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Methyleneation of Peptides by *N,N,N,N*-Tetramethylethylenediamine (TEMED) under Conditions Used for Free Radical Polymerization: A Mechanistic Study

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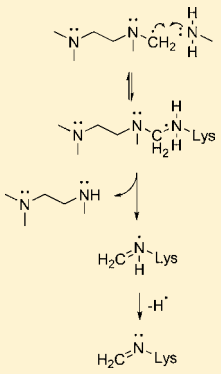
Supporting Information

ABSTRACT: Free radical polymerization is often used to prepare protein and peptide-loaded hydrogels for the design of controlled release systems and molecular imprinting materials. Peroxodisulfates (ammonium peroxodisulfates (APS) or potassium peroxodisulfates (KPS)) with *N,N,N,N*-tetramethylethylenediamine (TEMED) are frequently used as initiator and catalyst. However, exposure to these free radical polymerization reagents may lead to modification of the protein and peptide. In this work, we show the modification of lysine residues by ammonium peroxodisulfate (APS)/TEMED of the immunostimulant thymopentin (TP5). Parallel studies on a decapeptide and a library of 15 dipeptides were performed to reveal the mechanism of modification. LC−MS of APS/TEMED-exposed TP5 revealed a major reaction product with an increased mass (+12 Da) with respect to TP5. $LC-MS^2$ and $LC-MS^3$ were performed to obtain structural information on the modified peptide and localize the actual modification site. Interpretation of the obtained data demonstrates the formation of a methylene bridge between the lysine and arginine residue in the presence of TEMED, while replacing TEMED with a sodium bisulfite catalyst did not show this modification. Studies with the other peptides showed that the TEMED radical can induce methyleneation on peptides when lysine is next to arginine, proline, cysteine, aspargine, glutamine, histidine, tyrosine, tryptophan, and aspartic acid residues. Stability of peptides and protein needs to be considered when using APS/TEMED in *in situ* polymerization systems. The use of an alternative catalyst such as sodium bisulfite may preserve the chemical integrity of peptides during in situ polymerization.



INTRODUCTION

Peptide and protein bioactives ("biopharmaceuticals") are presently an important class of pharmaceuticals due their favorable properties compared with small molecule drugs. They possess high and selective activity, which opens their potential application for the treatment of a variety of diseases. Advances in biotechnology have now created the possibility to produce therapeutically active peptides and proteins on a commercial scale.[1] However, formulating biopharmaceuticals with the optimal therapeutic efficacy as well as optimal stability is highly challenging.[2] Recently, there has been a growing interest in polymeric delivery systems for peptides and proteins to achieve sustained therapeutic concentrations of biopharmaceuticals in the circulation. Polymeric matrices may also protect peptide and protein drugs from degradation and consequently enhance their efficacy.[3] Moreover, in the field of biomolecular analysis there is an increasing interest in peptide and protein imprinted polymers, due to their ability to selectively recognize and bind their targets in competitive media.[4−7]

Free radical polymerization is often used to prepare protein- and peptide-loaded hydrogels for controlled release systems and molecular imprinting.[8−10] For example, Verheyen et al. used free radical polymerization to copolymerize methacrylamide-modified lysozyme molecules with methacrylated dextran for covalent incorporation of the protein in the resulting hydrogel network and intracellular triggered release of this enzyme.[11] In a recent study, it was reported that human serum albumin (HSA) and immunoglobulin G (IgG) could be separated by molecularly imprinted hydrogels.[12]

Potassium peroxodisulfate (KPS) and ammonium peroxodisulfate (APS) with tetramethylenediamine (TEMED) are frequently used as initiator and catalyst for the preparation of hydrogels from hydrophilic building blocks. However, these reagents may cause unwanted modifications of the protein or peptide drugs during gel formation, which could result in structural changes and loss of biological activity.[13] Several studies investigated possible chemical modification of protein and peptide biopharmaceuticals that occur during the

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formulation.[14,15] For example, with the aim to obtain hydrogels that release recombinant human interleukin (rhIL-2) in a controlled manner, Cadée et al. initiated the cross-linking reaction of an aqueous solution of methacrylated dextrans containing the protein by the addition of KPS and TEMED. It was found that the methionine residues in rhIL-2 are susceptible to oxidation by KPS.[16] Schillemans et al. also found oxidation of cytochrome C when they added the protein to the hydrogel precursors and subsequently initiated network formation by the addition of APS/TEMED. On the other hand, postloading of preformed gels with protein did not result in oxidation of the protein.[9] Kafka et al.[17] prepared poly(ethylcyanoacrylate) (PECA) nanoparticles loaded with the bioactive D-Lys6-GnRH peptide by an *in situ* interfacial polymerization process using a w/o-microemulsion containing the peptide in the dispersed aqueous phase. The peptide D-Lys6-GnRH was reactive with the ethylcyanoacrylate (ECA) monomer, resulting in copolymerization of the peptide with the monomer. MALDI TOF/TOF (tandem) MS analysis revealed that the histidine residue of D-Lys6-GnRH interacts covalently with the ECA monomer during the polymerization process. The reaction mechanism suggested a nucleophilic attack of the histidine side chain of the peptide to the ECA monomer to initiate the reaction resulting in the covalent attachment of the peptide to the polymer.[17] In a more recent study, they showed that the C-terminal glutamic acid residue of the fragments of insulin-like growth factor 1 (IGF-1) covalently binds to poly(alkylcyanoacrylate) (PECA) during polymerization.[18]

The aim of the present study was to investigate the possible modification of the bioactive peptide thymopentin (TP5) under conditions used for preparation of imprinted hydrogels. Thymopentin is a well-known immunomodulatory drug that is the active segment 32−36 of thymopoietin (human thymus hormone, which contains 49 amino acids). TP5 is a synthetic pentapeptide with the following sequence: Arg−Lys−Asp−Val−Tyr.[19] This peptide is used for the treatment of autoimmune diseases such as chronic lymphatic leukemia, rheumatoid arthritis, and atopic dermatitis. Moreover the peptide causes the induction of T cell differentiation.[20−22] Potential therapeutic effects of TP5 as well as its sequence that contains a variety of functional groups that are present in other therapeutic peptides and proteins brought us to use it as a model for preparing peptide imprinted polymeric networks. In the present work, the effect of the initiator/catalyst (APS)/TEMED on the integrity of thymopentin under conditions used for preparation of imprinted hydrogels was investigated.[23]

Structural characterization of the peptide was carried out with LC−ion-trap mass spectrometry (ITMS) employing electrospray ionization (ESI). Using ion fragmentation by collision-induced dissociation (CID), ITMS is able to perform both MS/MS and MS^3 providing detailed structural information. Low energy CID yields predominantly b-ions and y-ions of peptides in MS/MS that can be further fragmented in MS^3 to reveal the nature and site of peptide modifications.[24,25] To gain more insight into the specificity and mechanism of peptide modification, similar experiments were carried out with other peptides, including one decapeptide and a library of 15 dipeptides.

