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Reflection on “Site-specific PEGylation of proteins by a Staudinger-phosphite reaction”: from protein modification to ADCs in the clinic

Karl T. Schuppe^{ab} and Christian P. R. Hackenberger^{*ab}

Chemoselective or bioorthogonal modification reactions resulted in several breakthrough studies that enabled the incorporation of functional modules into proteins and antibodies for basic and translational research. In 2010, we published a paper in *Chemical Science*, which described a chemoselective method for synthesizing branched PEGylated peptides and proteins using the Staudinger-phosphite reaction (R. Serwa, P. Majkut, B. Horstmann, J. M. Swiecicki, M. Gerrits, E. Krause and C. P. R. Hackenberger, *Chem. Sci.*, 2010, 1, 596–602, <https://doi.org/10.1039/C0SC00324G>). We discuss subsequent studies in using the protocol for the intracellular stabilization of peptides and the development of the P5-labeling platform, which we currently use in the generation of antibody–drug–conjugates (ADCs) as next-generation biopharmaceuticals in clinical studies, for which a first proof-of-concept study was also published in *Chemical Science* (P. Ochtrup, J. Jahzerah, P. Machui, I. Mai, D. Schumacher, J. Helma, M. A. Kasper and C. P. R. Hackenberger, *Chem. Sci.*, 2023, 14, 2259–2266, <https://doi.org/10.1039/D2SC05678J>).

Polyethylene glycol (PEG) has long been valued in chemical biology and pharmaceutical chemistry for its capacity to enhance the solubility, stability, and pharmacokinetic properties of biomolecules.¹ PEGylation has therefore become a widely used strategy in the development of therapeutic agents,² especially in the context of protein bioconjugation.³

Shortly after our first report of using the Staudinger-phosphite reaction⁴ for the chemoselective modification of proteins,^{5,6} our group used this strategy for PEGylating azide-containing peptides and proteins. In this transformation, published in *Chemical Science* in 2010, a phosphite carrying three PEG-moieties reacts with an azide-bearing peptide to form a phosphoramidate linkage (Fig. 1).⁷ The resulting phosphorus center bears two linear PEG chains and thus acts as a branching point, enabling branched

PEG architectures that can provide greater stabilization than linear polymer chains through the so-called “umbrella effect”.^{3,7} Importantly, the transformation proceeds efficiently under biologically relevant conditions, including physiological pH and even in crowded environments such as crude cell lysates. To further expand the method, we also introduced a photolabile nitrobenzyl-derivative between the phosphorus center and the PEG chain, enabling light-triggered de-PEGylation.⁷

After establishing the reliability and chemoselectivity of this PEGylation strategy, we examined its biological impact. Apoptosis-inducing BH3 peptides modified with solubilizing PEG chains using this approach showed enhanced hydrophilicity, increased half-life, and improved apoptotic activity in Jurkat cells, along with a homogeneous cytoplasmic distribution. These findings highlighted the advantages of employing phosphoramidate-PEG-linkages in the generation of bioactive peptides with pharmacological potential.⁸

In the years that followed, we broadened the scope of phosphorus-based

conjugation reactions,⁹ in particular the Staudinger-phosphite reaction using phosphonites instead of phosphites in the reaction with azides.¹⁰ Furthermore, we developed reagents by integrating unsaturated alkynes and alkenes into their design.^{11,12} This strategic expansion enabled us to use the Staudinger-phosphonite reaction to generate a new class of conjugation handles with high selectivity for native cysteine residues.¹³ These advances ultimately laid the groundwork for what became the P5-labeling platform, a powerful tool for the conjugation and modification of biomolecules and a more stable alternative to the widely used maleimide strategy (Fig. 2).^{14–17} This is particularly relevant in the field of antibody–drug conjugates (ADCs), where maleimide-based conjugation is commonly employed despite several known limitations such as thiol exchange, retro-Michael instability, payload loss in circulation, and associated off-target toxicity.¹³ The strategy to introduce PEG into biomolecules therefore evolved alongside our advances in phosphorus chemistry. By using P5 conjugation handles, PEG could be

^aLeibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Department Chemical Biology, Robert-Rössle-Straße 10, 13125 Berlin, Germany. E-mail: hackenbe@fmp-berlin.de

^bHumboldt-Universität zu Berlin, Department of Chemistry, Brook-Taylor-Straße 2, 12489 Berlin, Germany





