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Chemical cross-linking with NHS esters: a systematic study on amino acid reactivities

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Structure elucidation of tertiary or quaternary protein structures by chemical cross-linking and mass spectrometry (MS) has recently gained importance. To locate the cross-linker modification, dedicated software is applied to analyze the mass or tandem mass spectra (MS/MS). Such software requires information on target amino acids to limit the data analysis time. The most commonly used homobifunctional N-hydroxy succinimide (NHS) esters are often described as reactive exclusively towards primary amines, although side reactions with tyrosine and serine have been reported. Our goal was to systematically study the reactivity of NHS esters and derive some general rules for their attack of nucleophilic amino acid side chains in peptides. We therefore studied the cross-linking reactions of synthesized and commercial model peptides with disuccinimidyl suberate (DSS). The first reaction site in all cases was expectedly the α-NH_2-group of the *N*-terminus or the ε-NH_2-group of lysine. As soon as additional cross-linkers were attached or loops were formed, other amino acids were also involved in the reaction. In addition to the primary amino groups, serine, threonine and tyrosine showed significant reactivity due to the effect of neighboring amino acids by intermediate or permanent Type-1 cross-link formation. The reactivity is highly dependent on the pH and on adjacent amino acids.

Keywords: chemical cross-linking; NHS ester; amino acids; reactivity; disuccinimidyl suberate

Introduction

Chemical cross-linking in combination with mass spectrometry has emerged as a powerful tool for structural analysis of protein–protein interactions and protein three-dimensional structures. Intermolecular cross-linking stabilizes noncovalent complexes and thus allows fast identification of specific interactions and reliable determination of complex stoichiometry by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS).[1–3] In addition, interaction sites in complexes can be identified.[4–8] The cross-linker arm can also act as a ruler to map spatial proximities of amino acids in proteins. The derived distance constraints yield low-resolution tertiary structures of proteins.[9] Owing to its low sample consumption, short analysis time and broad applicability,[10] the MS-based approach is an interesting alternative to X-Ray crystallography, nuclear magnetic resonance spectroscopy (NMR) or cryo-electron microscopy.

Two principal strategies have been developed to locate cross-linker modifications: the *bottom-up* (enzymatic digestion followed by MALDI- or electrospray ionization-mass spectrometry (ESI-MS) analysis)[4–9] and the *top-down* approach (high-resolution MS analysis of fragmented protein ions).[11,12] An overview of these techniques was given in a recent review by Sinz.[10]

After proteolysis or fragmentation, three different types of modifications on peptides are obtained: so called 'dead ends', 'intralinks' and 'interlinks'. 'Dead end' is often used to describe the modification of a certain amino acid with a cross-linker (XL) whose other end is hydrolyzed. An 'intralink' describes a modification where both reactive ends reacted within a single peptide. If the two reactive ends of the cross-linker are attached to two different peptides, the product is often designated as 'interlink'. A confusing variety of other trivial names has been used in the literature. A systematic nomenclature introduced recently by Schilling *et al.*[13] proposes the assignment as Type-0 (dead end), Type-1 (intralink) and Type-2 (interlink) (Scheme 1). In the following, this systematic nomenclature is used to describe the different types of cross-linked peptides.

Since the number of unmodified peptides or fragments by far exceeds the number of modified ones, data analysis is often the crucial point for this method. With the use of cleavable cross-linkers or isotopic labeling, improvements in the detection of intramolecularly or intermolecularly cross-linked species can be obtained.[4,14–16]

Dedicated software is required to calculate all possible masses and/or to analyze the MS or tandem mass spectra (MS/MS). In addition to knowledge about the cross-linker mass and chemical identity of the product, customized programs, such as ASAP,[9] GPMAW,[17] SearchXLinks,[18] XQuest[19] or VIRTUALMSLAB,[20] require information on the target amino acids. To identify a high number of modified peptides and therefore extract the maximum information on protein conformation or complex interfaces, it is crucial to know all possible amino acids involved in the reaction with the cross-linker. Moreover, studies conducted without MS/MS confirmation might give false results if unexpected reactions occur. Therefore, it is not only necessary to know the main modified amino acids, but also possible side reactions.

One of the most common cross-linking strategies is the use of homobifunctional N-hydroxy succinimide (NHS) esters, which were introduced as highly amine reactive more than 30 years ago.[21] Instruction leaflets accompanying commercial products and also most application literature state that reaction occurs

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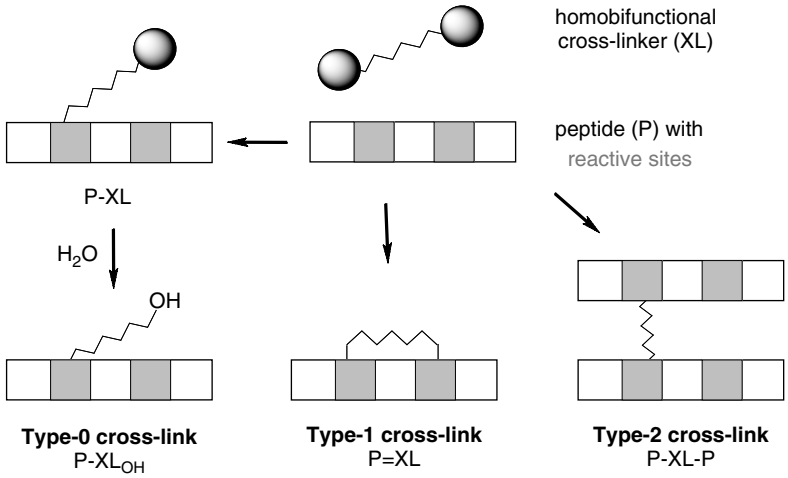

Scheme 1. Possible reaction products for chemical cross-linking and used nomenclature.

exclusively with the free *N*-terminus and the ε-amino group of lysine side chains.

However, some studies with certain homobifunctional NHS esters revealed unexpected side reactions. Swaim *et al*.[22] found several reaction products during their study on α-crystallin assemblies with the cleavable cross-linker 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP), which cannot be explained with cross-linking of primary amines. Besides side reactions with amine impurities in the buffer, they discovered modifications on serine and tyrosine side chains on some of the investigated model peptides. The results were confirmed by MS/MS measurements carried out on an electrospray quadrupole time-of-flight (ESI-Q-ToF) mass spectrometer. Cross-linking experiments with ethylene glycol bis[succinimidylsuccinate] (EGS) on oxidized insulin β-chain, performed by Leavell *et al*.,[23] confirmed the formation of intramolecular tyrosine–lysine and even tyrosine–tyrosine cross-links. The reaction of tyrosine was favored under acidic conditions (pH 6.0), whereas the *N*-terminus and lysine reacted preferentially under alkaline conditions (pH 8.4). Structural investigations on Prion protein fibrils from brains of Syrian hamsters infected with scrapie (PrP^{Sc}) confirmed a tyrosine modification on PrP 27–30 with bis[sulfosuccinimidyl] suberate (BS^3).[24] In the course of an epitope mapping study, tyrosine and serine were identified as cross-linker locations on bovine prion protein (bPrP) after cross-linking to the corresponding monoclonal antibody with disuccinimidyl suberate (DSS) and disuccinimidyl glutarate (DSG).[4] In a very recent study on laminin β1 and laminin γ1 *N*-terminal recombinant constructs using BS^3 and bis[sulfosuccinimidyl] glutarate (BS^2G) as a cross-linker, about 20% of all cross-linker containing species resulted from reaction products with hydroxyl-containing amino acids. Modifications occurred not only on serine (12.5% of all cross-linker containing species) and tyrosine (4.3%) but also on threonine (3%).[25]

Previous experiments mainly showed that besides amino groups, amino acids carrying a hydroxyl group are also reactive towards NHS esters. Our goal is to rationalize the findings in detail and eventually derive some general rules on the reactivity of NHS esters towards nucleophilic amino acid side chains. We therefore studied synthetic model peptides differing in only one amino acid to obtain reactivity information independent of the chemical environment. These amino acids carrying nucleophilic side chains were chosen according to the following considerations: since sulfhydryl groups are blocked by disulfide bridges in most of the native proteins under nonreducing conditions, cysteine was not considered in our investigations. Methionine and tryptophan as well as amino acids bearing amide side chains (glutamine, asparagines) are poor nucleophiles or buried in the core of the protein. The amino acids containing ionizable amine groups, such as lysine, arginine and histidine, are typically exposed on the surface and thus easily accessible. Owing to their hydrophilic character, threonine and serine are also often found at or near the surface of a protein. Therefore, lysine, arginine, histidine, serine, tyrosine and threonine were each embedded in a synthesized peptide as possible reaction target of the NHS ester. Results obtained from cross-linking experiments with the homobifunctional cross-linker DSS were compared with the results obtained after experiments on commercial peptides carrying several possible reactive amino acids. With the knowledge of all possible reaction targets, structure elucidation of proteins and protein complexes could be significantly improved due to the higher fraction of identified cross-linked species.