RESULTS

Analysis of Thymopentin Exposed to APS/TEMED. In an attempt to prepare a thymopentin (TP5) imprinted hydrogel using APS/TEMED as the initiator and catalyst,[23] after washing the hydrogel to extract the peptide template, the washing fractions showed new satellite peaks in the UPLC chromatogram (Supporting Information, Figure S1). However, preparing the imprinted TP5 hydrogel with KPS/$NaHSO_3$ did not show these extra chromatogram peaks in the extracts. To gain insight into this phenomenon, we decided to investigate the effect of the APS/TEMED on the integrity of TP5. Therefore, TP5 was incubated with APS with or without TEMED and subsequently analyzed by LC-MS. The LC-MS chromatograms of incubated TP5 with APS/TEMED showed two peaks, corresponding to TP5 and a degradation product, similarly to the observation of the washing fractions of the imprinted hydrogel. Figure 1 shows the base peak chromatogram of TP5 incubated with APS/TEMED. The mass spectrum of the first main peak (retention time 10.7 min, indicated in Figure 1 as A) showed intense signals at m/z 680.3 and 340.6, corresponding to singly and doubly protonated native TP5 (MW = 679.4 Da), respectively. The second main peak (degradation product, retention time 11.2 min; indicated in Figure 1 as B) exhibited intense signals at m/z 692.3 and 346.6, indicating that thymopentin modification encompassed a mass increase of 12 Da. Based on the exact mass measurement (Table S1, Supporting Information), the modified compound could be identified as thymopentin with an extra carbon atom. The extent of modification was temperature dependent: incubation of TP5 with APS/TEMED at room temperature for 16 h also resulted in modification of TP5, but the degradation (based on peak integrals) was 20% compared with 56% at 50 °C. In contrast, TP5 incubated only with APS (and thus in the absence of TEMED) at 50 °C did not show the extra peak at 11.2 min.

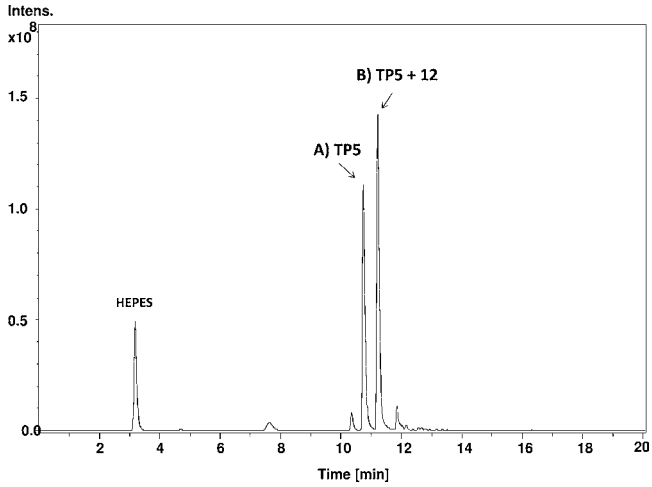

Figure 1. Base peak chromatogram obtained by LC−MS of TP5 incubated with APS/TEMED at 50 °C for 16 h. (A) TP5 $[M + H]^+$ = 680.3; (B) main degradation product $[M + H]^+$ = 692.3.

In order to investigate the identity of the main degradation product and obtain structural information about the nature of the observed peptide modification, the sample incubated with APS/TEMED was analyzed by LC−MS^2. MS^2 detection was performed on precursor ions with m/z 680.3 (TP5) and 692.3 (modified TP5). Figure 2 shows the resulting tandem mass spectra. The spectrum of TP5 (Figure 2A) nicely shows the expected b_2, b_3, and b_4 ions ascribed to cleavage of the peptide bonds that contain the N-terminus and y_1 ions that contain the C-terminus. The peak at m/z 517.4 is attributed to the

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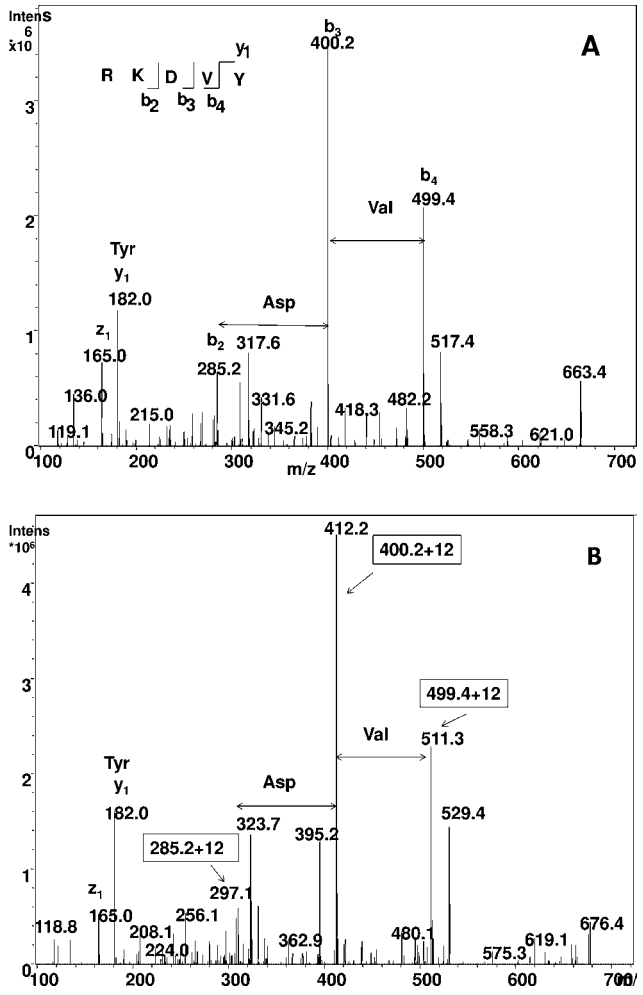

Figure 2. Tandem mass spectrum of (A) TP5 (precursor ion, $[M + H]^+$ = 680.4) and (B) modified TP5 (precursor ion, $[M + H]^+$ = 692.3).

formation of ($b_4 + H_2O$).[26] Figure 2B shows the tandem mass spectrum of the modified TP5 (precursor ion, m/z 692.3). In line with the mass of the protonated molecular ion, the m/z of all observed b ions (b_2–b_4) for the modified TP5 also increased by 12 Da compared with the native peptide. Also m/z 517.4 ascribed to ($b_4 + H_2O$) shows this mass increase of 12 Da. However, the m/z of the observed y_1 and z_1 ions, which contains the tyrosine residue, are unmodified. From these data, it is concluded that the modification that leads to the mass increase of 12 Da occurs either on the arginine or the lysine residue of TP5.

