 eq.). The reaction mixture was kept stirring overnight, diluted with water (10 mL) and acidified with an aqueous AcOH solution to a pH of 3. The mixture was extracted three times with DCM (5 x 5 mL). The combined organic layers were washed with water (10 mL), a saturated aqueous NaCl solution (10 mL), dried over MgSO$_4$, filtered and finally purified over FC (DCM $\rightarrow$ DCM:MeOH 97:3).

**axial-(4E)-5-(((cyclooct-4-en-1-yl)-oxy)carbonyl)amino)pentanoic acid (ax.-TCOs):**

colorless solid, 38 % (176 mg, 0.653 mmol)

1H NMR (500 MHz, CD$_2$OD) δ 5.64 – 5.55 (m, 1H), 5.51 – 5.43 (m, 1H), 4.33 – 4.25 (m, 1H), 3.07 (t, J = 6.8 Hz, 2H), 2.43 – 2.24 (m, 3H), 2.20 (t, J = 7.4 Hz, 2H), 2.02 – 1.84 (m, 4H), 1.77 – 1.66 (m, 2H), 1.64 – 1.54 (m, 3H), 1.53 – 1.45 (m, 2H).

**equatorial-(4E)-5-(((cyclooct-4-en-1-yl)-oxy)carbonyl)amino)pentanoic acid (eq.-TCOs):**

colorless solid, 27 % (126 mg, 0.467 mmol)

1H NMR (500 MHz, CDC$_3$) δ 5.62 – 5.46 (m, 2H), 5.02 – 4.79 (m, 2H), 3.28 – 3.16 (m, 2H), 2.37 (t, J = 7.2 Hz, 2H), 2.34 – 2.19 (m, 4H), 2.18 – 2.07 (m, 1H), 1.87 – 1.48 (m, 8H), 1.26 – 1.14 (m, 1H).

**((Z)-cyclooct-4-en-1-yl (4-nitrophenyl)carbonate (8)**

General synthesis procedure for 4-nitrophenyl carbonates starting from CCO-OH.

Colorless solid, 78 % (90 mg, 0.31 mmol).

1H NMR (300 MHz, CDCl$_3$) δ 8.36 – 8.20 (m, 2H), 7.43 – 7.31 (m, 2H), 5.76 – 5.59 (m, 2H), 4.89 – 4.77 (m, 1H), 2.50 – 2.33 (m, 1H), 2.24 – 1.87 (m, 6H), 1.84 – 1.53 (m, 3H).

13C NMR (75 MHz, CDCl$_3$) δ 155.8, 152.1, 145.4, 130.1, 129.4, 125.4, 121.9, 82.2, 33.8, 33.6, 25.7, 24.9, 22.2.

HR-ESI, calcd. for [C$_{15}$H$_{23}$NO$_4$+Na]$^+$: 292.1519, found: 292.1516.

HR-ESI, calcd. for [C$_{15}$H$_{23}$NO$_4$+Na]$^+$: 314.0999, found: 314.1004.
**Axial-(E)-4-((Cyclooct-2-en-1-yl)oxy)carbonyl)amino)butanoic acid (ax-TCO\textsuperscript{a}-)***

Colorless viscous resin, 53% (23 mg, 0.17 mmol)

\(^{1}\text{H} NMR (500 MHz, CDCl\textsubscript{3}) \delta 8.83 (s, br, 1H), 7.49 – 7.34 (m, 2H), 5.85 (m, 1H), 5.72 – 5.57 (m, 1H), 5.24 – 5.11 (m, 1H), 2.56 – 2.40 (m, 1H), 2.14 – 2.26 (m, 1H), 2.11 – 1.80 (m, 4H), 1.80 – 1.62 (m, 1H), 1.53 – 1.35 (m, 1H), 1.05 – 0.73 (m, 2H). \(^{13}\text{C} NMR (75 MHz, CDCl\textsubscript{3}) \delta 155.4, 156.9, 158.5, 159.2, 160.7, 175.6. HR-ESI, calcld. for [C\textsubscript{14}H\textsubscript{20}N\textsubscript{2}O\textsubscript{4}H\textsubscript{2}]\textsuperscript{+}: 254.1398, found: 254.1401.
**axial-(E)-6-(((Cyclooct-2-en-1-yl)oxycarbonyl)amino)hexanoic acid (ax.-TCO*)**: colorless solid, 60% (43.8 mg, 0.123 mmol)

1H NMR (500 MHz, CDCl3) δ 10.90 (s, br, 1H), 5.79 (dd, J = 15.8, 11.2, 3.7 Hz, 1H), 5.51 (dd, J = 16.4, 2.4 Hz, 1H), 5.31 (s, 1H), 4.80 (t, J = 5.9 Hz, 1H), 3.17 (q, J = 6.7 Hz, 2H), 2.52 – 2.40 (m, 1H), 2.34 (t, J = 7.4 Hz, 2H), 2.15 – 1.90 (m, 3H), 1.91 – 1.78 (m, 1H), 1.73 – 1.57 (m, 4H), 1.58 – 1.30 (m, 5H), 1.12 – 0.98 (m, 1H), 0.87 – 0.69 (m, 1H). 13C NMR (126 MHz, CDCl3) δ 179.2, 156.1, 131.7, 131.7, 74.0, 40.8, 40.8, 36.1, 36.0, 34.0, 29.8, 29.2, 26.3, 24.4, 24.2. HR-ESI, calcd. for [C11H28NO4H]: 282.1711; found: 282.1717.