Experimental Section

Peptides

N-terminal protected peptides with one or two reactive amino acids were synthesized in-house on a fully automized Peptide Synthesizer (ABI433, Applied Biosystems, Rotkreuz, Switzerland) with the sequences Fmoc-EGGGXGGGE, where X = Lys; Arg; His; Tyr; Thr or Ser, and Fmoc-EGGXGZGGE with (X, Z) = (His, Tyr); (His, Ser) or (Lys, Lys). Two glutamic acids were introduced to make the peptide water soluble. Calculated isoelectric point (pI) values for the unprotected peptide are in the range 4.2–4.9[26] and suggest that a permanent negative charge is maintained for pH values above 6. The peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a C18 preparative column (250–10 mm, LiChrospher®, 100 Å, RP-18, 10 µm, Merck KGaA, Darmstadt, Germany).

Deprotection was carried out on 3–9 mg of crude *N*-terminal protected peptide in a 17 mg/ml aqueous solution containing 33% (v/v) piperidine (Merck KGaA, Darmstadt, Germany) for 1 day at room temperature. The solutions were subsequently dried with a speedvac and the dried product was redissolved in 20%

trifluoroacetic acid (TFA). The reaction mixture was extracted five times with *n*-hexane : ethyl acetate (80 : 20, v/v). The aqueous phase was evaporated and the dried residue redissolved in water. Residual nonpolar products were separated on a solid phase extraction (SPE) cartridge (Sep-Pak®, Vac C18 1cc; Waters Corporation, Milford, U.S.A). The samples were then lyophilized und redissolved in water.

Bradykinin Fragment (2–9) (Brad), oxidized insulin α-chain and β-chain from bovine pancreas were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Leech osmoregulatory factor (LOF), mast cell degranulating peptide (MCD) HR2, Fmoc-(Glu^{70}, Ala^{71,72}, Lys^{74})-C3a (70–77) (C3a), myelin basic protein (MBP) (87–99), Angiotensin I/II (3–8) (Ang I/II), and Angiotensin I (1–9) (Ang I) were purchased from Bachem (Bachem AG, Bubendorf, Switzerland). All commercial peptides were used without further purification.

Abbreviations used to describe the cross-linked species

Reaction products of the peptides obtained after chemical reaction with the homobifunctional ester DSS are designated as 'cross-linked' peptides, regardless of their composition (Type-0, Type-1 or Type-2). For the reaction itself, the term 'cross-linking' is used. For simplicity, abbreviations where used to describe the different types of reaction products (Scheme 1). Peptides (P) modified with a cross-linker (XL) that is still active on the second reaction site are named as P–XL. Their hydrolyzed analogs are described as P–XL_{OH} (Type-0 cross-link). Intramolecularly linked peptides (Type-1 cross-link) are abbreviated as P═XL. For peptides modified with several cross-linkers the abbreviations are combined. For instance, P═$XLXL_{OH}$ describes a peptide that bears one Type-1 and one Type-0 cross-link (Type-0,1). A similar syntax was used to indicate the modification type of product ions in the product ion mass spectra. For instance, $b_6$2XL_{OH} describes a b-type ion fragmented at position 6 and modified with two hydrolyzed cross-linkers (Type-0,0).

Cross-linking experiments on synthesized peptides

For the cross-linking experiments, stock solutions of the protected peptides (about 1 mM) were diluted with 10-mM buffer solution (tetraethyl ammonium bicarbonate (TEAB) or sodium phosphate buffer (NaH_2PO_4/Na_2HPO_4)) to a final peptide concentration of about 25-µM. Disuccinimidyl suberate (DSS, Pierce, Rockford, USA) dissolved in dimethyl formamide (DMF) was added to an aliquot of the peptide solution at a 50-fold molar excess. Reactions with the synthesized peptides were conducted at four different pH values: pH 9.1 (TEAB), 6.1, 6.7 and 7.8 (NaH_2PO_4/Na_2HPO_4). To investigate the unprotected peptides, the lyophilized reaction products of the deprotection reaction were dissolved at 0.2 mg/ml with a final buffer concentration of 10 mM (pH 9.1, TEAB). For direct MALDI analysis, the cross-linking reactions were carried out at a volume ratio of 1/60 ($v_{peptide}/v_{XL}$), using a DSS concentration of 28 mg/ml to minimize the DMF amount in the solution. With the use of higher DMF volumes, low-quality spectra were obtained with MALDI-MS, probably caused by a change in the crystallization behavior of the matrix. For reactions with subsequent ZipTip purification (0.6 µL C18 resin, Millipore, Molsheim, France), a volume ratio of 1/10 ($v_{peptide}/v_{XL}$) with a DSS concentration of 5 mg/ml was applied. Reaction times were between 20 min and 1 day.

Cross-linking experiments on commercial peptides

The peptides were dissolved in water at 1-mM concentration and afterwards diluted with a 10-mM buffer solution of TEAB to reach a final pH of 9.1 and a peptide concentration of 25 µM. For the cross-linking experiments, the peptide solution was incubated with a 5 mg/ml solution of DSS in DMF in the volume ratio 1/10 ($v_{peptide}/v_{XL}$) for 40 h (1 h for the insulin peptides). The cross-linker concentration corresponded to a 50-fold molar excess. The long reaction times were chosen to maximize the reaction yield.

Mass spectrometric detection

Prior to the ESI measurements, the cross-linking solutions were acidified with TFA to yield a final acid concentration of 1% (v/v) and purified by ZipTip. The purified samples were diluted afterwards 1/2 (v/v) in MeOH/H_2O (50/50, v/v) containing 1% (v/v) acetic acid. MS and MS/MS measurements were performed on a quadrupole time-of-flight (Q-ToF) mass spectrometer (Q-ToF ULTIMA, Waters, Milford, USA) equipped with an automated chip-based nano-electrospray ionization (nanoESI) system (Nanomate 100, Advion Biosciences, Ithaka, USA). Experiments on this instrument were performed exclusively in the positive mode, since the Nanomate shows low sensitivity in the negative mode. Collision-induced dissociation (CID) experiments were conducted in a hexapole collision cell pressurized with argon (Purity 5.0, PanGas). The collision energy was optimized to give reasonable fragmentation patterns applying values between 35 and 80 eV. All mass spectra were baseline-corrected and smoothed using a Savitzky–Golay algorithm. A matrix-assisted laser desorption/ionization-time-of-flight (MALDI-ToF) mass spectrometer (Axima CFR, Kratos Analytics, Shimadzu Biotech, Manchester, UK) equipped with a nitrogen laser (λ = 337 nm) was used for direct analysis of reaction products. Measurements were performed in the reflectron mode of the instrument using delayed extraction and an acceleration voltage of 20 kV. Each mass spectrum was the average of 300 scans obtained at random sample positions. All mass spectra were baseline-corrected. For measurements in positive mode, α-cyano-4-hydroxycinnamic acid (CHCA, Sigma Chemical Company, Buchs, Switzerland) was used as a matrix, dissolved at 10 mg/ml in water/acetonitrile/TFA (50/50/0.1, v/v/v). For negative mode measurements, the matrix was dissolved in water/acetonitrile (50/50, v/v). The matrix solution was mixed with the reaction solution in a 1/2 (v/v) ratio. 0.5–1 µL of the mixture were spotted on a stainless steel plate and allowed to dry under ambient conditions. MS measurements were conducted directly after the specified reaction time. The reactions were quenched with the drop of the pH value after mixing with the acidic matrix solution for MALDI-MS or after acidification with TFA for nanoESI-MS experiments, respectively.

Results and Discussion

Synthetic peptides

N-terminal protected peptides containing a single potentially reactive amino acid (Fmoc-EGGGXGGGE, where X = Lys; Arg; His; Tyr; Thr or Ser) were subjected to reaction with the cross-linker DSS at four different pH values and analyzed by MALDI-MS in negative mode. Only for the peptide containing Lys (*MonoLys*, $[P-H]^-$, *m/z* 967 (Fig. 1), a mass increase of 156 u, corresponding to an amide linkage between the cross-linker (hydrolyzed on one site) and the ε-NH_2-group, was observed ($[P-XL_{OH}-H]^-$, *m/z* 1123). As expected,