To find out the site of modification (lysine or arginine) in the APS/TEMED exposed TP5, LC–MS^3 was performed, using the b_2 ions observed with tandem MS detection as precursor ions (m/z 285.2 and 297.2 for the native and modified peptide, respectively). Figure 3A shows the MS^3 spectrum of the Arg-Lys residue of native TP5. The peak at m/z 157.1 is due to the protonated arginine residue fragment. The peak at m/z 129.1 can be ascribed to either a fragment of Lys[27] or the Arg-immonium ion, whereas the main peak at m/z 268 is the Arg-Lys residue that lost ammonia. Figure 3B shows the MS^3 result on m/z 297.2 (b_2 ion) of the modified peptide. The overall spectral pattern is quite different from that of the corresponding fragment of the native peptide. The only similarity is a peak corresponding to the loss of ammonia at

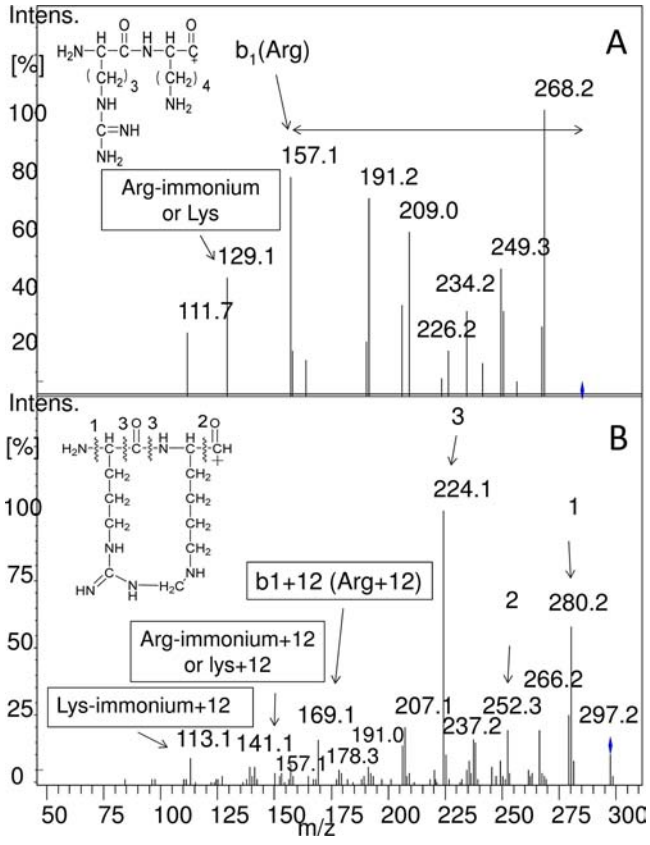

Figure 3. MS^3 spectrum of (A) TP5 (precursor ions, 680.3 → 285.2), (B) modified TP5 (precursor ions, 692.3 → 297.2); peak 1 at m/z 280.2, Δm −17, corresponds to loss of ammonia; peak 2 at m/z 252.2, Δm −45, loss of ammonia and carbonyl; peak 3 at m/z 224.1, Δm −45, loss of ammonia and 2× carbonyl.

m/z 280 (Δm = −17). The peaks at m/z 157.1 and m/z 169.1 are ascribed to the protonated arginine residue and increment of +12 on arginine residue, respectively. On the other hand, the peak at m/z 113 is ascribed to the modified immonium ion of lysine.[28] In other words, the +12 Da mass extra can be tagged both on arginine and lysine residues, which suggests that the modification is not the result of simple modification of either of the two amino acids by a group of 12 mass units. The mass at m/z 252.2 (Δm = −45) is related to loss of ammonia and the terminal carbonyl group (fragmentations 1 and 2, see inset of Figure 3B). The mass at m/z 224.1 can be explained by cleavage of the peptide bond between arginine and lysine and subsequent loss of the carbonyl group of that peptide bond (fragmentations 3, Figure 3B), while the arginine and lysine remained connected to each other with the extra mass of 12. We therefore conclude that a methylene bridge is formed between the two residues, as shown in the inset of Figure 3B. This will be further explained in the Discussion section (*vide infra*).

Analysis of the Decapeptide Exposed to APS/TEMED. To further investigate whether the peptide modification occurs either on the lysine or on the arginine residue or on both, another peptide (a decapeptide with the sequence QKSLSLSPGK) containing two lysines but lacking an arginine residue was incubated with APS/TEMED at 50 °C overnight and analyzed with LC–MS. Figure 4A shows the LC–MS results of the native decapeptide. Actually, two peaks are observed in the chromatogram showing the presence of an

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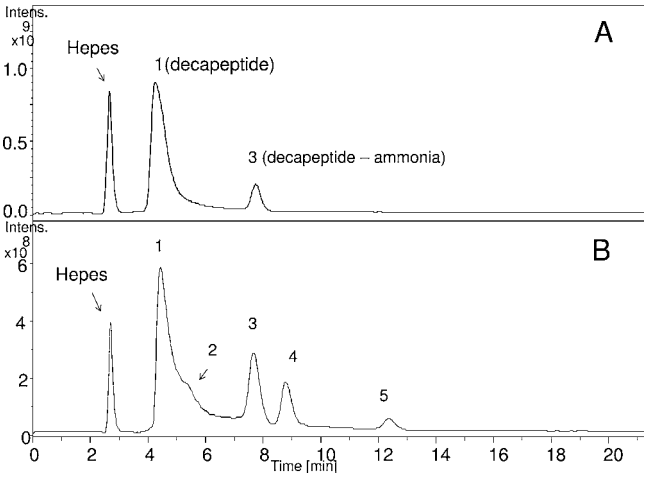

Figure 4. Base peak chromatogram of (A) native decapeptide obtained by LC–MS and (B) the decapeptide incubated with APS/TEMED at 50 °C overnight obtained by LC–MS.

impurity with a mass that is 17 Da less than that of the original decapeptide, indicating elimination of ammonia. Indeed it is known that the N-terminal glutamine of peptides undergoes NH_3 elimination in solutions to form a cyclic pyroglutamyl residue.[29]

Figure 4B and Table 1 shows the LC–MS result of the decapeptide incubated with APS/TEMED. Again, a peak was

Table 1. Identification of the Major Peaks Shown in Figure 4

peak	$[M + H]^+$ m/z	$[M + 2H]^{2+}$ m/z	Δm (Da)
1	1044.7	523.0	native decapeptide
2	1068.8	529.0	+12
3	1027.8	514.5	−17 (NH_3)
4	1068.8	535.0	+24
5	1039.8	520.5	−17 (NH_3) + 12

observed with additional mass of +12 Da (peak 2, appearing as a shoulder of the native peptide) and a peak that could be attributed to the pyroglutamyl impurity with an additional mass of +12 Da (peak 5). Even a peak with an extra mass of +24 was observed (peak 4).