Fig. 1 PEGylation of azide-containing proteins via the Staudinger-phosphite reaction, forming a phosphoramidate linkage using a PEG-substituted phosphite.⁷

incorporated as an alkoxy residue, allowing its addition simultaneously with the functional conjugation of a biomolecule.¹⁴

Antibody–drug conjugates (ADCs), inspired by Paul Ehrlich's vision of “magic bullets” for chemotherapy, represent a rapidly growing class of targeted cancer therapies.^{18,19} As the name suggests, ADCs consist of a potent cytotoxic payload that is bound *via* a linker to a monoclonal antibody that specifically targets antigens on the surface of cancer cells. These biopharmaceuticals aim to combine the advantages of both components; the precise specificity of these protein molecules with the cytotoxic potency of chemotherapy. The field has become increasingly dynamic, with 15 ADCs now approved by the FDA,

including three blockbuster drugs: Kadcyla (Roche), Adcetris (Seagen), and Enhertu (Daiichi Sankyo).²⁰ Today, ADCs are no longer merely a conceptual postulate but have evolved into precision-guided chemotherapeutics that combine extreme potency with molecular accuracy. They offer an expanded therapeutic window, the potential to overcome resistance mechanisms, and a modular platform that can be rationally tailored to different tumor types.²¹

The combination of a water-soluble antibody with hydrophobic cytotoxic small molecules is an inherent problem that leads to aggregation, precipitation, off-target effects, and accelerated clearance from systemic circulation. To counterbalance the hydrophobicity of the payload, PEG chains can be built into the

construct to improve its physicochemical properties. This also opens the possibility to attach more payload molecules to the antibody, increasing the conjugate's potency.¹⁹ However, when PEG chains are used as a linear spacer between the antibody and payload, they can increase unwanted solvent exposure, promoting nonspecific hydrophobic interactions.²²

In our 2023 publication, also published in *Chemical Science*, we directly incorporated a PEG sidechain into our P5-labeling reagent, combining our previous PEGylation strategy with the efficiency and stability of the P5-labeling platform for cysteine-selective bioconjugation of antibodies (Fig. 3).²³ We used this approach to conjugate brentuximab with MMAE *via* a valine–citru-line *p*-aminobenzylcarbamate (VC-PAB)

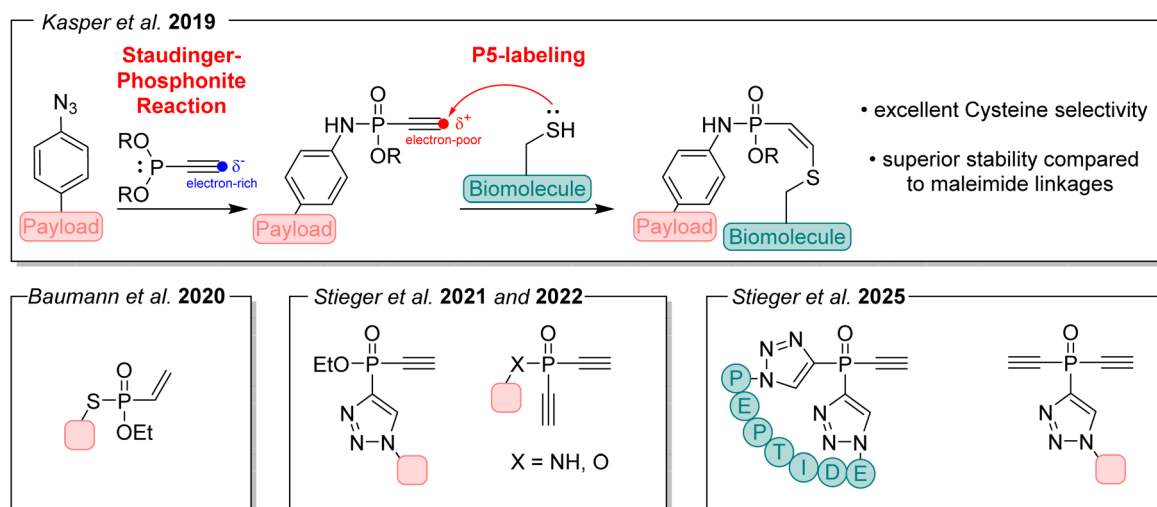


Fig. 2 Synthesis of a P5-electrophile (ethynyl-phosphonamidate) followed by cysteine-selective conjugation. Various P5 reagents developed subsequently are shown underneath.^{8,14–17}



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