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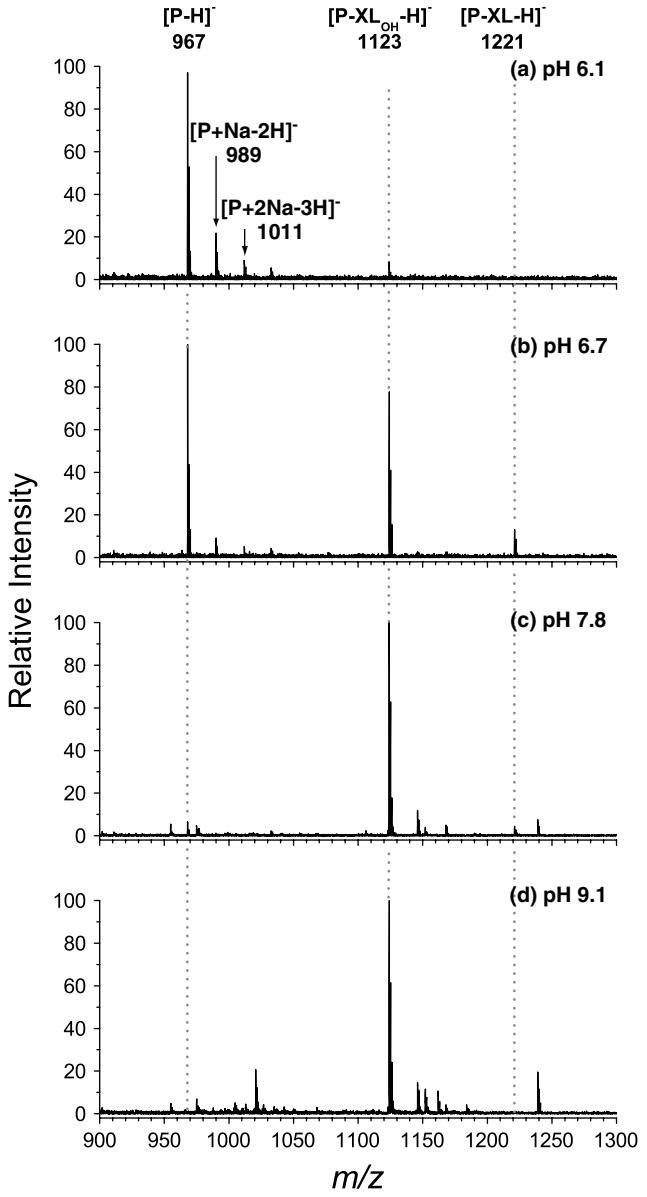


Figure 1. Negative mode MALDI mass spectra of the cross-linking products of *MonoLys* (Mw = 969 Da) after 1 day reaction time at different pH values. (a) At pH 6.1 the peptide did not show any reaction; (b) at pH 6.7 the main species appearing were the deprotonated ions of a Type-0 cross-link (+156 u, $P-XL_{OH}-H]^-$) at *m/z* 1123; (c) at pH 7.8 and (d) pH 9.1 the spectra were dominated by the cross-linked peptide.

the yield of $P-XL_{OH}$ increased with increasing pH. Thus, at pH 7.8 and pH 9.1 the reaction was complete for a reaction time of one day. Since only the hydrolyzed species ($[P-XL_{OH}-H]^-$, *m/z* 1123) was detected with negative mode MALDI-MS, the data suggests complete hydrolysis of the other end of the cross-linker. A minor amount of the intact succinimidyl site ($[P-XL-H]^-$, *m/z* 1221) and a large fraction of unmodified peptide was observed at pH 6.7, indicating incomplete hydrolysis and cross-linking reaction. At pH 6.1, no reaction took place within one day. The product identity of the Type-0 cross-linked peptide was confirmed by MS/MS measurements with nanoESI-MS in positive mode (Fig. 2). Regardless of the pH, no reaction of Arg (*MonoArg*), His (*MonoHis*), Ser (*MonoSer*), Tyr (*MonoTyr*) and Thr (*MonoThr*) could be detected under the described conditions (Table 1).

More than twenty years ago, researchers studied the reaction of 'monofunctional' NHS esters with *N*-terminal protected single amino acids by liquid scintillation[27] or HPLC.[28] For His, Lys and Cys, Cuatrecasas and Parikh[27] observed an interference with the coupling of unprotected [^{3}H]-Ala to the NHS ester of agarose. Anjaneyulu and Staros[28] ranked the determined reaction rates in the following order: imidazole $>\varepsilon$-amino $\sim\alpha$-amino $\gg$ thiolate $\sim$ phenolate. Reaction on the more nucleophilic His side chain yielded N-acylated imidazole, which hydrolyzed rapidly. In recent acylation studies on free amino acids, Tyr showed O-acylation additional to N-acylation on the *N*-terminus in the pH range 7.5–8.5.[29] With increasing pH, hydrolysis of the O-acylate was amplified and therefore the N-acylated product dominated the reaction products. No significant labeling was observed for the side chains of Arg, Gln, Phe, Trp, Met, Ser and Thr in all of these studies.

For our model peptides, the lack of modified His imidazole was not surprising owing to the assumed hydrolysis of the N-acylimidazole. Since no O-acylation on the phenyl group of tyrosine was detected, the chemical environment must have an effect on the reactivity of amino acids. Therefore, we also analyzed the cross-linking reactions of three *N*-terminal protected peptides with two reactive sites (Fmoc-EGGXGZGGE with (X, Z) = (His, Tyr); (His, Ser); (Lys, Lys)). The presence of cross-linked species at different pH values was checked by MALDI-MS in negative mode. Contrary to *MonoTyr* and *MonoSer*, Tyr and Ser showed significant reactivity towards DSS in the presence of His. High intensities of $P-XL_{OH}$ were observed for both *HisSer* and *HisTyr* at pH 7.8 and 9.1. The spectra for *HisTyr* are given in Fig. 3. MS/MS (CID) measurements with nanoESI-MS in positive mode proved the modification of the hydroxyl-containing amino acids (data not shown). We also detected a minor product with a mass increase of 138 u in the MALDI spectra after 20 min reaction time for pH values 6.7, 7.8 and 9.1, indicating a Type-1 cross-link between His and Ser or Tyr, respectively (inset in Fig. 3).

The presence of His close to Ser or Tyr in the triads His-Gly-Xxx (with Xxx = Ser, Tyr) increased the reactivity of the hydroxyl-containing side chains significantly. This observation has been previously reported for biotinylation experiments with NHS esters on Ser, Tyr and Thr,[30–35] but has been largely ignored in previous cross-linking studies. Miller *et al*. hypothesized that hydrogen bonding with the histidyl imidazole base-catalyzes the reaction by increasing the nucleophilicity of the hydroxyl oxygen. Alternatively, the imidazole could act as a nucleophilic catalyst by forming an N-acylated imidazole as intermediate and subsequent transfer of the cross-linker chain onto the hydroxyl group of the adjacent hydroxylic side chain. Acylation with N-acetylimidazole has been reported as selective strategy for the labeling of tyrosine.[36]

With the use of homobifunctional NHS esters, another mechanism becomes likely. The elevated reactivity can be explained by the formation of a transient Type-1 cross-link between His and Ser or Tyr. Thus the reaction of the hydroxyl groups would be more favored due to the high local concentration of the cross-linker and due to entropic effects (Scheme 2). After hydrolysis of the N-acylimidazole, a Type-0 cross-link in the form of an O-acylate is obtained. Our results support the theory of an intermediate Type-1 cross-link formation, since an intermediate species with a mass loss corresponding to water was observed in the MALDI spectra. In comparison to *MonoLys, HisSer* and *HisTyr* showed a much higher extent of hydrolyzed cross-linker at pH 9.1. Whereas for *MonoLys*

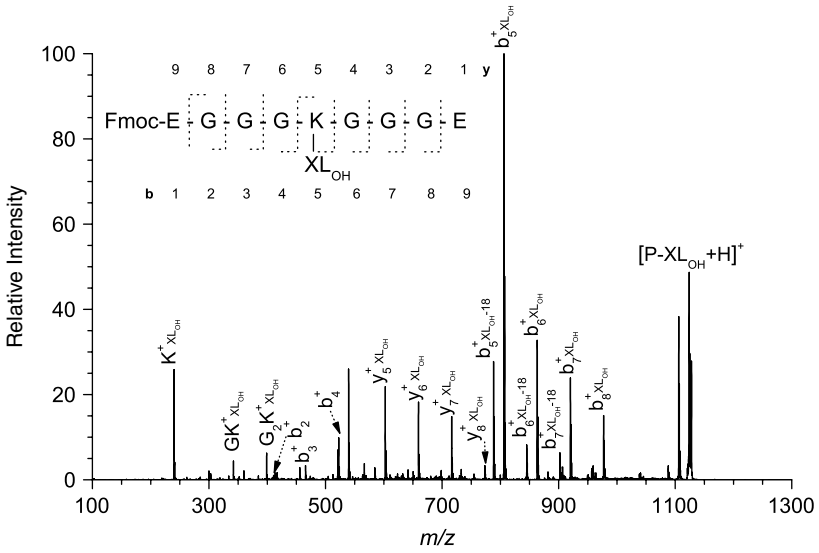


Figure 2. Investigation of the sequence of the cross-linking product of *MonoLys* after reaction with DSS. Product ion mass spectrum (positive mode, nanoESI-MS) of *MonoLys* modified with a hydrolyzed cross-linker (Type-0 cross-link) ($[P-XL_{OH}+H]^+$, *m/z* 1125).