Similar to that for TP5, MS^2 was performed for revealing the site of modification of the decapeptide incubated with APS/TEMED. Figure 5A,B,C shows the MS^2 of native decapeptide, decapeptide + 12, and decapeptide + 24, respectively. The fragments that were obtained from the peaks of the decapeptide impurity (peaks 3 and 5, loss of ammonia) were the same as that of the native one and are therefore not discussed further. Figure 5A provides information about the sequence of the native decapeptide. Most of the y-ions can be seen in the spectrum and the peak at m/z 240 is ascribed to the residue of the amino acids glutamine and lysine minus ammonia (ion b2 − 17). Comparing the spectrum of the modified peptide (Figure 5B) with that of the native decapeptide reveals a striking mass at m/z 252.1, which indicates that the +12 adduct occurred on fragment b_2 − 17. In Figure 5C, the mass at m/z 264.1 suggests that the +24 adduct occurred on the same residues of lysine and glutamine. There are no indications that the C-terminal lysine has been modified because all original y ions until y_8 remained in all spectra of both the unmodified and modified peptide. These observations strongly suggests that the

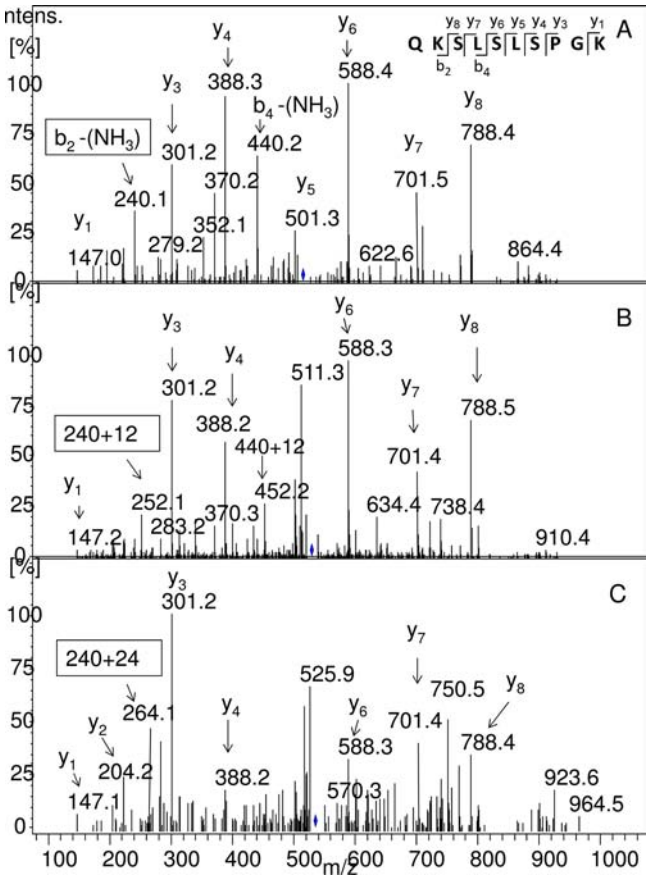

Figure 5. Tandem mass spectrum of (A) native decapeptide (precursor ion, $[M + 2H]^{2+}$ = 523.0), (B) modified decapeptide (precursor ion, $[M + 2H]^{2+}$ = 529.0), and (C) modified decapeptide (precursor ion, $[M + 2H]^{2+}$ = 535.0).

lysine modification is dependent on the nature of the neighboring amino acid.

Incubation of Dipeptides from a Library with APS/TEMED. In order to gain more insight into the neighboring effect of lysine modification when exposed to APS/TEMED, 15 dipeptides of a library with C-terminal lysine and different N-terminal amino acids were incubated with APS/TEMED at 50 °C overnight. Table 2 shows that +12 Da adducts were detected to different extents when lysine was next to arginine, glutamine, proline, cysteine, aspargine, glutamine, histidine, tyrosine, tryptophan, and aspartic acid.

For a detailed comparison, MS/MS experiments were carried out on the modified dipeptides to determine the position of modification. Figure 6A,B shows the tandem mass spectra of native Arg-Lys (precursor ion m/z 303.0) and modified Arg-Lys (precursor ion m/z 315.0), respectively. Close inspection of these MS^2 spectra shows that the native Arg-Lys b_2 ion (m/z 285) underwent neutral loss of ammonia and water, whereas the modified b_2 ion (m/z 297) did not undergo loss of ammonia. Interestingly, further fragmentation of the modified one lead to production of an ion at m/z 224.0, which was also observed in modified TP5 (*vide supra*) but was absent in the native dipeptide. This again suggests that modified Arg-Lys remains connected after full fragmentation of the peptide backbone.

The tandem mass spectra of native and modified dipeptides 3, 5, 6, 7, 8, 9, 11, 12, and 14 from Table 2 are shown in the Supporting Information (Figures S2–S10). The MS/MS data

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Table 2. Extent of Modification of Dipeptides from a Library That Contains C-Terminal Lysines When Exposed to APS/TEMED

deptide no.	dipeptide sequence	+12 modification	relative intensity, %, of +12	+24 modification
1	Lys-Lys	no		no
2	Arg-Lys	yes	10	yes
3	Pro-Lys	yes	43	no
4	Thr-Lys	no		no
5	Cys-Lys	yes	68	no
6	Asn-Lys	yes	75	no
7	Gln-Lys	yes	29 for native and 14 for -NH3	no
8	Glu-Lys	yes	5	no
9	His-Lys	yes	9	no
10	Phe-Lys	no		no
11	Tyr-Lys	yes	5	no
12	Trp-Lys	yes	90	no
13	Ser-Lys	no		no
14	Asp-Lys	yes	1.5	no
15	Met-Lys	no		no

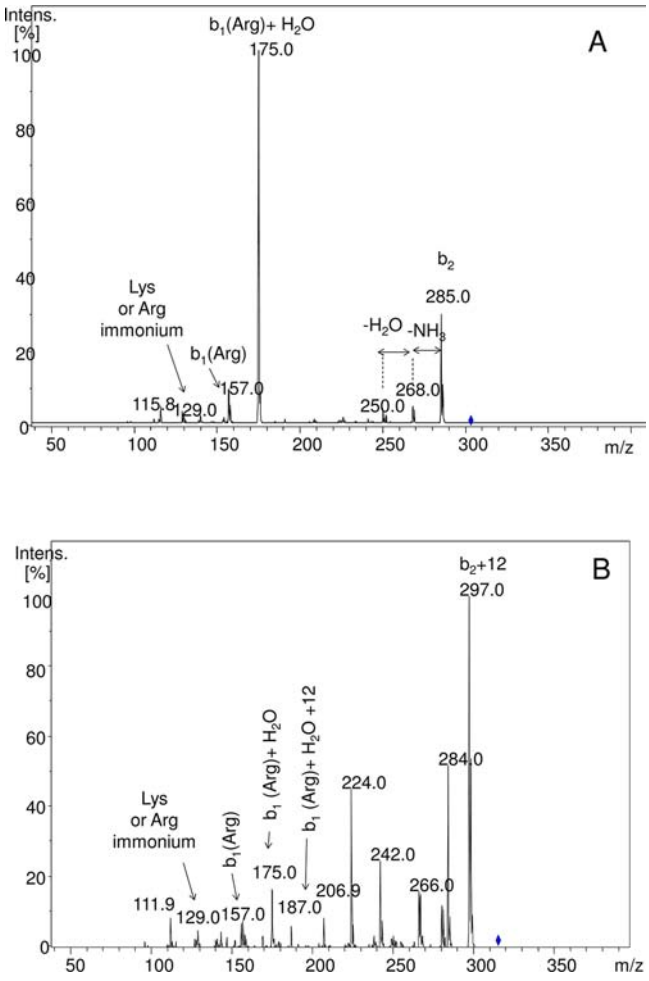

Figure 6. Tandem mass spectra of dipeptide no. 2 (Arg-Lys): (A) native, precursor ion m/z 303.0, (B) modified, precursor ion m/z 315.1.

indicated +12 addition on the lysine residue of the Pro-Lys, Asn-Lys, Gln-Lys, Glu-Lys, Tyr-Lys, and Asp-Lys dipeptides and not on the lysine residues of Cys-Lys, His-Lys, and Trp-Lys.