**axial-(E)-7-(((Cyclooct-2-en-1-yl)oxycarbonyl)heptanoic acid (ax.-TCO*)**: colorless solid, 82% (49.9 mg, 0.168 mmol)

1H NMR (500 MHz, CDCl3) δ 11.26 (s, br, 1H), 5.79 (dd, J = 15.8, 11.2, 3.7 Hz, 1H), 5.51 (dd, J = 16.5, 2.4 Hz, 1H), 5.40 – 5.27 (m, 1H), 4.78 (t, J = 6.0 Hz, 1H), 3.16 (q, J = 6.7 Hz, 2H), 2.55 – 2.39 (m, 1H), 2.33 (t, J = 7.5 Hz, 2H), 2.15 – 1.92 (m, 3H), 1.92 – 1.78 (m, 1H), 1.74 – 1.41 (m, 6H), 1.41 – 1.28 (m, 3H), 1.11 – 0.97 (m, 1H), 0.88 – 0.69 (m, 1H). 13C NMR (126 MHz, CDCl3) δ 179.3, 156.1, 131.7, 131.7, 73.0, 40.9, 40.8, 36.1, 36.0, 34.1, 29.9, 29.2, 28.8, 26.5, 24.7, 24.3. HR-ESI, calcd. for [C11H29NO4H]: 296.1867; found: 296.1876.

**equatorial-(E)-1-(((Cyclooct-2-en-1-yl)oxycarbonyl)amino)pentanoic acid (eq.-TCO*)**

General carbamate synthesis procedure 1 with 9b and 5-a-minopentanoic acid. Elution of the title compound during FC: 10 - 40% EtOAc in DCM + 0.5% AcOH.

Colorless solid, 82% yield (41.1 mg, 0.16 mmol). 1H NMR (500 MHz, CDCl3) δ 5.79 – 5.70 (m, 1H), 5.57 – 5.45 (m, 1H), 5.16 – 5.00 (m, 1H), 4.74 (t, J = 5.2 Hz, 1H), 3.18 (q, J = 6.6 Hz, 2H), 2.44 – 2.39 (m, 1H), 2.37 (t, J = 7.3 Hz, 2H), 2.27 – 2.16 (m, 1H), 2.01 – 1.91 (m, 2H), 1.91 – 1.74 (m, 2H), 1.71 – 1.61 (m, 2H), 1.59 – 1.52 (m, 2H), 1.52 – 1.35 (m, 2H), 0.93 – 0.73 (m, 2H). 13C NMR (126 MHz, CDCl3) δ 178.6, 156.5, 133.2, 132.0, 78.9, 41.2, 40.5, 35.7, 35.6, 33.6, 29.5, 29.2, 27.6, 21.9. HR-ESI, calcd. for [C11H29N2O4H]: 268.1554; found: 268.1555.

**(Z)-Cyclooct-2-ene-1-yl(4-nitrophenyl)carbonate (10)**

General synthesis procedure for 4-nitrophenyl carbonates with CCO*-OH.

Colorless viscous resin, 81% (186 mg, 0.639 mmol). 1H NMR (300 MHz, CDCl3) δ 8.32 – 8.21 (m, 2H), 7.45 – 7.32 (m, 2H), 5.88 – 5.70 (m, 1H), 5.70 – 5.53 (m, 2H), 2.32 – 1.95 (m, 3H), 1.77 – 1.36 (m, 7H). 13C NMR (75 MHz, CDCl3) δ 155.8, 152.0, 145.4, 131.1, 129.2, 125.3, 121.9, 78.4, 34.9, 28.8, 26.5, 25.9, 23.3. HR-ESI, calcd. for [C13H11NO5Na]+: 314.09999; found: 314.09999.

**(Z)-5-(((Cyclooct-2-ene-1-yl)oxycarbonyl)amino)pentanoic acid (CCO*)**

General carbamate synthesis procedure 2 (paragraph Fehler! Verweisquelle konnte nicht gefunden werden.) with 10 and 5-a-minopentanoic acid. Elution of the title compound during FC: 10 - 40% EtOAc in DCM + 0.5% AcOH.

Colorless solid, 84% (44 mg, 0.16 mmol). 1H NMR (500 MHz, CDCl3) δ 5.69 – 5.60 (m, 1H), 5.60 – 5.50 (m, 1H), 5.51 – 5.42 (m, 1H), 4.73 (t, J = 5.2 Hz, 1H), 3.19 (q, J = 6.7 Hz, 2H), 2.37 (t, J = 7.3 Hz, 2H), 2.32 – 2.20 (m, 1H), 2.14 – 2.05 (m, 1H), 1.99 – 1.87 (m, 1H), 1.71 – 1.62 (m, 3H), 1.63 – 1.33 (m, 8H). 13C-NMR (126 MHz, CDCl3) δ 178.7, 156.4, 131.2, 129.6, 72.9, 40.6, 35.5, 33.9, 29.5, 29.0, 26.5, 26.0, 23.5, 21.9. HR-ESI, calcd. for [C16H27NO5Na]: 268.1554; found: 268.1573.
Cycloalkyne substrate candidates (synthesis overview)

Scheme S32. Synthesis of strained cyclooctyne substrates for Lpl<sup>ERTY</sup>. a: 4-Nitrophenyl chloroformate, Py, DCM, RT, 2 h, 38%; b: 1) 5-aminopentanoic acid, DPEA, DMF, RT, o/n, 2) AcOH, 62%; c: 1) Rh(OAc)<sub>2</sub>, DCM, 0 °C, 2) RT, 3 d, 20% (12a) and 37% (12b); d: 1) Dibal-H, Et<sub>2</sub>O, 0 °C, 30 min, 2) RT, o/n, 3) Br<sub>2</sub>, DCM, 5 min, 4) i-Pr<sub>2</sub>NEt, THF, 0 °C, 5) reflux, 1.5 h, 68%; e: 4-nitrophenyl chloroformate, Py, DCM, RT, 1 h, 59%; f: 1) for endo-BCN<sub>b</sub>: 4-aminobutanoic acid, for endo-BCN<sub>c</sub>: 5-aminopentanoic acid, for endo-BCN<sub>d</sub>: 6-aminohexanoic acid, DPEA, DMF, RT, o/n, 2) AcOH, 62% (endo-BCN<sub>b</sub>), 73% (endo-BCN<sub>c</sub>), 53% (endo-BCN<sub>d</sub>).