Table 1. Summary of the results of cross-linking experiments on synthesized peptides with DSS at pH 6.1, 6.7, 7.8 and 9.1, measured with MALDI–MS in negative mode. The possible reaction sites are marked in bold in the sequence. '+' indicates that ions with a mass increase of 156 u ($P-XL_{OH}$) or 138 u (P═XL) were observed, '–' indicates that no modified peptide ions were detected. 'n.a.' indicates that the corresponding experiment was not conducted. Amino acids modifications that could be identified by product ion mass measurements are underlined

			Cross-linking at pH			
Abbreviation	Sequence	Reactive group	6.1	6.7	7.8	9.1
MonoLys	Fmoc-EGGG**K**GGGE	NH_2	–	+	+	+
MonoArg	Fmoc-EGGG**R**GGGE	Guanidino	–	–	–	–
MonoHis	Fmoc-EGGG**H**GGGE	Imidazole	–	–	–	–
MonoSer	Fmoc-EGGG**S**GGGE	OH (aliphatic)	–	–	–	–
MonoThr	Fmoc-EGGG**T**GGGE	OH (aliphatic)	–	–	–	–
MonoTyr	Fmoc-EGGG**Y**GGGE	OH (aromatic)	–	–	–	–
DiLys	Fmoc-EGG**K**G**K**GGE	2 NH_2	+	+	+	+
HisTyr	Fmoc-EGG**H**G**Y**GGE	−NH+ OH (aromatic)	+	+	+	+
HisSer	Fmoc-EGG**H**G**S**GGE	−NH+ OH (aliphatic)	–	+	+	+
MonoArg	**E**GGG**R**GGGE	Guanidino	n.a.	n.a.	n.a.	–
MonoSer	**E**GGG**S**GGGE	OH (aliphatic)	n.a.	n.a.	n.a.	–
MonoThr	**E**GGG**T**GGGE	OH (aliphatic)	n.a.	n.a.	n.a.	–
MonoTyr	**E**GGG**Y**GGGE	OH (aromatic)	n.a.	n.a.	n.a.	+

the integral ratio $P-XL : P-XL_{OH}$ was about 4 : 1 after 20 min, *HisTyr* shows a $P-XL : P-XL_{OH}$ ratio of 1 : 5 after the same reaction time.

In the context of studies on the effect of a second reactive amino acid on the reactivity, *DiLys* was also studied. Reaction products at *m/z* 1177 (+138 u) and *m/z* 1239 (+156 u) were detected at all studied pH values in the range 6.1–9.1 (Table 1) by MALDI-MS in negative mode. The mass increase of 138 u indicated the presence of a Type-1 cross-link, which involves both Lys residues. With increasing pH, the ratio between $[P-XL_{OH}-H]^-$ and $[P{=}XL-H]^-$ shifted from the Type-0 cross-linked species towards the Type-1 cross-linked species (Fig. 3). This implies that the increase in reactivity of the ε-amino group substantially exceeds the increase in hydrolysis rate with increasing pH. Labeling on single amino acids at different pH values gave similar results.[29] The product identity of $[P{=}XL + H]^+$ was confirmed by MS/MS measurements with nanoESI-MS in positive mode (Fig. 4).

To investigate the effect of a reactive primary amino group in close proximity, we conducted cross-linking experiments on deprotected, synthesized peptides with the sequence EGGGXGGGE, where X = Arg; Tyr; Thr or Ser. The experiments were conducted at pH 9.1, since the highest reaction yields were obtained for the protected peptides at this pH. Reaction products were analyzed with MALDI–MS in negative mode. The free *N*-terminus was assumed to have a considerably higher reactivity than the nucleophilic amino acids in the sequence. Therefore, a modification of Arg, Tyr, Thr or Ser was mainly expected to occur as a Type-1 cross-link. The presence of a highly reactive amino group promoted the formation of a Type-1 cross-link exclusively in the case of *MonoTyr*. No adducts of +138 u were observed for the remaining deprotected peptides.

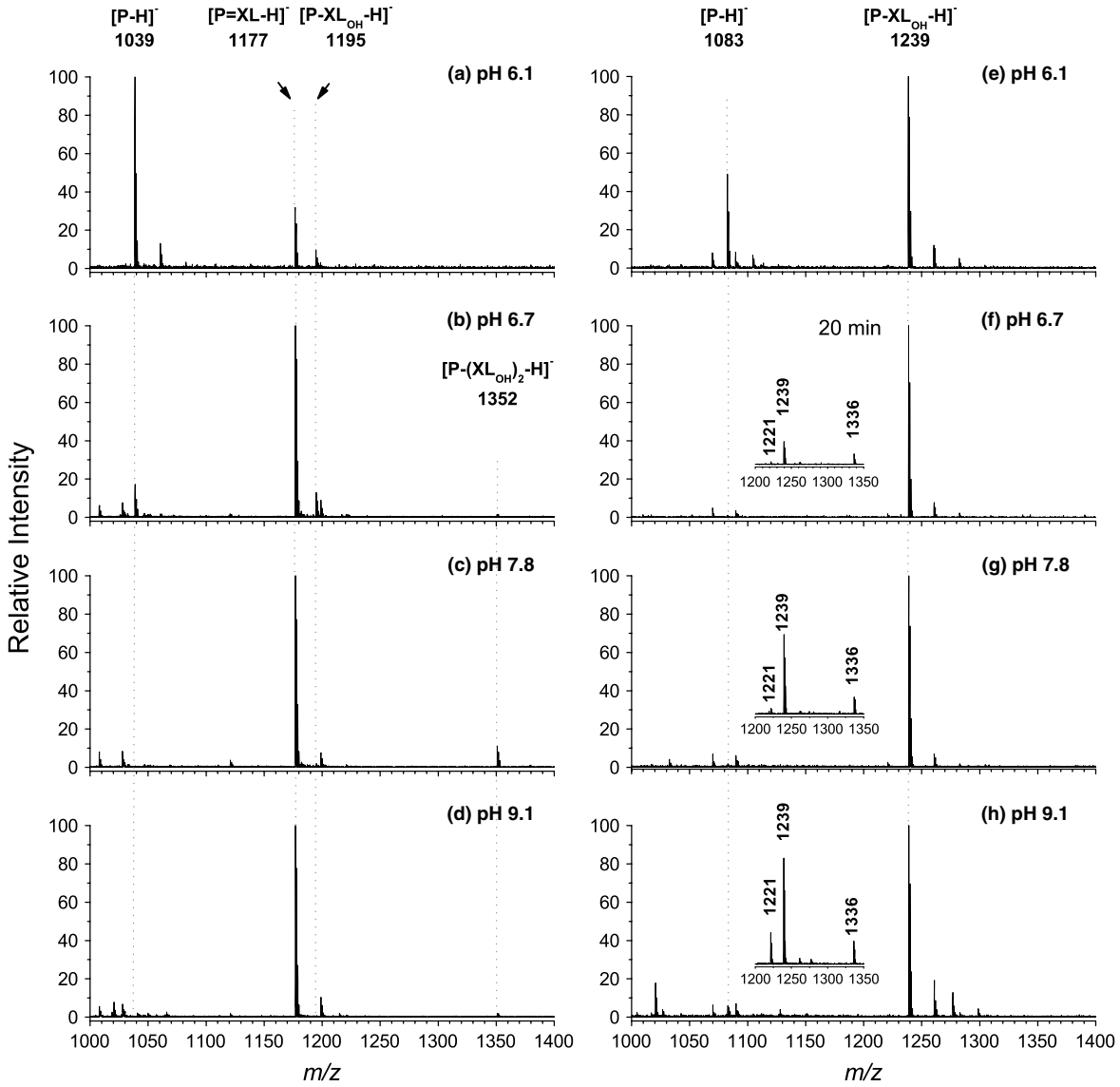
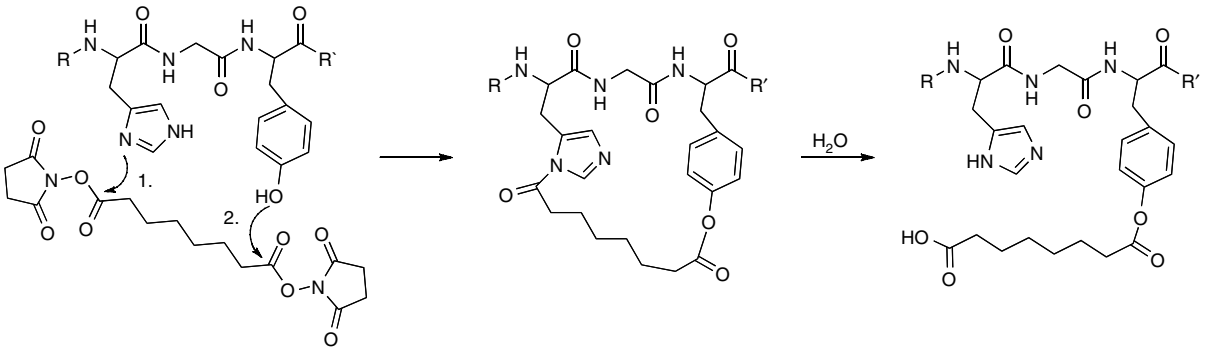


Figure 3. Negative mode MALDI mass spectra of the reaction products of *DiLys* (Mw = 1040 Da) and *HisTyr* (Mw = 1084 Da) with DSS after 1 day reaction time at different pH values. For *DiLys* at (a) pH 6.1 and at (b) pH 6.7 the Type-1 $[P{=}XL-H]^-$ and Type-0 cross-linked species $[P-XL_{OH}-H]^-$ were observed. For *DiLys* at (c) pH 7.8 and at (d) pH 9.1 only the Type-1 cross-linked species was present. (e) For *HisTyr* at pH 6.1 the reaction was not complete within 1 day. The spectra for *HisTyr* at (f) pH 6.7, (g) pH 7.8, and (h) pH 9.1 showed mainly the peptide modified with a hydrolyzed cross-linker $[P-XL_{OH}-H]^-$. The inset spectra (20-min reaction time) show the formation of an intermediate Type-1 cross-link $[P{=}XL-H]^-$ at *m/z* 1221 and a Type-0 cross-link at *m/z* 1336.