^{1}H NMR Spectroscopic Analysis Modified Trp-Lys. The dipeptide that had the highest degree of modification, Trp-Lys (see Table 2, peptide no. 12), was isolated by HPLC in sufficient quantity for NMR analysis. The ^{1}H NMR spectrum of this compound was compared with that of native Trp-Lys (Figure 7).

The most striking observation that emerges from the ^{1}H NMR spectra is peak 5, which is ascribed to the chemical shift of the CH in the pyrrole ring (δ 7.25 ppm, 1 proton singlet) of the native dipeptide. This peak was not observed in the modified dipeptide spectrum, which showed a new peak with a chemical shift of 4.3 ppm (peak 13; 2 protons) that overlapped with the chemical shift of the α CH of the backbone lysine (peak 8). Moreover, peak 6 (δ 3.05 ppm) of the CH_2 of native tryptophan shifted slightly upfield in modified Trp-Lys (δ 2.91 ppm) and peak 7 (δ 4.02 ppm) of the α CH of the backbone of native tryptophan shifted slightly downfield in modified tryptophan (δ 4.48 ppm). It is remarkable that peaks assigned to the lysine residue did not change position in the modified peptide.

DISCUSSION

It is clear from the presented results that the 12 Da mass increase happened for TP5 on the Arg/Lys sequence. We observed 56% of TP5 being modified at the site of Arg-Lys when incubated overnight by APS/TEMED at 50 °C. Importantly, the same modification (although to a somewhat lower extent) was also observed in the Arg-Lys dipeptide of the peptide library (Table 2) when incubated under the same conditions.

The major peak observed in the LC−MS^3 spectrum (Figure 3B) of the modified TP5 fragment at m/z 224.1 can be explained by breaking of the peptide bond with loss of ammonia and a carbonyl group, while the arginine and lysine still remain covalently connected involving a gain of mass of 12 Da. Interestingly, this only happened when the catalyst TEMED was present and not when TEMED was absent or replaced by $NaHSO_3$, which convincingly demonstrates the involvement of TEMED in the modification of the peptide. We propose that the TEMED acts as a carbon source providing the extra mass difference, by the formation of a methylene bridge between arginine and lysine caused by TEMED radicals. The proposed mechanism of this reaction will be discussed further on (*vide infra*).

Our observations that TP5 and the decapeptide are methylated at the lysine residues led us to investigate a library of dipeptides each containing at least one lysine. For the different dipeptides studies in this paper (Table 2), under the same conditions, the relative intensity of +12 modification varied from 1.5% (Asp-Lys) up to 90% (Trp-Lys), which suggests that the reactivity of the amino acid residues for methyleneation is different. For example, we observed that the presence of the amide group of asparagine or glutamine next to lysine gives higher reactivity than with the carboxylic acid group of aspartate or glutatmate. The guanidine group of arginine is also a reactive site in the dipeptide, although to a lesser extent than in TP5 (approximately 10% and 50% of Arg-Lys sequence were modified, respectively). On the other hand, the Gln-Lys sequence, which was present both in the dipeptide library and in the decapeptide, showed a higher degree of modification in the dipeptide than in the decapeptide. Apparently, there is an

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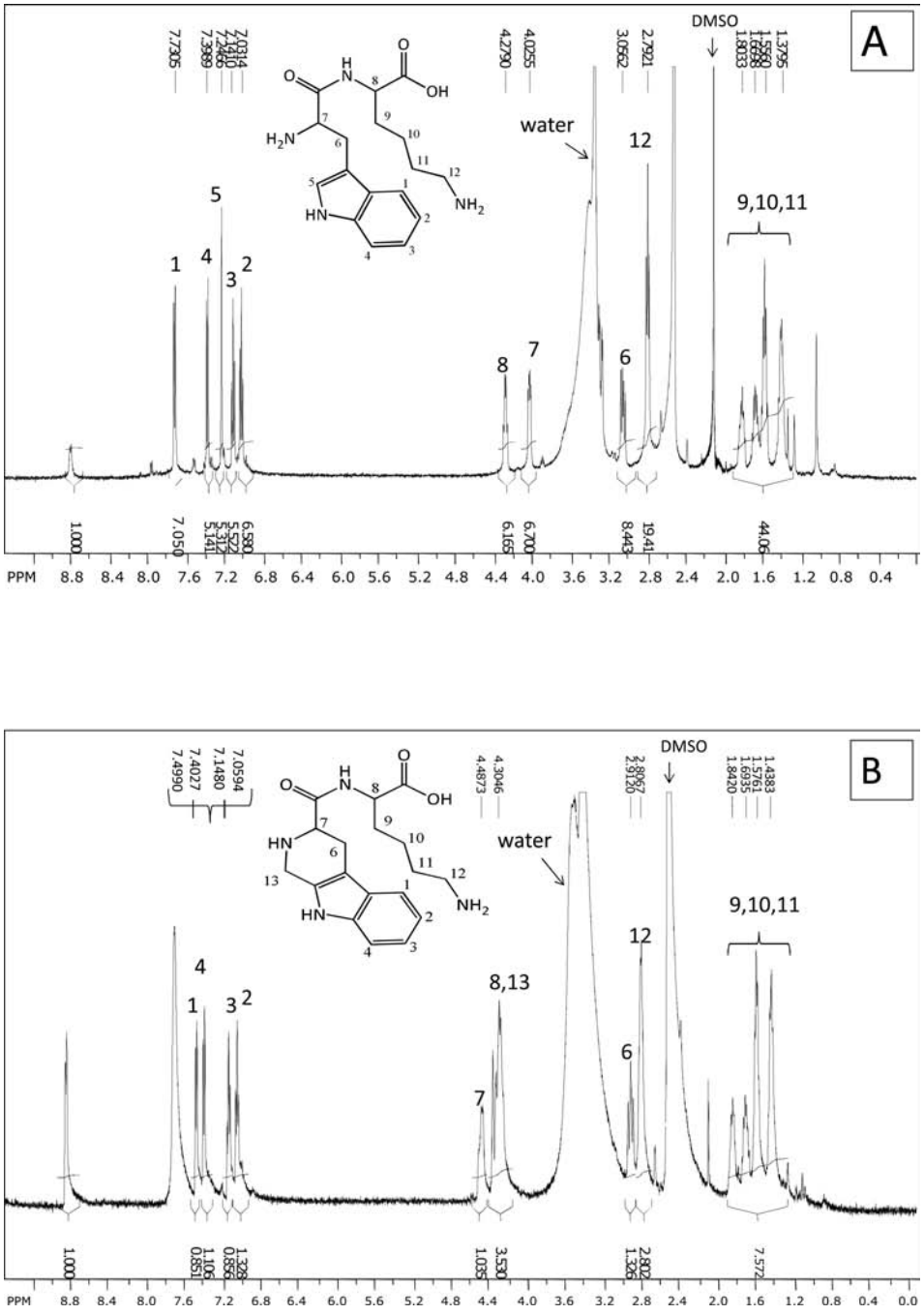