Cyclooct-2-yn-1-yl-(4-nitrophenyl)carbonate (11)

General synthesis procedure for 4-nitrophenyl carbonates starting from SCO-OH. FC: CH<sub>2</sub>→ CH:EtOAc 39:1.

Colorless viscous liquid, 36% (30 mg, 0.10 mmol). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.30 – 8.23 (m, 2H), 7.43 – 7.36 (m, 2H), 5.36 – 5.29 (m, 1H), 2.38 – 2.28 (m, 1H), 2.29 – 2.15 (m, 3H), 2.01 – 1.89 (m, 2H), 1.89 – 1.74 (m, 2H), 1.68 – 1.59 (m, 2H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 155.6, 151.8, 145.5, 125.4, 121.9, 104.2, 89.3, 72.1, 41.5, 34.2, 29.6, 26.1, 20.8. Ar<sub>t</sub>(CH:EtOAc 3:1) = 0.43.

5-((Cyclooct-2-yn-1-yl)oxy)carbonyl)(amino)pentanoic acid (SCOS)

General carbamate synthesis procedure 1 starting from 11 and 5-aminopentanoic acid. Elution of the title compound during FC: DCM:EtOAc 17.3 + 0.5 % AcOH

Colorless solid, 62% (8.6 mg, 32 μmol). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 5.37 – 5.26 (m, 1H), 4.84 – 4.66 (m, 1H), 3.25 – 3.14 (m, 2H), 2.39 (t, J = 7.2 Hz, 2H), 2.33 – 2.25 (m, 1H), 2.25 – 2.11 (m, 2H), 2.05 – 1.75 (m, 4H), 1.75 – 1.62 (m, 4H), 1.61 – 1.51 (m, 3H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 177.9, 155.7, 101.7, 91.2, 67.2, 42.1, 40.6, 34.4, 33.4, 29.8, 29.4, 26.3, 21.9, 20.9.

HR-ESI, calcd. for [C<sub>26</sub>H<sub>22</sub>N<sub>5</sub>O<sub>2</sub>Na<sup>+</sup>] = [M+Na]<sup>+</sup>: 290.1363, found: 290.1389; calcd. for [C<sub>26</sub>H<sub>22</sub>N<sub>5</sub>O<sub>2</sub>Na<sup>+</sup>] = [2M+Na]<sup>+</sup>: 557.2833, found: 557.2838.

endo-Bicyclononyne substrate candidates (endo-BCN<sub>a</sub>- endo-BCN<sub>c</sub>)

General carbamate synthesis procedure 1 starting from 13. Following linear amino acids were used: endo-BCN<sub>b</sub>: 4-aminobutanoic acid; endo-BCN<sub>c</sub>: 5-aminopentanoic acid; endo-BCN<sub>d</sub>: 6-aminohexanoic acid; 1.5 eq. each. For elution of the target compounds during FC, the following solvent mixture was used: DCM:EtOAc 9:1 + 0.5 % AcOH

4-(((1R,8S,9a)-Bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)(amino)butanoic acid (endo-BCN<sub>b</sub>):

colorless solid, 62% (8.6 mg, 32 μmol)

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 4.93 – 4.72 (m, 1H), 4.15 (d, J = 8.1 Hz, 2H), 3.35 – 3.18 (m, 2H), 2.41 (t, J = 7.1 Hz, 2H), 2.36 – 2.14 (m, 6H), 1.90 – 1.79 (m, 2H), 1.66 – 1.51 (m, 2H), 1.39 – 1.29 (m, 1H), 1.01 – 0.89 (m, 2H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 177.5, 151.7, 99.0, 63.1, 40.3, 31.2, 29.2, 25.3, 21.6, 20.3, 17.9. HR-ESI, calcd. for [C<sub>31</sub>H<sub>28</sub>N<sub>5</sub>O<sub>2</sub>Na<sup>+</sup>] 302.1363, found: 302.1359.

5-(((1R,8S,9a)-Bicyclo[6.1.0]non-4-yn-9-yl)hydroxy)carbonyl)(amino)pentanoic acid (endo-BCN<sub>c</sub>):

colorless solid, 73% (24.5 mg, 83.5 μmol)

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 4.92 – 4.65 (m, 1H), 4.14 (d, J = 8.2 Hz, 2H), 3.27 – 3.12 (m, 2H), 2.38 (t, J = 7.2 Hz, 2H), 2.34 – 2.13 (m, 6H), 1.74 – 1.49 (m, 6H), 1.43 – 1.27 (m, 1H), 1.06 – 0.83 (m, 2H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 178.4, 157.0, 99.0, 62.9, 40.7, 33.6, 29.5, 29.2, 21.9, 21.6, 20.3, 17.9. HR-ESI, calcd. for [C<sub>36</sub>H<sub>31</sub>N<sub>5</sub>O<sub>4</sub>Na<sup>+</sup>] 316.1519, found: 316.1526.
6-(((1R,8S,9a)-Bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)-hexanoic acid (endo-BCN):

colorless solid, 53 % (12 mg, 39 µmol)