Comparison of reactivity

To quantify the elevated reactivity of Ser and Tyr due to the presence of His, we studied the yield of the cross-linking reaction of *HisSer, HisTyr, DiLys* and *MonoLys* after 20 min at four different pH values at 26 °C. The short reaction time was chosen to maintain a significant amount of unmodified peptide for a proper comparison of reaction yields. Samples were analyzed by MALDI–MS, since nanoESI measurements required purification of the reaction mixtures by ZipTip, which could potentially perturb the product distribution owing to different retention properties on the filling material (C18-silica). The reaction yields were calculated by dividing the sum of the peak areas of the cross-linked ions $([P-XL_{OH}+H]^+/[P-XL_{OH}-H]^-$, $[P{=}XL+H]^+/[P{=}XL-H]^-$ and/or $[P-XL+H]^+/[P-XL-H]^-)$ by the sum of unreacted and modified peptides. Except for *MonoLys*, which gave only the cationized species $[P-XL_{OH}+Na]^+$ in positive mode, the protonated (positive mode) or deprotonated (negative mode) ions were considered. The values shown in Fig. 5 were the average of six measurements (300 scans each) of one experiment. The measured reaction yields were highly dependent on the detection mode, especially for the peptides containing one or two Lys. Negative mode experiments (Fig. 5(b)) lead to significantly higher amount of cross-linked peptides than positive mode measurements (Fig. 5(a)).

To evaluate mass spectrometric data for semi-quantitative analysis, ionization efficiencies have to be considered. Since the cross-linker reacts with the most nucleophilic and often most basic sites in the case of *MonoLys* or *DiLys*, it blocks these sites for protonation. Moreover, an additional acid is introduced in the hydrolyzed state of the cross-linker. Therefore a change in ionization efficiency is expected to occur after the cross-linking reaction. Although His is the most basic and most nucleophilic site, the free His residue was recovered after reaction of the



Scheme 2. Possible catalysis mechanism for Tyr by intermediate Type-1 cross-link formation with the His imidazole. According to this hypothesis, the reaction of Tyr is favored due to entropic effects and the high local concentration of the cross-linker after formation of an N-acylated intermediate. The N-acylate is assumed to hydrolyze rapidly.

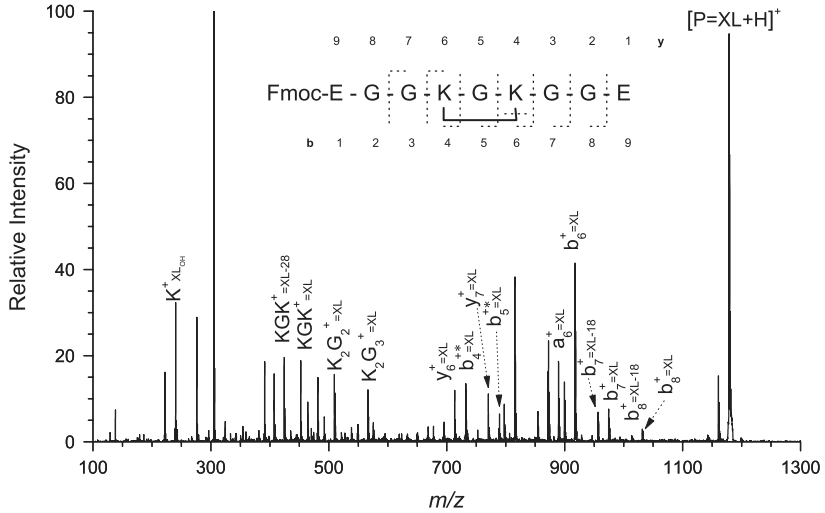


Figure 4. Investigation of the sequence of a cross-linking product of *DiLys* after reacting with DSS. Product ion mass spectrum (positive mode, nanoESI-MS) of the Type-1 cross-linked species of *DiLys* ($[P{=}XL + H]^+$, *m/z* 1179). Peaks marked with an asterisk indicate the fragmentation of the cross-link.

cross-linker with *HisTyr* and *HisSer,* as was mentioned above. A direct comparison of peak integrals between *MonoLys* or *DiLys* and the His containing peptides is therefore probably prone to errors for the positive detection mode. Since the glutamic acids are easily deprotonated and retained during the cross-linking reaction, measurements in the negative mode should give more reliable results.

The results confirm that the reactivity of the nucleophilic side chains is strongly pH dependent. No reaction products were observed after 20 min reaction time at pH 6.1. At pH 6.7 and 7.8, *HisTyr* showed an even higher reactivity than the Lys containing peptides, whereas at pH 9.1, cross-linking of *MonoLys* and *DiLys* was faster. The pKa values of the nucleophilic side chains in model peptides were determined to be 6.5 for His, 10.4 for Lys, and 9.8 for Tyr.[37] At pH values 6.7 and 7.8, Lys is thus predominately present in its protonated form and shows low nucleophilicity. For histidine, the percentage of deprotonated molecules is much higher at these pH values due to its lower pKa, and therefore its nucleophilicity is expected to be higher at these pH values. Thus, O-acylation of Tyr and Ser was favored by catalysis of the imidazolyl group. The high reaction yield of 100% for *DiLys* illustrates that the formation of Type-1 cross-links was faster than the formation of Type-0 cross-links.

For the biotinylation of the *N*-terminal protected peptides ENHASFV and EKWAP at pH 8.0, Miller and Kurosky noticed a 14-fold faster reaction for the Lys side chain in comparison with the serine hydroxyl group, based on HPLC measurements.[32,33] Our results demonstrate clearly that with the presence of His in close proximity to Tyr and Ser, the reactivity of Ser and Tyr is on the same order of magnitude as the reactivity of Lys. The same effect is expected to occur for Thr. Thus, the hydroxyl-containing amino acids must be taken into account as reactive amino acids for cross-linking experiments.

Reactive sites on commercial peptides

To apply the newly gained knowledge of amino acid reactivities in well-defined model peptides, we analyzed seven commercial peptides with nanoESI–MS in positive mode. Reaction mixtures were analyzed after 40 h. Thus, no transient species were detected by MS measurements and the stability of the reaction products was proven simultaneously. Control experiments with different cross-linkers and shorter reaction times (1 h) yielded the same types of reaction products, but with lower signal intensity of cross-linked peptides. Since in-source fragmentation of the cross-linked species $P-XL_{OH}$ leads to *m/z* values corresponding to the loss of water, a spectrum of the isolated precursor ion $P-XL_{OH}$ (when present)

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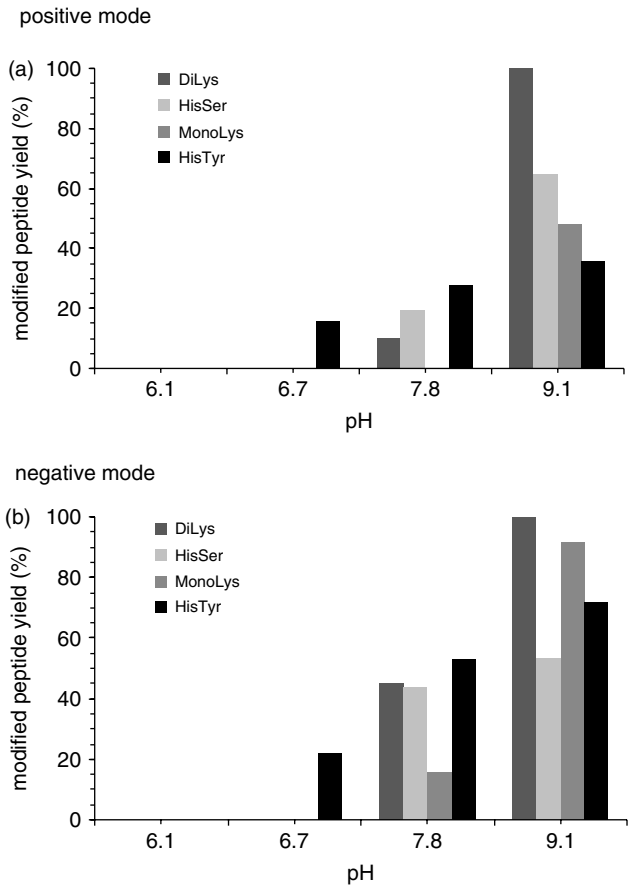

Figure 5. Comparison of reaction yields after 20-min reaction time for protected peptides at pH 6.1, 6.7, 7.8, and 9.1 with a 50-fold molar excess of DSS (volume ratio 1/60, v/v) by MALDI-MS in (a) positive mode and (b) negative mode.

was taken under low collision energy values and the intensity ratio of P–XL_{OH}–18 to P–XL_{OH} was compared with the ratio of P═XL to P–XL_{OH} in the full mass spectrum. The assignment of P═XL was only made if P═XL showed a significantly higher intensity ratio than P–XL_{OH}–18. The maximum number of modified amino acids per detected species and the identified reaction sites are illustrated in Table 2.