Figure 7. [1]H NMR spectrum of native (A) and modified (B) Trp-Lys (dipeptide no. 12, see Table 2) in deuterated DMSO.

effect of the total sequence or the accessibility of residues in the peptides. It is important to note that the second (C-terminal) lysine of the decapeptide was not modified, which also indicates that methyleneation of a lysine residue depends on the nature of the neighboring amino acids. Primary aliphatic alcohols (serine and threonine), primary amine and the thioether of methionine next to lysine are not reactive.

Mechanism of Methyleneation. Interestingly, methylene bridge formation between amino acids has been reported before to occur from the reaction of peptides with formaldehyde, where the latter acts as a carbon source for additional masses of 12 Da.[28,30] It was demonstrated that lysine is methylated by formaldehyde to form $-N{=}CH_2$ units, followed by the nucleophilic addition of a second amino acid to the N= C double bond. However, as discussed above, in our case the methylene source must be TEMED, and also here we propose that methyleneation of lysine is the first step in the reaction sequence. According to the known formation of radicals from peroxodisulfate and TEMED (Figure 8),[31] the most probable modification is the addition of a methylene group from the TEMED radical, by the mechanism that we propose in Figure 9. First, attack of a TEMED radical on the primary amine of lysine occurs, followed by a hydrogen atom shift from the amine of the lysine to the amine of TEMED. Importantly, this hydrogen atom shift, which is followed (or preceded) by a hydrogen radical shift, is essential to cause the liberation of a methylated lysine. In principle, these reactions are all reversible and may be independent of the neighboring amino acids. The

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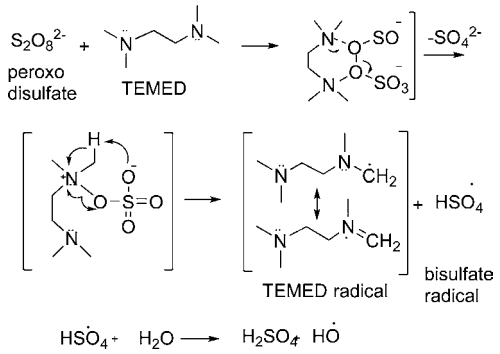

Figure 8. Formation of radicals from peroxodisulfate and TEMED[31]

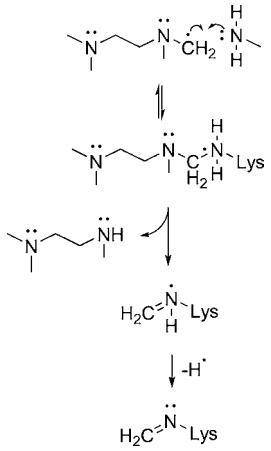

Figure 9. Proposed mechanism of methyleneation of Lys by the TEMED radical.

question now is, why the extent of methyleneation is dependent on the type of neighboring amino acid. Two explanations can be given: (1) the shift of the H atom is catalyzed by appropriate neighboring functional groups, or (2) the driving force that leads to completion of the reaction, rather than reversing to the original situation, could be the subsequent methylene bridge formation with the neighboring amino acid (if the neighboring amino acid is prone to that).

Metz et al.[28,30] reported that no bridges were formed between two primary amino groups, which is in accordance with the lack of modification of Lys-Lys (peptide no. 1, Table 2) from our dipeptide library. We observed methyleneation of the peptide when the following functional groups were present next to the lysine: (1) guanidine (from Arg); (2) amide (from Gln and Asn); (3) carboxylic acid (from Glu and Asp); (4) phenol (from Tyr); (5) thiol (from Cys); or (6) secondary amine in a ring structure (from Pro, His, Trp).

Therefore, if catalyzing the H atom shift is the driving force for methyleneation, it seems that most protic functionalities that are able to add and at the same time release protons might be responsible for the catalytic effect, except for nonconjugated alcohols (Thr, Ser) and primary amines (Lys). Interestingly, for all the amino acids Arg, GLn, Asn, Glu, Asp and Tyr, the H atom shift can be explained by a concerted mechanism with the formation of an intermediate eight-membered ring transition state according to Figure 10 and is thus thermodynamically quite favorable. The fact that no methyleneation with Thr-Lys, Ser-Lys, or Lys-Lys was observed could be due to insufficient catalytic effect of primary aliphatic alcohols or primary amine in

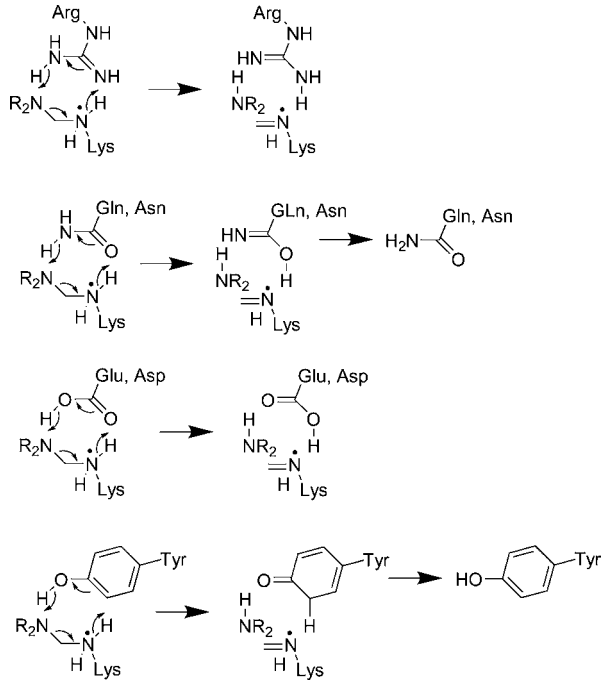

Figure 10. Proposed mechanism of H atom shift in TEMED−Lys radical adduct, induced by different neighboring amino acid residues, with the liberation of a methylated lysine radical.

the proton transfer reaction that would otherwise drive the methyleneation of lysine to completion. Although it should be stressed that the proposed mechanism of Figure 10 is hypothetical and not yet experimentally proven (e.g., by stable isotope experiments for H shifts or by radical trapping), it can very well explain the observed neighboring amino acid selectivity of the reaction.

