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Site-selective three-component reaction for dual-functionalization of peptides†

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A site-selective dual-functionalization of peptides is presented, involving readily available maleimides as well as *N*-hydroxylamines. The modification proceeds through a three component 1,3-dipolar cycloaddition, forming a stable product. This was exemplified by the one-pot attachment of two molecular imaging moieties to a tumor binding cyclic peptide, and was extended to the conjugation of a DOTA chelator to a 12 kDa protein.

Site-selective strategies for chemical modification of peptides and proteins are essential tools for many areas of research and for the development of biopharmaceuticals, yet they remain a chemical challenge due to the multiplicity of functional groups present and the requirement for mild, aqueous reaction conditions. Several innovative methods have been developed to address these challenges,[1] either targeting specific low-abundance surface accessible amino acids or through N-terminal modifications.[2] Cycloaddition reactions in particular have proven versatile for site-selective modifications, due to their practical simplicity and general orthogonality to functional groups found in most biomolecules, exemplified by their use in Huisgen alkyne–azide[3] and Diels–Alder based[4] modification methods. Other recently reported cycloadditions include the strain promoted alkyne-nitrone 1,3-dipolar cycloaddition (SPANC),[5] as well as alkyne-diazocarbonyl (SPADC) and -nitrile oxide (SPANOC) cycloadditions,[6] providing important additional tools for chemoselective reactions.[7]

However, these methods aim at introducing a single functionality at each reaction site. Modifying biomolecules with multiple functionalities would thus normally require two or more chemoselective reactions. Recent advances in multimodal molecular imaging techniques,[8] for instance, target a synergistic combination of multiple imaging modes[9] through covalent attachment of moieties such as a metal ligand, nanoparticle, and fluorophore to a single multi-functional targeting molecule. A facile 'one-site multifunction' chemical strategy would be beneficial as it would reduce the number of modifications.

Here we present a one-pot, three-component peptide modification method based on a 1,3-dipolar cycloaddition (DCA) of (1) a functionalized maleimide, (2) an *N*-hydroxylamine, and (3) a peptide with an oxidizable serine residue at the N-terminus or potentially other aldehyde moieties, and its extension to proteins (Fig. 1). Maleimides are among the most efficient dipolarophiles in DCA.[10] Conveniently, the majority of labeling compounds commonly attached to proteins and peptides are commercially available as maleimides.

Nitrones, in turn, are easily obtained from aldehydes and primary *N*-hydroxylamines under aqueous conditions, converting the excellent nucleophile and electrophile into a new reactive dipole set-up for a subsequent cycloaddition. Nitrones have already been applied in bioorthogonal reactions and with rates comparable to those found for azide-alkyne DCA;[5] however, strained alkynes requiring multi-step syntheses were used as dipolarophiles and only a single functionality was added although the authors allude to the possibility of dual modification. The peptide and protein aldehydes can be obtained by initial oxidation with $NaIO_4$ of an N-terminal serine residue to form a 2-oxoacetamide.[11] The chemoselective DCA between the

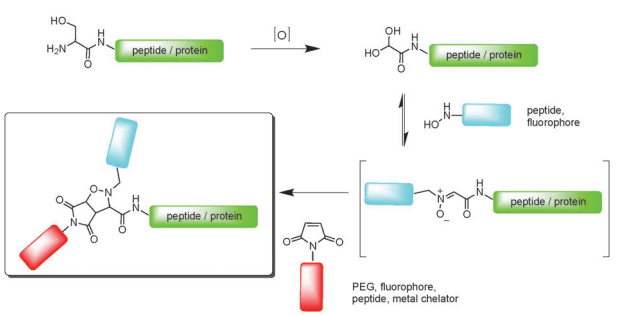

Fig. 1 An N-terminal serine provides an anchor for attaching multiple functionalities *via* a three-component one-pot modification method.

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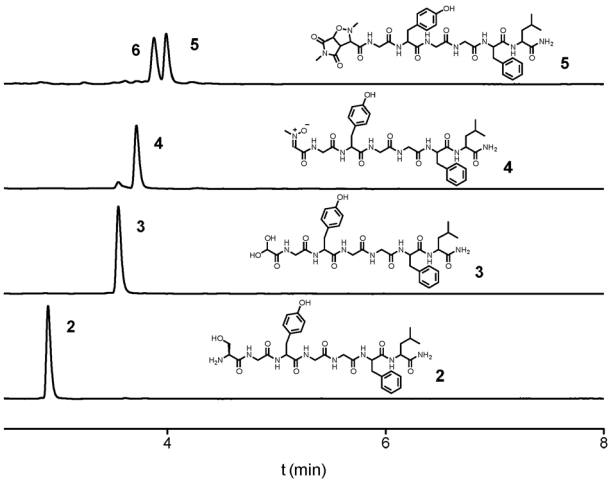
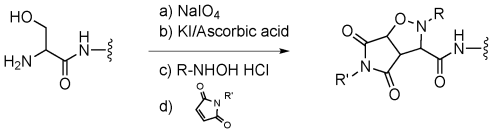


Fig. 2 HPLC monitoring of a model 3-component modification. Peptide **2** is oxidized to 2-oxoacetamide **3**, subsequently forming nitrone **4** by addition of *N*-methylhydroxylamine. DCA with *N*-methylmaleimide yields the isoxazolidine product **5**, as well as the byproduct **6** formed by reaction of aniline with excess maleimide.