In most cases, the peptide sequence included one Lys and/or one free *N*-terminus and additionally Ser, Tyr, Thr, Arg and/or His. The only exception MCD (**F**LPLILG**K**LV**K**GLL-NH_2) was chosen to examine the completeness of the cross-linking reaction for a higher number of primary amines. The MS spectrum was dominated by a peak at *m/z* 831 corresponding to $[P{=}XL+2H]^{2+}$. The Type-1 cross-link could be mainly attributed to an intramolecular link connecting the two Lys. The fully cross-linked species $[P{=}XLXL_{OH} + 2H]^{2+}$ at *m/z* 909 occurred as minor species (data not shown).

For some of the examined peptides, namely Brad, LOF and MBP, the hydroxyl containing-amino acids Ser, Tyr and Thr were present without any His in close proximity. Additionally, two peptides bearing the triad Tyr-Ile-His, namely Ang I/II and Ang I, were investigated. The studied peptide MBP (**VH**FF**K**NIV**T**P**RT**P) contained the primary amino group of the free *N*-terminus, the ε-amino group of lysine and additionally two Thr, one His, and one Arg. We observed four peaks corresponding to reaction products with at least two modified amino acids and a maximum of three attached cross-linkers. For the doubly charged species containing one Type-1 cross-link ($[P{=}XL + 2H]^{2+}$, *m/z* 848) or two hydrolyzed cross-linkers ($[P-(XL_{OH})_2 + 2H]^{2+}$, *m/z* 779), the α- and the ε-NH_2 groups were identified as the main reaction sites by MS/MS measurements (data not shown). Additionally, two doubly charged species with three reaction sites were observed: $[P-(XL_{OH})_3 + 2H]^{2+}$ at *m/z* 1013 and $[P{=}XLXL_{OH} + 2H]^{2+}$ at *m/z* 926. For both species, the primary amino groups were involved in the cross-linking reaction. In the case of $[P-(XL_{OH})_3 + 2H]^{2+}$ (Fig. 6(a)), the complete b-series of b_2XL_{OH} to b_4XL_{OH} modified with a hydrolyzed cross-linker (*m/z* 393, 540, 688), and b_5**2**XL_{OH} to b_8**2**XL_{OH} modified with two hydrolyzed cross-linkers (*m/z* 972, 1086, 1199, 1298) could be identified. The third connecting point of the cross-linker was localized in the peptide range of T9 to T12 (**T**P**RT**) due to the presence of y_5XL_{OH} to y_8XL_{OH} (*m/z* 728, 827, 940, 1054). The high intensity of the unmodified y_4-ion (*m/z* 470) indicated a cross-linker position at T9. However, the presence of a peak at *m/z* 627 corresponding to y_4XL_{OH} (designated with an asterisk) points to a minor species with a modification at R11 or T12. For the species $[P{=}XLXL_{OH} + 2H]^{2+}$ (*m/z* 926, Fig. 6(b)), a Type-1 cross-link connecting K5 and T9 and a Type-0 cross-link at the *N*-terminus V1 were identified as the main modifications. The position of the Type-1 cross-link was deduced from the presence of $y_9{=}XL$ to $y_{12}{=}XL$ (*m/z* 1164, 1311, 1458, 799) and the high intensity of unmodified y_4 (*m/z* 470). Moreover, the b-series of b_2XL_{OH} to b_4XL_{OH} (*m/z* 393, 540, 688) was observed. Since the y-series y_4XL_{OH} to y_8XL_{OH} (*m/z* 627, 728, 827, 940, 1054) was also detected in very low intensity, the fragmentation pattern suggested the presence of an additional minor species (indicated with an asterisk). This included a Type-1 cross-link connecting V1 and K5 and a Type-0 cross-link located on R11 or T12. Summing up the cross-linking

Table 2. Summary of the results of the cross-linking experiments on commercial peptides with DSS at pH 9.1, measured with ESI. The possible reaction sites are marked in bold on the sequence. The amino acids modifications that could be identified by MS/MS measurements are underlined. For Ang I (Angiotensin I) one reaction site could not be unambiguously determined, since the particular species appeared with very low intensity in the MS spectra

Abbreviation	Sequence	Max. number of modified sites	Modified amino acids
MCD	**F**LPLILG**K**LV**K**GLL-NH_2	3	F1, K8, K11
Brad	**P**PGF**S**PF**R**	2	P1, S5
LOF	**I**PEP**Y**VWD	2	I1, Y5
MBP	**VH**FF**K**NIV**T**P**RT**P	3	V1, K5, T9 (evt. R11 or T12)
C3a	FMOC-EAAL**K**LA**R**	2	K5 (R8)
Ang I/II	**VY**I**H**PF	2	V1, Y2
Ang I	**D**R**V**Y**I**H**F**H**L**	3	D1, Y4 (evt. R2)

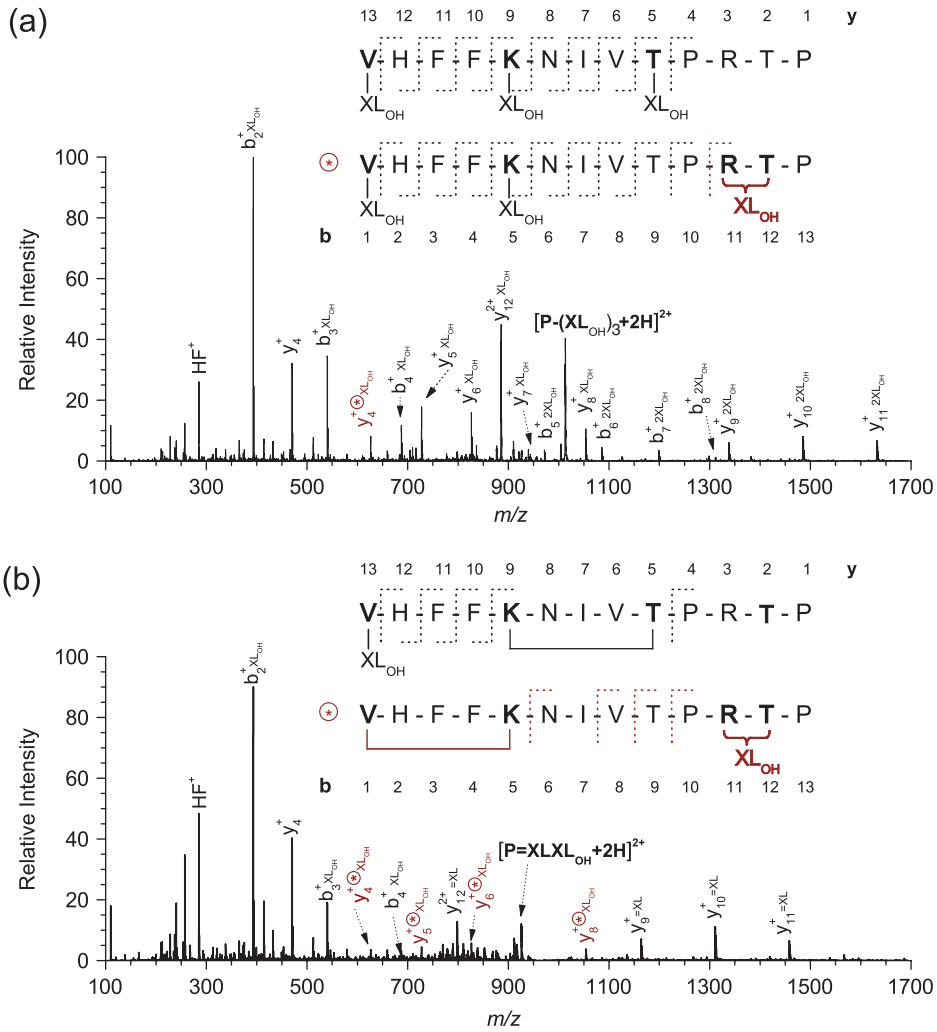

Figure 6. Cross-linking experiment of MBP with DSS. (a) Product ion mass spectrum (positive mode, nanoESI-MS) of MBP modified with three hydrolyzed cross-linkers $[P-(XL_{OH})_3+2H]^{2+}$ at *m/z* 1012 indicated the presence of one main species, where V1, K5 and T9 are modified with a hydrolyzed cross-linker. Owing to the presence of y_4XL_{OH} (marked with an asterisk), the cross-linking of R11 or T12 cannot be excluded. (b) Product ion mass spectrum (positive mode, nanoESI-MS) of MBP modified with one hydrolyzed cross-linker and one Type-1 cross-link $[P{=}XLXL_{OH}+2H]^{2+}$ at *m/z* 926 indicated the presence of two different species. The first structure with a cross-link connecting Lys and Thr was clearly evident from the measured data, since several y═XL-ions were observed. Moreover, the high intensity of $b_{2,3}XL_{OH}$ cannot be explained by fragmentation of the cross-link.