1H NMR (300 MHz, CDCl₃) δ 4.87 – 4.50 (m, 1H), 4.15 (d, J = 8.2 Hz, 2H), 3.25 – 3.10 (m, 2H), 2.36 (t, J = 7.3 Hz, 2H), 2.35 – 2.12 (m, 6H), 1.72 – 1.47 (m, 6H), 1.45 – 1.30 (m, 3H), 1.01 – 0.88 (m, 2H). 13C NMR (75 MHz, CDCl₃) δ 177.9, 157.1, 99.0, 62.9, 40.9, 33.7, 29.8, 29.2, 26.3, 24.4, 21.6, 20.3, 17.9. HR-ESI, calcd for [C₁₁H₁₆NO₄Na⁺]: 330.1676, found: 330.1686.

Methyltetrazinylalkanoic acids (synthesis overview)

Scheme S33. Synthesis of methyltetrazinylalkanoic acid substrate candidates for LptATSTR, a: tert-Butyl 2,2,2-trichloroacetimidate, BF₃·OEt₂, CH, RT, o/n, for 24a, n = 5: 47 %, for 24b, n = 6: 37 %, for 24c, n = 7: 63 %, for 24d, n = 8: 47 %, 24e, n = 9: 53 %; b: NaCN, DMF, RT, o/n, for 25a, n = 5: 80 %, for 25b, n = 6: 73 %, for 25c, n = 7: 81 %, for 25d, n = 8: 78 %, 25e, n = 9: 86 %; c: 1) MeCN, Na₂H₅O₂, Zn(OH)₂, 60 °C, o/n, 2) NaNO₃, HCl(aq), 0.5 % (Me₂ZAl), 5 % (Me₂ZAl), 1 % (Me₂ZAl), 6 % (Me₂ZAl), 2 % (Me₂ZAl).

Bromoalkanoic acid tert-butyl esters 12a-12f

6-Bromohexanoic acid (1.00 g, 5.13 mmol, 1.00 eq.), 7-Bromoheptanoic acid (856 mg, 4.09 mmol, 1.00 eq.), 8-Bromocinanoic acid (745 mg, 3.34 mmol, 1.00 eq.), 9-Bromononanoic acid (651 mg, 2.75 mmol, 1.00 eq.) or 10-Bromodecanoic acid (1.01 g, 4.02 mmol, 1.00 eq.) was added to a solution of tert-Butyl 2,2,2-trichloroacetimidate (1.11 eq.) in CH (−0.5 M solution). BF₃·Et₂O (5 mol-%) was added dropwise during which a colorless precipitate formed. The reaction mixture was stirred overnight, diluted with CH (25 mL) and filtered into a separatory funnel. The organic layer was washed three times with an aqueous NaCl solution (5 % w/v, 3 × 10 mL), once with a saturated aqueous NaCl solution (10 mL) and dried over MgSO₄. After filtration, the solvent was removed in vacuo to obtain the desired tert-butyl ester. The alkaline aqueous layer was acidified with concentrated hydrochloric acid to pH 1 and extracted three times with CH. The organic layer was washed with a saturated aqueous NaCl solution and dried over MgSO₄. After filtration, the solvent was removed in vacuo and residual starting material was recovered.

6-Bromohexanoic acid tert-butyl ester (12a):
colorless liquid, 47 % (610 mg, 2.43 mmol), recovery of 17 % starting material (166 mg, 0.851 mmol)

1H NMR (300 MHz, CDCl₃) δ 3.40 (t, J = 6.8 Hz, 2H), 2.22 (t, J = 7.3 Hz, 2H), 1.95 – 1.80 (m, 2H), 1.67 – 1.55 (m, 2H), 1.53 – 1.37 (m, 11H). 13C NMR (75 MHz, CDCl₃) δ 173.0, 80.3, 35.5, 33.7, 32.6, 28.3, 27.8, 24.4. HR-ESI, calcd. for [C₁₁H₁₆BrO₂Na⁺]: 273.0461, found: 273.0465.

7-Bromoheptanoic acid tert-butyl ester (12b):
colorless liquid, 37 % (403 mg, 1.52 mmol), recovery of 36 % starting material (307 mg, 1.47 mmol)

1H NMR (300 MHz, CDCl₃) δ 3.40 (t, J = 6.8 Hz, 2H), 2.21 (t, J = 7.4 Hz, 2H), 1.92 – 1.80 (m, 2H), 1.63 – 1.56 (m, 2H), 1.51 – 1.40 (m, 11H), 1.40 – 1.28 (m, 2H). 13C NMR (75 MHz, CDCl₃) δ 173.2, 80.2, 35.6, 34.0, 32.7, 28.3, 28.0, 25.0. HR-ESI, calcd. for [C₁₂H₁₄BrO₂Na⁺]: 287.0617, found: 287.0596.

8-Bromocinanoic acid tert-butyl ester (12c):
colorless liquid, 63 % (585 mg, 2.10 mmol), recovery of 21 % starting material (127 mg, 0.596 mmol)

1H NMR (300 MHz, CDCl₃) δ 3.40 (t, J = 6.8 Hz, 2H), 2.20 (t, J = 7.4 Hz, 2H), 1.96 – 1.76 (m, 2H), 1.57 (q, J = 7.0 Hz, 2H), 1.49 – 1.38 (m, 11H), 1.38 – 1.24 (m, 4H). 13C NMR (75 MHz, CDCl₃) δ 173.3, 80.1, 35.7, 34.1, 32.9, 29.0, 28.6, 28.3, 28.1, 25.1. HR-ESI, calcd. for [C₁₂H₁₂BrO₂Na⁺]: 301.0774, found: 301.0776.