In principle, the resulting methylene-substituted lysine units (with mass increment of 12 Da compared with the unmodified lysine) could exist as such; however, as explained above, the LC−MS[3] results of TP5 suggest that a methylene bridge had been formed between arginine and lysine. Interestingly, methylene bridge formation between methylated lysine and neighboring arginine has been reported previously by Metz et al. (methylated lysine being a reaction product with formaldehyde in that case).[28] Indeed, methylene bridge formation can be easily explained by addition of arginine to the C═N double bond of the methylated lysine according to the mechanism proposed in Figure 11. Likewise, the study of

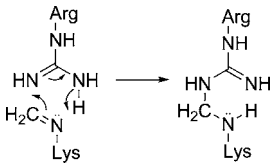

Figure 11. Arg-Lys bridging.

Metz et al. showed that in the presence of formaldehyde and glycine, coupling of glycine to peptides via methylene bridge occurred with the following amino acids: His, Arg, Gln, Asn, Tyr, and Trp. Interestingly, we observed methyleneation in all dipeptides containing these amino acids as well.

With the proposed mechanism of methyleneation and possible bridge formation, we can now analyze the mass

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spectra of the individual dipeptides. Generally, fragmentation of lysine-containing peptides in MS/MS produces a peak with m/z 147 (C-terminal y_1 as the result of the splitting of the peptide bond) and two immonium ions, a major one at 84 Da (which lacks the ε-NH_2 group) and a minor peak at 101 Da (Figure 12).[28] Several dipeptides after incubation with APS and

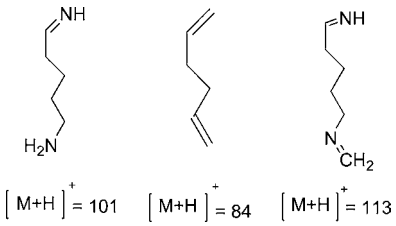

Figure 12. Structure of lysine immonium ion at m/z 84 and 101 and methylated lysine immonium ion at m/z 113.[28]

TEMED showed fragmentation peaks in the MS/MS spectra that are indicative of methyleneation of lysine, that is, at m/z 159 and at m/z 113, which are related to Lys + 12 (y_1 + 12) and the immonium ion of Lys + 12 (101 + 12) respectively. These fragment ions with increment of 12 Da can be found for the methylated dipeptides that contain proline (peptide 3, Figure S2B, Supporting Information), aspargine (peptide 6, Figure S4B, Supporting Information), glutamine (peptide 7, Figure S5B, Supporting Information), glutamic acid (peptide 8, Figure S6B, Supporting Information), tyrosine (peptide 11, Figure S8B, Supporting Information), and aspartic acid (peptide 14, Figure S10B, Supporting Information). However, we cannot conclude whether either single methyleneation happened on lysine without bridge formation, or the increment of 12 Da on lysine fragment ions is a result of cleavage in CID of a possible methylene bridge. It should be noted that the covalent bond of the methylene bridge can break during MS/MS analysis, resulting in a 12 Da mass shift tag on one of the two bridged residues, depending on the stability of the ions produced in CID.[32] For example, in dipeptide 14, +12 adduction can be found both on lysine and on aspartic acid (Asp + 12, b_1 at m/z 127.9), which supports the methylene bridge formation between these two amino acids (Figure S10B, Supporting Information).

In dipeptide no. 3 (Pro-Lys), methyleneation occurred to a relatively large extent (43%), but tandem mass data (Figure S2B, Supporting Information) shows that the +12 can be tagged on lysine only. Proline was also present in the middle of the decapeptide, but this residue was not methylated. Methylene bridge formation was not reported by Metz et al.[28,30] for formaldehyde and glycine treated peptides that contain proline. However, Toews et al.[33] pointed to single modification of N-terminal proline in a peptide with the sequence of PGHDPPISYYETN-NH_2 after treating with formaldehyde. They observed a fragment ion of b_2 + 12, which localizes on Pro-Gly. They concluded that, because glycine is not susceptible to formaldehyde modification, the 12 Da increment was exclusively present on the amino-terminal proline.[33] However, our study does not support single methyleneation on proline because the +12 modification was tagged on lysine. Yet, we cannot conclude whether methyleneation happened exclusively on lysine or is a result of cleavage of a methylene bridge by collision induced dissociation during the MS analysis.

For dipeptide 7 (Gln-Lys, Figure S5, Supporting Information), the peak at m/z 113.0 that is ascribed to the immonium ion of Lys + 12 can be detected, but because the lysine and glutamine have isobaric masses, it is not possible to conclude whether the peak at 140.9 is due to Lys + 12 or Glu + 12. However, because of the m/z 113.0, we can conclude that at least lysine is methylated.

From the reaction of tryptophan with formaldehyde, Metz et al. reported the formation of a single methyleneation product (imine formation on the nitrogen atom of the pyrrole ring without bridge formation) in a peptide with the sequence of Ac-VELWVLL−OH.[28] They observed that the typical immonium ion of tryptophan (159 Da) was lost after the reaction with formaldehyde and a new fragment appeared (171 Da), indicating that the tryptophan residue was modified. They did not detect any other new mass related to cross-linked peptide. However, when the mentioned peptide was treated with glycine and formaldehyde, coupling of glycine to tryptophan via a methylene bridge occurred. In our dipeptide 12 (Trp-Lys), also the fragment at m/z 159.0 ascribed to the immonium ion of native tryptophan vanished after treating with APS/TEMED, while a new fragment at m/z 171.0 ascribed to the methylated immonium ion of tryptophan was observed (Figure S9, Supporting Information). These MS data alone are not strong enough to draw a conclusion for single methyleneation. However, our NMR data from the modified Trp-Lys (Figure 7B) are indicative of methylene bridge formation, not with the lysine but with the amine terminus of the peptide through a ring closure with the CH in the pyrrole ring.

For the dipeptide 9 (His-Lys), the fragment of histidine + 12 was detected (Figure S7B, Supporting Information). No other fragments related to methyleneation of lysine or cross-linking between lysine and histidine were found. Because the NMR data for tryptophan showed the bridge formation between the N terminus and the pyrrole ring, it may be anticipated that bridge formation between with the N terminus and the imidazole ring in histidine could have been occurred as well, to form a thermodynamically stable six-membered ring (Figure 13).

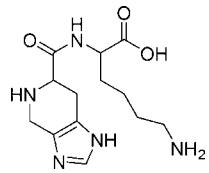

Figure 13. Proposed structure of methyleneation for His-Lys dipeptide.

The tandem mass spectra of native dipeptide 5 (Cys-Lys) and modified Cys-Lys are shown in Figure S3, Supporting Information. All ions related to lysine at m/z 84, 129, and 147 (y_1) can be detected in both the native and modified peptide, which may indicate that methyleneation occurred preferentially at the cysteine residue, but these MS data alone are not strong enough to draw a definite conclusion for single methyleneation. Methyleneation was not observed in Met-Lys and can be explained due to the absence of free SH group in methionine.