Table 1 Model study of N-terminal one-pot modification with varying *N*-hydroxylamines and maleimides



Entry	pH	Peptide	R	R′	*T* (°C)	Conv. (%)
1[a]	4.5	**1**	CH_3	CH_3	rt	86
2[a]	4.5	**1**	CH_3	CH_3	50	84[d]
3[b]	4.5	**1**	CH_3	MDCC	50	>98
4	7.0	**1**	CH_3	CH_3	rt	89
5	7.0	**1**	CH_3	CH_3	50	95[d]
6	4.5	**2**	Cy	CH_3	37	97
7[c]	4.5	**2**	Ph	CH_3	37	>98
8	4.5	**2**	Bn	CH_3	37	92
9	4.5	**2**	CH_3	CH_3	37	>98
10	4.5	**2**	**17**	CH_3	37	95
11	4.5	**1**	**18**	CH_3	50	>98

3-Component modification of model peptides according to procedure A for 15 hours. [a] 10 eq. aniline. [b] 5 eq. MDCC (*N*-(2-(1-maleimidyl)ethyl)-7-(diethylamino)coumarin-3-carboxamide). [c] Additional 6 eq. of phenylhydroxyl-amine was added. [d] 2 hours.

nitrone and a functionalized maleimide thus forms a stable isoxazolidine core which combines the three components (Fig. 2).

We initially studied the reaction on model peptide H-Ser-Gln-Trp-Phe-NH_2 (**1**) in acetate buffer at pH 4.5, near the reported optimal pH range for nitrone formation.[12] The rate limiting step under acidic conditions is the initial nucleophilic attack forming the carbinolamine,[7] suggesting the possibility to employ nucleophilic catalysis.

A one-pot protocol would require quenching of the iodate formed as a byproduct in the initial oxidation step, as iodate has been shown to oxidize the hydroxylamine component, forming undesirable nitroso compounds.[5] We also observed that the DCA was hampered without this quenching step (data not shown). This problem has been addressed by other groups by quenching with hydrophobic scavengers such as 4-methoxybenzenethiol.[5] In our hands, this led to the formation of large amounts of precipitates which necessitated removal by filtration. To circumvent this problem, we found that excess iodate could be successfully reduced by treatment with potassium iodide and sodium ascorbate. We observed no interference or side-reactions from this reductive work-up. This scavenging strategy thus enabled the one-pot formation of the nitrone from the serine peptide by subsequent treatment with *N*-methylhydroxylamine, using aniline or *p*-anisidine as the nucleophilic catalyst.[13] Subsequent DCA with *N*-methylmaleimide formed the isoxazolidine product in excellent conversion over three steps (see Table 1).§

The reaction proceeded at room temperature overnight, but could be significantly accelerated by gentle heating to 50 °C, lowering reaction times to just 2 hours (entries 2 and 3, Table 1).

The reaction proceeded equally well in phosphate buffer at pH 7 (entries 4 and 5, Table 1). A second peptide SGYGGFL (**2**) was modified according to procedure A (entry 9, Table 1), giving the desired isoxazolidine product **5** in 58% isolated yield over three steps. This represents a combined yield of two diastereoisomers formed during the cycloaddition. NMR revealed a 48 : 52 ratio of the two diastereoisomers by integration of the doublets at 4.92 and 4.88 ppm originating from the two H5 signals in the formed isoxazolidine rings (ESI†).

The same 8.1 Hz coupling constant of the overlapping H3 signals indicates that both diastereoisomers are *exo* products originating from the re- and si-face cycloaddition between the maleimide and the nitrone. Varying the amino acid adjacent to the N-terminal serine (Glu, Lys, Phe, and Gly) did not noticeably influence conversion rates for the condensation nor the DCA (see Table S1, ESI†). However, the chiral environment on a protein may likely induce the preferred formation of one isomer.[14] Using the maleimide-functionalized fluorophore MDCC,[15] full conversion to the desired isoxazolidine product was obtained at 50 °C (entry 3, Table 1). Expanding our method to incorporate a functionalized nitrone, we employed several commercially available *N*-substituted hydroxylamines to give the corresponding nitrones in good to excellent conversion. A 1,5-hydroxylamino-AEDANS fluorescent reagent (**17**) was prepared from commercially available 1,5-IAEDANS[16] by treatment with NH_2OH (see ESI†). Subsequent reaction of the resulting peptide-AEDANS nitrone with *N*-methylmaleimide afforded the fluorescent peptide in 95% conversion (entry 10, Table 1).