9-Bromononanoic acid tert-butyl ester (12d):
colorless liquid, 47 % (380 mg, 1.30 mmol), recovery of 30 % starting material (196 mg, 0.827 mmol)

1H NMR (300 MHz, CDCl₃) δ 3.50 – 3.33 (m, 2H), 2.20 (t, J = 7.5 Hz, 2H), 1.94 – 1.75 (m, 2H), 1.64 – 1.49 (m, 2H), 1.49 – 1.35 (m, 11H), 1.35 – 1.25 (m, 6H). 13C NMR (75 MHz, CDCl₃) δ 173.4, 80.1, 35.7, 34.1, 32.9, 29.2, 29.1, 28.7, 28.3, 28.2, 25.2.
10-Bromodecanoic acid tert-butyl ester (12e):
colorless liquid, 53 % (648 mg, 2.11 mmol), recovery of 44 % starting material (301 mg, 1.20 mmol)

^1^H NMR (500 MHz, CDCl\textsubscript{3}) \( \delta \) 3.40 (t, \( J = 6.8 \) Hz, 2H), 2.20 (t, \( J = 7.5 \) Hz, 2H), 1.89 – 1.80 (m, 2H), 1.60 – 1.53 (m, 2H), 1.52 – 1.36 (m, 11H), 1.35 – 1.23 (m, 8H). ^1^C NMR (126 MHz, CDCl\textsubscript{3}) \( \delta \) 173.4, 80.1, 35.7, 34.1, 33.0, 29.4, 29.3, 29.2, 28.9, 28.3, 25.2. HR-ESI, calcd. for [\( \text{C}_{14}\text{H}_{27}\text{BrO}_{2}+\text{Na} \]^+: 329.087, found: 329.1096.

Cyanoaalkanoic acid tert-butyl esters 13a-13e

6-Cyanohexanoic acid tert-butyl ester (13a):
colorless liquid, 80 % (376 mg, 1.91 mmol)

^1^H NMR (300 MHz, CDCl\textsubscript{3}) \( \delta \) 2.34 (t, \( J = 7.1 \) Hz, 2H), 2.23 (t, \( J = 7.2 \) Hz, 2H), 1.74 – 1.56 (m, 4H), 1.56 – 1.32 (m, 11H). ^1^C NMR (75 MHz, CDCl\textsubscript{3}) \( \delta \) 172.8, 119.7, 80.4, 35.2, 28.2, 28.2, 25.3, 24.3, 17.2. HR-ESI, calcd. for [\( \text{C}_{15}\text{H}_{20}\text{NO}_{2}+\text{Na} \]^+: 220.1308, found: 220.1322. \( R_f \) (CH\textsubscript{3}EtOAc 9:1) = 0.09.

7-Cyanoheptanoic acid tert-butyl ester (13b):
colorless liquid, 73 % (235 mg, 1.11 mmol)

^1^H NMR (300 MHz, CDCl\textsubscript{3}) \( \delta \) 2.33 (t, \( J = 7.1 \) Hz, 2H), 2.21 (t, \( J = 7.4 \) Hz, 2H), 1.72 – 1.53 (m, 4H), 1.44 (s, 13H). ^1^C NMR (75 MHz, CDCl\textsubscript{3}) \( \delta \) 173.1, 119.8, 80.3, 35.5, 28.5, 28.3, 25.3, 24.8, 17.2. HR-ESI, calcd. for [\( \text{C}_{16}\text{H}_{22}\text{NO}_{2}+\text{Na} \]^+: 234.1465, found: 234.1470. \( R_f \) (CH\textsubscript{3}EtOAc 9:1) = 0.10.

8-Cyanoctanoic acid tert-butyl ester (13c):
colorless liquid, 81 % (383 mg, 1.70 mmol)

^1^H-NMR (300 MHz, CDCl\textsubscript{3}) \( \delta \) 2.33 (t, \( J = 7.1 \) Hz, 2H), 2.20 (t, \( J = 7.4 \) Hz, 2H), 1.72 – 1.52 (m, 4H), 1.51 – 1.39 (m, 11H), 1.39 – 1.27 (m, 4H). ^1^C-NMR (75 MHz, CDCl\textsubscript{3}) \( \delta \) 173.2, 119.9, 80.1, 35.6, 34.1, 33.0, 29.4, 29.3, 29.2, 28.9, 28.3, 25.3, 24.3, 17.3. HR-ESI, calcd. for [\( \text{C}_{17}\text{H}_{24}\text{NO}_{2}+\text{Na} \]^+: 248.1621, found: 248.1623. \( R_f \) (CH\textsubscript{3}EtOAc 9:1) = 0.12.

9-Cyannonanoic acid tert-butyl ester (13d):
colorless liquid, 78 % (242 mg, 1.01 mmol).