experiments with MBP, threonine was clearly identified as reaction site, but no unambiguous indication was found for a modification of arginine. The reaction product $[P{=}XLXL_{OH}+2H]^{2+}$ occurred with an 17-fold higher intensity than $[P-(XL_{OH})_3+2H]^{2+}$ in the MS spectrum. Assuming comparable ionization efficiencies, the formation of a Type-1 cross-link is thus of higher importance for the reaction behavior of Thr than the formation of Type-0 cross-links. For the peptides Brad (**P**PGF**S**PF**R**) and LOF (**I**PEP**Y**VWD), Ser and Tyr were exclusively modified by a Type-1 cross-link. MS/MS measurements on the Type-0 cross-linked species merely yielded product ions indicating a modification of the *N*-terminus, but not Ser or Tyr, respectively (data not shown).

In the commercial peptides Brad, MBP, and LOF, the amino acids Ser, Tyr and Thr generally were not in close proximity to His. Nevertheless, we detected O-acylation for all of them, mainly as Type-1 cross-links involving Lys or the *N*-terminus on the other side of the cross-linker. Thus, the high local concentration of a cross-linker molecule induced an elevated reactivity. An increased reactivity of Tyr due to Type-1 cross-link formation with the *N*-terminus was also observed with the deprotected synthesized peptide *MonoTyr*, as discussed before. However, the deprotected peptides *MonoSer* and *MonoThr* with the sequences EGGGXGGGE (X = Ser, Thr) did not exhibit any reactivity on Ser or Thr. Thus, additional effects of adjacent amino acids seem to be existent. An assumed interaction of the protonated Arg, as present for Brad and MBP, with the oxygen atoms of the cross-linker in the function of a two-point activator could not be fully supported with the reaction sites observed by Swaim *et al.*[22] or Kalkhof and Sinz.[25] However, a detailed proof of the activation by Arg is far beyond the scope of this study.

To approve the previously discussed His-effect on commercial peptides, Ang I (**DR**V**Y**I**H**PF**H**L) and Ang I/II (**VY**I**H**PF) were studied. In cross-linking experiments, the reaction of Tyr was not only restricted to Type-1 cross-link formation, but also yielded Type-0 cross-links with a similar intensity of $[P{=}XL+2H]^{2+}$ and $[P-(XL_{OH})_2+2H]^{2+}$ (data not shown). The occurrence of $[P-(XL_{OH})_2+2H]^{2+}$ suggests a reaction course analogous to the mechanism proposed in Scheme 2. Hence, the reactivity increase

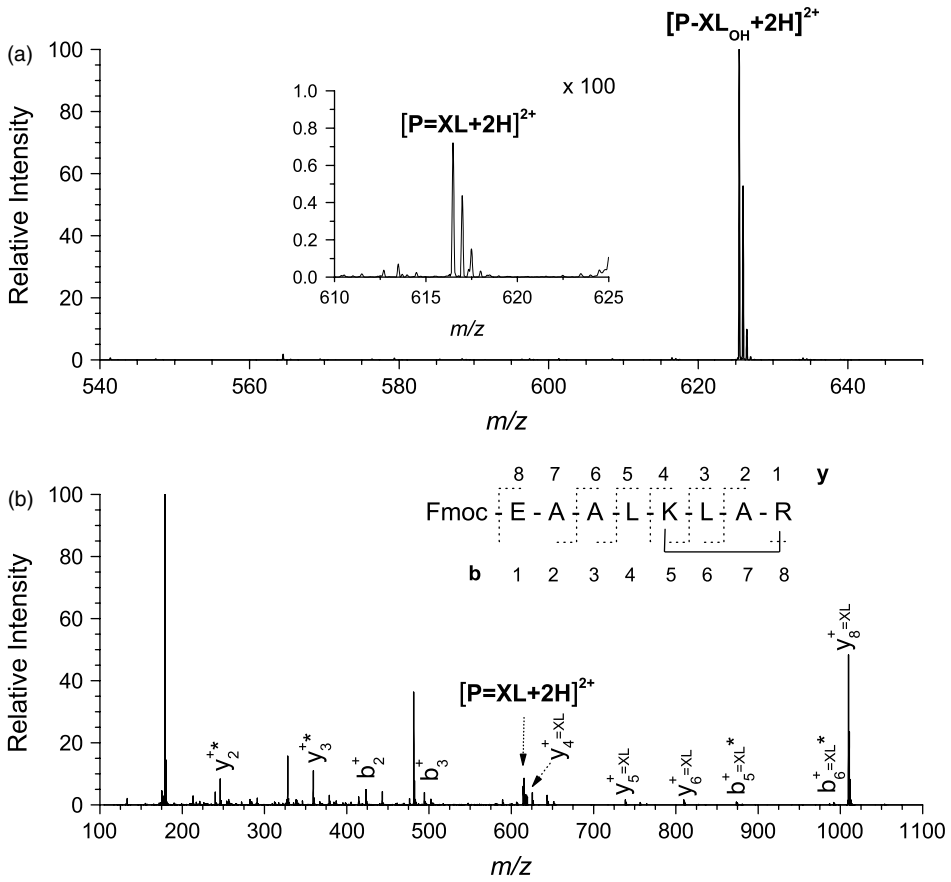

Figure 7. Analysis of the cross-linking products of C3a (Fmoc-EAALKLAR) with positive mode nanoESI-MS (a) MS spectra after 40-h reaction time. The inset spectrum shows the 100-fold enlarged peak of the possible Type-1 cross-link $[P{=}XL + 2H]^{2+}$. (b) Product ion spectra of the Type-1 cross-linked species $[P{=}XL + 2H]^{2+}$. The product ions with an asterisk are expected to result from fragmentation of the link. However, an overlap with $[P - XL_{OH} - H_2O + 2H]^{2+}$ is very likely.

due to the presence of His affects also the product distribution of peptides containing a primary amine as well. For Ang I, a present Type-0,1 cross-linked product could only be identified due to its *m/z* values and characteristic fragment peaks. The position of the cross-linker could not be determined exactly because of the low intensity of the precursor ion and its product ions in the product ion mass spectra.

In an attempt to analyze the reactivity of arginine, the cross-linking products of C3a (Fmoc-EAAL**K**LA**R**) were studied with nanoESI–MS in positive mode (Fig. 7(a), (b)). The Type-0 cross-linked species $[P - XL_{OH} + 2H]^{2+}$ at *m/z* 625 dominated the spectra. MS/MS experiments revealed the modification of K5 with a hydrolyzed cross-linker. Additionally, a minor peak at *m/z 616* corresponding to the mass-to-charge ratio of $[P{=}XL + 2H]^{2+}$ was observed (inset Fig. 7(a)). MS/MS measurements suggested the modification of R8 (Fig. 7(b)). However, the peak intensity of the Type-1 cross-linked species $[P{=}XL + 2H]^{2+}$ was less than 1% of the intensity of $[P - XL_{OH} + 2H]^{2+}$. Moreover, an overlap with a fragment ion of $[P - XL_{OH} + 2H]^{2+}$ after neutral loss of water is probable. As a consequence of the low intensity, the detection of arginine modifications in *bottom-up* or *top-down* experiments on proteins is not expected.

In summary, we could identify Ser, Tyr and Thr as potential reaction targets of the cross-linker in addition to Lys and the *N*-terminus. The identification of a modified guanidino group was only possible for one Type-1 cross-linked species (C3a) and occurred in very low intensity. For all examined peptides both Type-0 and Type-1 modifications were observed. The first reaction site in all cases was expectedly the α-NH_2-group of the *N*-terminus (H_N) or the ε-NH_2-group of lysine. As soon as Type-1 cross-links were formed or additional cross-linkers attached, other amino acids were also involved in the reaction. Minor side products with the same *m/z* values for the precursor ion cannot be excluded, since no chromatographic separation was conducted prior to mass spectrometric detection.