This strategy was furthermore applied[17] to the peptide ligation of two short peptide fragments in the N → N′ direction. Nitrone condensation of a peptide hydroxylamine HO-GQWF-NH_2 (**18**) with the oxidized peptide **1** and subsequent DCA at 50 °C with *N*-methylmaleimide provided the ligation product in 98% yield (entry 11, Table 1).

The transmembrane glycoprotein integrin $\alpha_v\beta_3$ receptor is an attractive target for diagnostic cancer imaging as the protein is overexpressed in several tumor forms such as melanomas,

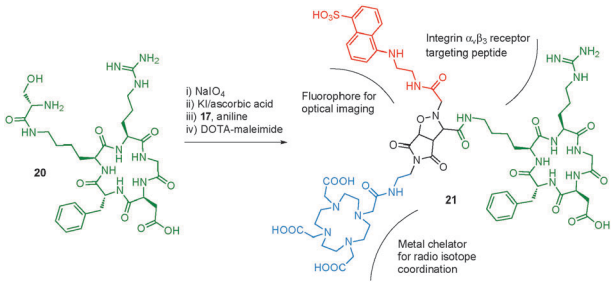


Fig. 3 Peptide **20** modified according to procedure A with 1,5-hydroxylamino-AEDANS and 5 eq. DOTA-maleimide giving product **21**, as a model for potential modifications of targeting peptides.

ovarian and lung carcinoma, neuroblastomas, glioblastomas, and breast cancer.[18] The receptor specifically recognizes Arg-Gly-Asp domains in peptides and several linear and cyclic RGD targeting peptide analogs have been designed, such as c(RGDfK), **19**. As a model for a bimodal radio- and optical imaging agent, we attached a fluorophore and the radio-isotope chelator DOTA to a c(RGDfK) peptide in a one-pot reaction. Boc-Ser(tBu)-OH was coupled to the Lys side-chain of **19** to provide **20**. Subsequent three-component modification with *N*-hydroxylamine-AEDANS fluorophore (**17**) and DOTA-maleimide then gave the desired bimodal compound **21** in 79% isolated yield (Fig. 3).

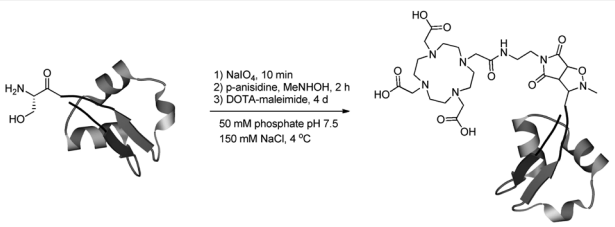


Fig. 4 Site-selective modification of OXR1 at pH 7.5, forming the desired DOTA-functionalized protein.

In a first demonstration of this protocol for protein modification, we conjugated a DOTA-maleimide chelator to the N-terminus of the 12 kDa LysM domain of human oxidation resistance protein 1 (OXR1). OXR1 is highly conserved among eukaryotes and is believed to be involved in the repair of oxidative DNA damage.[19,20] To avoid aggregation, the protein concentration was lowered to 700 μM and the temperature kept at 4 °C, which necessitated longer reaction times. Rewardingly, after 4 days, the product was observed by ESI-LCMS in approximately 50% yield (see ESI†). Thus, our protocol proved effective for site-selective introduction of a ligand by a stable linkage onto the protein OXR1. Likely, it can be further extended to introduce two ligands on some proteins (Fig. 4).

The presented site-selective three-component peptide and protein modification method proceeds with excellent conversions under mild aqueous conditions. The advantages over oxime formation include that three components are linked and that the resulting isoxazolidine products provide a chemically robust linkage between the conjugated components. The wide selection of commercially available maleimides, functionalized with metal ligands, PEGs, fatty acids, and fluorophores is a distinctive advantage of this strategy. Peptide and protein aldehydes were conveniently prepared by chemical oxidation of precursors with an N-terminal Ser; however, they could potentially also be accessed by a variety of other methods. The three-component reaction can be used for introduction of one or two functionalities in a peptide or for ligation of two peptide chains. We also demonstrated its value for site-specific protein modification. We envisage this one-pot protocol to have wide applicability beyond the exemplifications reported here.

Notes and references

§ Experimental: General modification procedure A/B: All components are freshly prepared prior to each reaction (A: 200 mm NaOAc pH 4.5/CH_3CN 1 : 1; B: 100 mm phosphate buffer pH 7.0/CH_3CN 1 : 1). A 10 mm peptide solution is treated with $NaIO_4$ (1.2 eq.). Oxidation proceeds immediately and the formed iodate is quenched with sodium ascorbate (4 eq.) and KI (6.6 eq.). After 15 min, aniline (3 eq., at pH 4.5) or *p*-anisidine (3 eq., at pH 7.0) and the appropriate *N*-hydroxylamine (3 eq. if not otherwise stated) are added, and the solution is monitored until nitrone formation is complete. The appropriate maleimide (25 eq.) is then added and the solution kept at rt or with gentle shaking in a heater shaker for a specified amount of time.

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