^1^H-NMR (300 MHz, CDCl\textsubscript{3}) \( \delta \) 2.32 (t, \( J = 7.1 \) Hz, 2H), 2.19 (t, \( J = 7.4 \) Hz, 2H), 1.72 – 1.50 (m, 4H), 1.50 – 1.39 (m, 11H), 1.39 – 1.22 (m, 6H). ^1^C-NMR (75 MHz, CDCl\textsubscript{3}) \( \delta \) 173.3, 119.9, 80.1, 35.7, 29.1, 29.1, 28.7, 28.7, 28.3, 25.5, 25.1, 17.3. HR-ESI, calcd. for [\( \text{C}_{18}\text{H}_{26}\text{NO}_{2}+\text{Na} \]^+: 262.1778, found: 262.1785. \( R_f \) (CH\textsubscript{3}EtOAc 9:1) = 0.13.

10-Cyanoodecanoic acid tert-butyl ester (13e):
colorless liquid, 86 % (450 mg, 1.78 mmol).

^1^H NMR (300 MHz, CDCl\textsubscript{3}) \( \delta \) 2.33 (t, \( J = 7.1 \) Hz, 2H), 2.19 (t, \( J = 7.5 \) Hz, 2H), 1.72 – 1.53 (m, 4H), 1.50 – 1.38 (m, 11H), 1.37 – 1.23 (m, 8H). ^1^C NMR (75 MHz, CDCl\textsubscript{3}) \( \delta \) 173.4, 120.0, 80.1, 35.7, 29.3, 29.3, 29.1, 28.8, 28.8, 28.3, 25.5, 25.2, 17.3. HR-ESI, calcd. for [\( \text{C}_{19}\text{H}_{28}\text{NO}_{2}+\text{Na} \]^+: 276.1934, found: 276.1930. \( R_f \) (CH\textsubscript{3}EtOAc 9:1) = 0.14.

Methyltetrazinylalkanoic acids MeTzAl\textsubscript{3}-MeTzAl\textsubscript{6}

Tetrazine synthesis was performed according to Yang et al.\textsuperscript{[16]}

\[
\text{MeTzAl}\textsubscript{n} \quad n = 5 \quad \text{MeTzAl}\textsubscript{n} \quad n = 6 \quad \text{MeTzAl}\textsubscript{n} \quad n = 7 \quad \text{MeTzAl}\textsubscript{n} \quad n = 8 \quad \text{MeTzAl}\textsubscript{n} \quad n = 9
\]
6-(Methyltetrazinyl)hexanoic acid (MeTzAl₆):
magenta-colored solid, 0.5% (3.1 mg, 15 µmol)

1H NMR (500 MHz, CDC₁₃): δ 3.03 (t, J = 7.7 Hz, 2H), 3.04 (s, 3H), 2.36 (t, J = 7.4 Hz, 2H), 2.04 – 1.92 (m, 2H), 1.76 – 1.69 (m, 2H), 1.57 – 1.46 (m, 2H). 13C NMR (126 MHz, CDCl₃): δ 178.0, 170.0, 167.6, 34.6, 33.6, 28.6, 28.0, 24.4, 21.2. HR-ESI, calcd. for [C₈H₁₅N₄O₂+Na⁺]: 233.1009, found: 233.1010.

7-(Methyltetrazinyl)heptanoic acid (MeTzAl₇):
magenta-colored solid, 5% (45.5 mg, 203 µmol)

1H NMR (300 MHz, CDC₁₃): δ 3.28 (t, J = 7.7 Hz, 2H), 3.04 (s, 3H), 2.36 (t, J = 7.4 Hz, 2H), 2.05 – 1.86 (m, 2H), 1.78 – 1.59 (m, 2H), 1.52 – 1.38 (m, 4H). 13C NMR (75 MHz, CDCl₃): δ 178.5, 170.1, 167.5, 34.7, 33.8, 28.9, 28.8, 28.2, 24.6, 21.2. HR-ESI, calcd. for [C₉H₁₆N₄O₂+Na⁺]: 247.1165, found: 247.1168.

8-(Methyltetrazinyl)octanoic acid (MeTzAl₈):
magenta-colored solid, 1% (5.7 mg, 24 µmol)

1H NMR (500 MHz, CDC₁₃): δ 3.28 (t, J = 7.7 Hz, 2H), 3.04 (s, 3H), 2.35 (t, J = 7.5 Hz, 2H), 2.00 – 1.85 (m, 2H), 1.68 – 1.60 (m, 2H), 1.46 – 1.32 (m, 6H). 13C NMR (126 MHz, CDCl₃): δ 178.3, 170.2, 167.5, 34.8, 33.8, 29.0, 29.0, 24.7, 21.2. HR-ESI, calcd. for [C₁₀H₂₀N₄O₂+Na⁺]: 261.1322, found: 261.1324.

9-(Methyltetrazinyl)nonanoic acid (MeTzAl₉):
magenta-colored solid, 6% (15.0 mg, 59.5 µmol)

1H NMR (300 MHz, CDC₁₃): δ 3.28 (t, J = 7.7 Hz, 2H), 3.04 (s, 3H), 2.35 (t, J = 7.5 Hz, 2H), 2.00 – 1.85 (m, 2H), 1.70 – 1.57 (m, 2H), 1.51 – 1.27 (m, 8H). 13C NMR (75 MHz, CDCl₃): δ 178.7, 170.3, 167.5, 34.8, 33.9, 29.2, 29.2, 29.1, 28.4, 24.8, 21.2. HR-ESI, calcd. for [C₁₁H₂₂N₄O₂+Na⁺]: 275.1478, found: 275.1475.