Reactive sites on insulin

Cross-linking experiments on oxidized insulin α-chain (**G**IVEQCCA**S**VC**S**L**Y**QLEN**Y**CN) and β-chain (**F**VNQHLCG**S**HLVEAL**Y**LVCGE**R**GFF**YT**P**K**A) were conducted at pH 9.1 to apply our findings to a more complex system. The reaction products were analyzed by MS/MS experiments (nanoESI–MS, positive mode). For the α-chain, which includes two Ser and two Tyr, the only reaction site detected after 1 h was the α-amino group on the *N*-terminal G1.

For the β-chain, which includes one Lys (K29), one Ser (S9), one Arg (R22) and two Tyr (Y16, Y26), several species with up to four modified sites were identified. No clear evidence of reaction sites other than the primary amino groups was found for products with up to two chemical modifications, $P - XL_{OH}$, $P{=}XL$, and $P - (XL_{OH})_2$. Thus, the *N*-terminus (F1) and the side chain of Lys (K29) were the most reactive sites for the present reaction conditions.

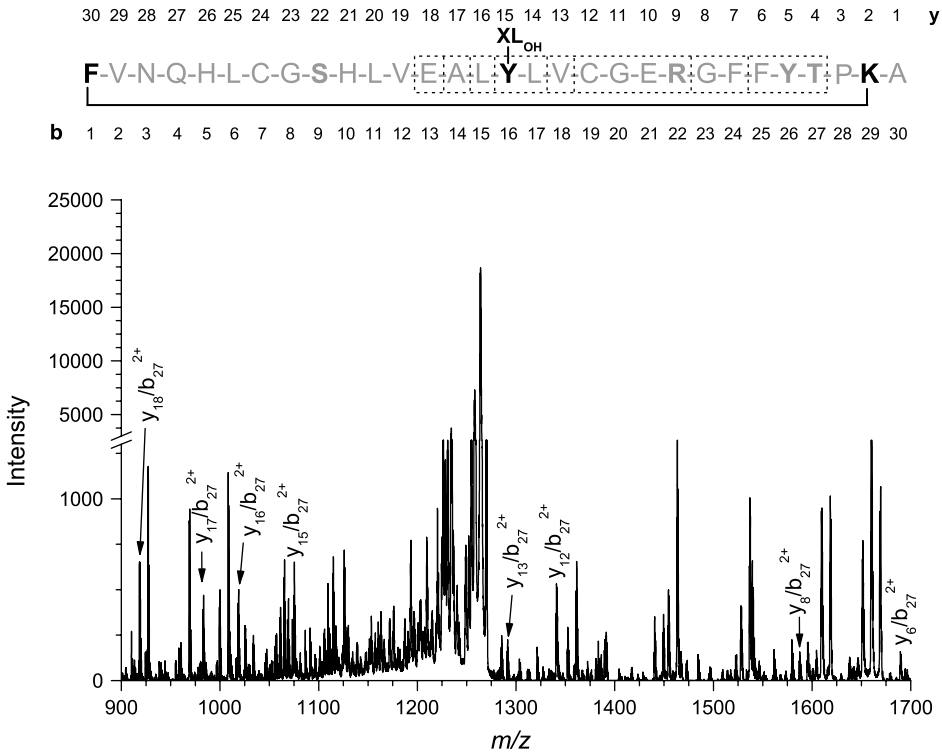

Figure 8. Analysis of oxidized β-chain of insulin after cross-linking at pH 9.1 for 1 h. Product ion mass spectrum (positive mode, nanoESI-MS) of $[P = XLXL_{OH} + 3H]^{3+}$ at *m/z* 1264. The assigned product ions resulted from 'ring-opening' dissociation and indicated a Type-1 cross-link at K29 and F1 and a Type-0 cross-link at Y16.

The most abundant triply charged ion of a cross-linking product with three modified sites (*m/z* 1264; P═$XLXL_{OH}$) was selected and fragmented by CID (Fig. 8). Assuming F1 and K29 as fixed modification sites, 15 different species are theoretically possible, involving either S9, Y16, Y26 or maybe R22 as third reaction site. The determination of the exact position of the Type-1 and the Type-0 cross-link was very difficult because both Type-1 cross-linked product ions (+138 u) and Type-0 cross-linked product ions (+156 u) after neutral loss of H_2O (−18 u) that are isobaric have to be considered. Water loss is common for the amino acids Ser, Thr or Glu, but was also observed for ions still containing the hydrolyzed cross-linker, i.e. P−XL_{OH} and P−$(XL_{OH})_2$. Some of the peaks in the product ion spectra were assigned to species with a Type-1 cross-link between K29 and F1 (Fig. 8). The formation of a Type-1 cross-link between K29 and F1 proposes a solution conformation where these amino acids are in close proximity. Structural studies on solution conformations of oxidized insulin β-chain conducted with NMR spectroscopy suggested an α-helix for residues 9–19, a β-turn (20–23) and an extended conformation for the *C*-terminal region.[38,39] The conformation of the *N*-terminal residues (1–8) was described to be extremely flexible. In molecular dynamics simulations based on NMR distance constraints, the most frequented conformations showed a close proximity between the *N*- and *C*-terminus[40] which is in agreement with the formed Type-1 cross-link between F1 and K29.

Product ions resulted mainly from 'ring-opening' dissociation of the cyclized peptide *N*-terminally to the most labile site P28[41] and additional y-type fragmentation. The doubly charged P═XL−Z ions with Z = EALYLVCGERGFFYT (y18/b27, *m/z* 918), ALYLVCGERGFFYT (y17/b27, *m/z* 983), LYLVCGERGFFYT (y16/b27, *m/z* 1018), and YLVCGERGFFYT (y15/b27, *m/z* 1075), were identified. The Type-0 cross-link was found to be located on Y16, as derived from the presence of doubly charged ions of P═$XLXL_{OH}$−Z with Z = VCGERGFFYT (y13/b27, *m/z* 1291), CGERGFFYT (y12/b27, *m/z* 1341), GFFYT (y8/b27, *m/z* 1587), and FYT (y6/b27, *m/z* 1689). Prior studies on oxidized insulin β-chain at pH 8.4 yielded Type-1 cross-links between Y16 and F1 or Y26 and K29.[23] Product ions corresponding to these species were also observed in the product ion spectra, but could not be assigned unambiguously due to the discussed loss of water. The presence of other species is very likely due to the complexity of the spectra. Nevertheless, no clear evidence was found for a modification of S9, T27 and R22. Although S9 is situated next to His (H10), the lack of cross-linked Ser (S9) is not surprising. In experiments conducted with monofunctional NHS esters,[30–35] a clear dependence of the reactivity on the position of the His was shown. Significant effects were only observed in the sequences His-Gly-Xxx and His-Gly-Gly-Xxx (with Xxx = Ser, Tyr), but not for His-Xxx.

In summary, our results demonstrate that in a more complex system additional effects have to be taken into account. For example, Y16 reacts with the cross-linker even without a base in close proximity and without intralink formation, as opposed to the findings for the linear peptides. Larger biomolecules usually have a folded structure with preferential conformations. Thus, cross-linking of amino acid residues that are far from each other in the primary structure, but close in the secondary or tertiary structure, can occur. Hydrogen bonding and other noncovalent interactions can decrease the pKa of an amino acid side chain and thus increase or decrease its reactivity, as was shown by Guo *et al.*[42]

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Conclusions

We investigated the reactivity of amino acids towards the NHS ester DSS in detail. In addition to the primary amino groups, Ser, Thr and Tyr are of significant importance as reactive sites, in particular, as a result of other amino acids in close proximity, by intermediate or permanent formation of a Type-1 cross-link. For the hydroxyl-containing amino acids with a His in close proximity, reactivities comparable to Lys are obtained at pH 7.8 and 9.1. Decreasing the pH gives reaction yields that are even above that of Lys. For peptides containing several reactive sites, the primary amino groups are the first to react at pH 9.1. As soon as Type-1 cross-links are formed or additional cross-linkers attached, other amino acids can begin to react.

From our results and the results of earlier studies,[22,23,25] structural elucidation of proteins based on chemical cross-linking should be significantly improved. For example, for their conformational studies on PrP^{Sc}, Onisko *et al.*[24] obtained some cross-linked species that could not be assigned when only primary amines and Tyr were considered as reactive site. We suggest that additionally Ser and Thr are taken into account for a more complete assignment.

The cross-linking experiments on synthesized model peptides with a free *N*-terminus did not yield Type-1 cross-linked species involving Ser, Thr or Arg. However, a reaction of these amino acids was observed for the commercial peptides. Therefore, additional effects are supposed to influence the reactivity of the amino acid side chains. We assume that the basic amino acid Arg can increase the reactivity of amino acids by catalysis. To confirm this assumption, further research has to be carried out.

Our investigations focused on small peptides with a linear solution conformation. For more complex proteins, additional interactions, for example, hydrogen bonding, are present. These interactions can induce changes of pKa values and therefore affect the nucleophilicity of some residues. Thus, a much richer pattern of reactivity is expected for larger proteins, and restriction to primary amines (*N*-terminus, Lys residues) as the only sites that react with NHS esters is incorrect.

Acknowledgements

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