10-(Methyltetrazinyl)decanoic acid (MeTzAl₁₀):
magenta-colored solid, 2% (8.2 mg, 31 µmol)

1H NMR (500 MHz, CDC₁₃): δ 3.28 (t, J = 7.7 Hz, 2H), 3.03 (s, 3H), 2.34 (t, J = 7.5 Hz, 2H), 2.00 – 1.87 (m, 2H), 1.66 – 1.58 (m, 2H), 1.49 – 1.22 (m, 10H). 13C NMR (126 MHz, CDCl₃): δ 178.9, 170.3, 167.5, 34.8, 34.0, 29.3, 29.3, 29.3, 29.2, 29.1, 28.4, 24.8, 21.2. HR-ESI, calcd. for [C₁₂H₂₄N₄O₂+Na⁺]: 289.1635, found: 289.1634.

MeTzBnNH-TAMRA

5-TAMRA (50 mg, 0.12 mmol, 1.0 eq.) was suspended in DMF (2 mL). DIPEA (101 µL, 0.58 mmol, 4.8 eq.) and HATU (44 mg, 0.12 mmol, 1.0 eq.) were added and stirred for 10 min. MeTzBnNH·HCl (33 mg, 0.14 mmol, 1.2 eq.) was added and the reaction mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure and the residue was taken up in DCM (20 mL). The organic layer was washed three times with an aqueous LiCl solution (5% w/v, 3 × 10 mL), three times with a saturated aqueous NH₄Cl solution (3 × 5 mL), once with a saturated aqueous NaCl solution (4 mL) and dried over MgSO₄. After filtration and removal of the solvent in vacuo, the residue was purified with FC (CHCl₃:MeOH:NEt₃ 99:1 → CHCl₃:MeOH:NEt₃ 94:5:1) and with preparative RP-HPLC (9:1 H₂O:MeCN + 1% TFA → 1:9 H₂O:MeCN + 1% TFA). After lyophilization, the target compound was obtained as a dark violet solid with golden shine in 8% yield (6.0 mg, 9.8 µmol, TFA salt).

1H NMR (500 MHz, CD₂OD): δ 8.84 (d, J = 1.8 Hz, 1H), 8.62 – 8.49 (m, 2H), 8.32 (dd, J = 7.9, 1.8 Hz, 1H), 7.68 (d, J = 8.3 Hz, 2H), 7.55 (d, J = 7.9 Hz, 1H), 7.17 (d, J = 9.5 Hz, 2H), 7.06 (dd, J = 9.5, 2.5 Hz, 2H), 6.99 (d, J = 2.4 Hz, 2H), 4.79 (s, 2H), 3.31 (s, 12H), 3.04 (s, 3H). 13C NMR (126 MHz, CD₂OD): δ 168.8, 168.3, 167.7, 165.2, 160.8, 159.1, 159.0, 144.9, 138.2, 137.4, 133.2, 132.5, 132.2, 132.0, 131.3, 129.4, 129.1, 115.5, 114.7, 97.4, 44.5, 40.9, 21.1. HR-ESI, calcd. for [C₂₀H₁₉N₂O₅+H⁺]: 614.2510, found: 614.2531.
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Appendix I: $^1$H and $^{13}$C NMR spectra

5-(((2,3-Dimethylcycloprop-2-en-1-yl)methoxy)carbonyl)amino)pentanoic acid (DMCyb)

$^1$H NMR:

APT NMR ($^{13}$C):

APT NMR ($^{13}$C):
5-(((2-Methylcycloprop-2-en-1-yl)methoxy)carbonyl)amino)pentanoic acid (MMCy₆)

¹H NMR:

APT NMR (¹³C):

APT NMR (¹³C):
equatorial-(4E)-5-(((cyclooct-4-en-1-yloxy)carbonyl)amino)pentanoic acid (eq.-TCO₅)

¹H NMR:

APT NMR (¹³C):
(Z)-5-(((cyclooct-4-en-1-yloxy)carbonyl)amino)pentanoic acid (CCO₅)

$^1$H NMR:

APT NMR ($^{13}$C):

APT NMR ($^{13}$C):
equatorial-(2E)-5-(((Cyclooct-2-en-1-yloxy)carbonyl)amino)pentanoic acid (eq.-TCO's)

$^1$H NMR:

APT NMR ($^{13}$C):

$^{13}$C NMR (APT):
(Z)-Cyclooct-2-en-1-yl(4-nitrophenyl)carbonate (10)

$^1H$ NMR:

APT NMR ($^{13}C$):

![APT NMR spectrum](image1)

![APT NMR spectrum](image2)
(Z)-5-(((Cyclooct-2-en-1-yloxy)carbonyl)amino)pentanoic acid (CCO*)

$^1$H NMR:

APT NMR ($^{13}$C):

****
5-((Cyclooct-2-yn-1-yloxy)carbonyl)amino)pentanoic acid (SCO₅)

**¹H NMR:**

![NMR Spectrum](image)

**APT NMR (**¹³C**):**

![APT NMR Spectrum](image)
5-(((1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)-pentanoic acid (endo-BCN₉)

¹H NMR:

APT NMR (¹³C):
7-Cyanoheptanoic acid tert-butyl ester (13b)

$^1$H NMR:

APT NMR ($^{13}$C):

...
7-(Methyltetrazinyl)heptanoic acid (MeTzAl₈)

$^1$H NMR:

APT NMR ($^{13}$C):

[Chemical structures and NMR spectra images]
MeTzBnNH-TAMRA

$^1$H NMR:

APT NMR ($^{13}$C NMR):

Solvent signal (quintet) is superimposed by tetramethyl signal
Appendix II: DNA sequences

Sequence for ds(mRuby3-LAP):
TAGGTCTCAGTCAGCAAAGGCGAGGAGCTTATCAAGGAAAATATGCGCATGAAGGTTGTTATGGAGGGGTCCGTAAATGGGACACCAATTCAAATGTACGGGGGAAGGTGAAGGCCGTCCTTACGAGGGGGTACAAACCATGCGCATTAAAGTAATCGAGGGTGAGACCATTGCCCTTCGCCTTCGACATTCTGGCTACATCATTTATGTACGGCTCACGTACCTTCATTAAGTATCCGGCAGATATTCCTGACTTCTTCAAACAAAGCTTTCCAGAAGGTTTTACTTGGGAGCGTGTGACACGTTATGAAGATGGTGGAGTAGTCACTGTGACTCAAGACACCAGCTTAGAAGATGGTGAAATTGGTCTATAACGTGAAGGTGCGCGGTGTCAATTTTCCCAGCAACGGCCCGGTAATGCAGAAGAGACGAGGAGTGGAACCTAACACGGAGATGATGTATCCTGCTGACGGCGGCTTACGCGGTATACAGATATTGCTTTGAAGGTGGATGGTGGCGGCCATCTGCATTGCAATTTTGTTACAACCTATCAGTAAGAAGACA

Note: codon optimized for E. coli K12

Appendix III: Protein sequences

Wildtype LplA, LplA\textsuperscript{W37V} and LplA\textsuperscript{W37V/C85A}

MKHHHHHHHMSLRLSIDSYPDWFNFLAVEECIFSRPMTATQRVFLWNADTVIGRAQRPWKECNTRRMEDNRVLARRSGGGAGFHDNLGNETFMAGKPEYKTISITVSNLNAVGSAEASGRNDDLWVKTVEGDRKVSANYRETRKDRKGHGHTLLNNADLSRLANYLPDKKLAAKIGITSVRSVRTNLTELOPGITHEQCEAIETAAFFAHYGERVVEAIEISPNKTDPDLNPFAETFARQSSWEWFQPAPAFLSHLDERFTWGVGELHFDVEKUGHTRAVQFTDSNLPAPEALAGRLQGLYRMLQCEALLVDPEPEKEKELRELKSAWMAGAVR*

Note: LplA coding sequence

Trp37 (processed full-length chain) substituted by Val for LplA\textsuperscript{W37V} and LplA\textsuperscript{W37V/C85A}

Cys85 (processed full-length chain) substituted by Ala for LplA\textsuperscript{W37V/C85A}

EGFP\textsuperscript{Q157::LAP}

GSSHHHHHHSSTKGEELFTGVVPILELDVDGNGHFSVREGECEGDATNKLTLKFICTTGKLPW PTLVTTLYGVCFSRYPDHKQHDFKSAMPEGVRQERTISFKDDGTYKTRAEVKFEVDTLVNIELKGIDFKEDGNLHGKLEYNFSNHSNVTIDAKCGFEIDKVWYDLDAKNGIKAFKIRHNVEDGVSQVLADYQQNTPIGDPGVLPDNHLYSTQSKLSDPNKEREKRDHMLVLEFVTAAGITLGMDELYK*

Note: EGFP coding sequence, Cln157, LAP

EGFP\textsuperscript{Q157::LAP}

GSSHHHHHHSSTKGEELFTGVVPILELDVDGNGHFSVREGECEGDATNKLTLKFICTTGKLPW PTLVTTLYGVCFSRYPDHKQHDFKSAMPEGVRQERTISFKDDGTYKTRAEVKFEVDTLVNIELKGIDFKEDGNLHGKLEYNFSNHSNVTIDAKCGFEIDKVWYDLDAKNGIKAFKIRHNVEDGVSQVLADYQQNTPIGDPGVLPDNHLYSTQSKLSDPNKEREKRDHMLVLEFVTAAGITLGMDELYK*

Note: EGFP coding sequence, Cln172, LAP
MBP-LAP
MKSSHHHHHGSSMKIEEGKLVIWINGDKYNGLAEVGKFEKDTGKVTEHPDKLEEKFPQVAATGDPDIIFWANTDFGGYAGQSLAEITPDFQDQLKLPFTWDAVRYNGKLIAYPIAVEALSILYNYKDLLPnPPTWEPIALDKELKAKGKSALMFNLQEPYFTWPLIAADGTYAFYENGFYDIDKVDVGVDNAGAKAGLTFLVDLIKNKHMMADTYDIAEAFNKGGETANTINGPWAWSNIIDTSKVNYGVTVLPTFKGQPSKPFGVGLSAGINAAASPNEKELAKEFLENYLTDLEGHEAVNKDKPLGAVALKSYEEELAKDPIAATMENAOKGEIMPNIPOMSAFHYAWRTAVIAASGRQTVDALKDAQTNGIEENLYFQSNAGGGGS6FEIDKVWYDLAGGGGSYPYDVPDYA*

Note: Maltose binding-protein (MBP) coding sequence, LAP, HA

mRuby3-LAP
GSSHHHHHSHSGVSKGEELIKENRMKVMGSSGVNGHQKCTGEGGEPYEGVQTMRIKVIIEGGPLPAFDILATSFGMSTFRTFIPADIPDFKQSFPEGTWERVTRYEDGGVVTQDTSLEDGELVYNYKVRGVNFPSNPVMKKTGQPTNMPSADGGLRGYTDIALKVDGGHLHCPFVNTYRSTKTVGNIKMPGVHAVDHRLEIESDNETYVQQREVAVAKYSLNGLGMDLYKGSGFEIDKVWYDLAGGSYPYDVPDYA*

Note: mRuby3 coding sequence, LAP, HA