In the Arg-Lys dipeptide, a peak with a 24 Da mass increase, although with very low intensity, was detected. Interestingly, dimethylene bridge formation has been reported by Metz et al.

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to occur between lysine and arginine upon reaction with formaldehyde, showing the great similarity between the quite different reaction conditions of TEMED and formaldehyde induced methyleneations. Besides, a mass increase of 24 Da was also observed in the Gln-Lys fragment of the decapeptide. However, this was not the case for the dipeptide Gln-Lys, which only showed the +12 Da modification. Unfortunately, subsequent fragmentation of the +24 Da modified decapeptide did not lead to the generation of a key fragment ion, which may be partly due to the isobaric mass of lysine and glutamine, which differ just 0.04 Da in mass.[34]

CONCLUSION

A practical method for identifying methylated peptides was provided, and we hypothesized the mechanism by which the methyleneation occurs in the presence of TEMED radicals. Mass spectrometric experiments have proven useful in detecting and identifying the modification sites and determining their relative reactivity. The studies on the simple peptide models presented here provided valuable insight into TEMED radical reactions.

It is shown that the TEMED radical is a carbon source to produce the methylated products. Taken together, the data from this work suggests that in most cases primary amines (lysine or N-terminus) are the initial reaction sites with the TEMED radical. In the case of lysine, the reaction with TEMED radical is driven to completion by a proton shift possibly catalyzed by a proper neighboring amino acid residue and at least for Arg-Lys followed by methylene bridge formation. In the case of N-terminal tryptophan, NMR analysis indicated that methyleneation was followed by methylene bridge formation between the amino terminus of the peptide and the pyrrole ring of tryptophan. Although identification of all possible intramolecular cross-links or single methyleneation products of TEMED-treated peptides still remains a tremendous job, the data from this study can be useful to predict the modifications that could happen on peptides and proteins in the presence of TEMED radicals.

Because the stability of peptide and protein payloads or templates is crucial in in situ polymerization for the preparation of drug delivery systems or molecularly imprinted polymers, avoiding the use of TEMED and replacing this catalyst with sodium bisulfite is recommended.

EXPERIMENTAL PROCEDURES

Chemicals. Thymopentin of 99.0% purity was purchased from PROSPEC (Ness Ziona, Israel). Ammonium peroxodisulfate (APS) was obtained from Fluka (Zwijndrecht, the Netherlands). Potassium peroxodisulfate (KPS) was purchased from Merck (Darmstadt, Germany). Sodium bisulfite ($NaHSO_3$) was from Acros Organics (Geel, Belgium). *N,N,N,N*-Tetramethylethylenediamine (TEMED), formic acid, trifloroacetic acid (TFA), and HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were purchased from Sigma-Aldrich Co (Zwijndrecht, the Netherlands). HPLC and MS grade acetonitrile were purchased from Biosolve (Valkenswaard, the Netherlands). A decapeptide (Gln-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly-Lys), which is a fragment of human immunoglobulin G,[35] was from LifeTein (New Jersey, USA). Fifteen dipeptides with C-terminal lysine and different N-terminal amino acids were synthesized by Chinapeptides Co., Ltd.

Thymopentin Imprinting. Thymopentin imprinted hydrogels were prepared by dissolving TP5 (4 μmol) in 600 μL of HEPES buffer (20 mM, pH 7.4) containing 400 μmol of total monomers (methacrylamide as functional monomer and methylenebis(acrylamide) as a cross-linker), which polymerized by addition of 4 μmol of TEMED and 4 μmol of APS. Subsequently the vials were seal and incubated at 50 °C for 16 h. Alternatively the monomers were polymerized after addition of 4 μmol of $NaHSO_3$ and 4 μmol of KPS followed by incubation at room temperature for 16 h. Extraction of the template was performed using sodium chloride solution.[23,36]

Incubation of Peptides with APS/TEMED. The different peptides (4 μmol) were incubated for 16 h with 4 μmol of TEMED and 4 μmol of APS in an Eppendorf vial containing 600 μL of HEPES buffer (20 mM, pH 7.4) at 50 °C. In addition, thymopentin was also incubated at room temperature under otherwise similar conditions. Solutions of each peptide (4 μmol) in 600 μL in the same buffer were used as controls. Next, the samples were diluted with MQ water to reach a concentration of 0.33 μmol of peptide/mL for TP5 and decapeptide and 1.33 μmol of peptide/mL for the different dipeptides prior to analysis.

Peptide Analysis. Thymopentin solutions were analyzed by ultraperformance liquid chromatography (Waters ACQUITY UPLC) using a column (length, 50 mm; internal diameter, 2.1 mm) packed with 1.7 μm ACQUITY BEH 300 C18 material. A gradient method was performed at 30 °C. The mobile phase A was water−acetonitrile−trifloroacetic acid (95:5:0.1, v/v/v) and phase B was acetonitrile−trifloroacetic acid (100:0.1, v/v). Eluent A linearly changed from 100% to 30% in 5 min with a flow rate of 0.25 mL/min. UV detection was done at 275 nm, and the sample injection volume was 5 μL.

LC−MS experiments were performed using a Shimadzu 10A HPLC system (Kyoto, Japan) coupled to an Agilent Technologies 6300 series LC/MSD ion-trap mass spectrometer (Santa Clara, CA, USA). A HPLC column (150 mm × 4.6 mm) packed with 3.5 μm XBridge BEH130 C18 material was used at ambient temperature. For analysis of the TP5 samples, a gradient method was used with the mobile phase A water−acetonitrile−formic acid (95:5:0.1, v/v/v) and mobile phase B acetonitrile−formic acid (100:0.1, v/v). The eluent A after 5 min linearly changed from 100% to 50% in 25 min with a flow rate of 0.5 mL/min. For the decapeptide, an isocratic method was used with a mobile phase of water−acetonitrile−formic acid (70:30:0.1, v/v/v). The sample injection volume was 5 μL. For the different dipeptides, the mobile phase was changed to water−methanol−trifloroacetic acid. The gradient method was the same as mentioned above, but the sample injection volume was 10 μL. Electrospray ionization (ESI) was performed in the positive ion mode using an Agilent Technologies ion source and interface. The MS settings were capillary voltage of 2 kV, nebulizer pressure of 60 psi, dry gas flow of 11 L/min, dry gas temperature of 350 °C, and scan range of m/z 50−1500. MS^2 and MS^3 experiments were performed using an isolation width of 2 Da and a fragmentation amplitude of 1.0 V. Accurate mass spectra of thymopentin and modified thymopentin were obtained by direct infusion of a sample incubated with APS/TEMED into a Bruker (Bremen, Germany) ESI-time-of-flight mass spectrometer.

The Trp-Lys from the dipeptide library, treated with APS/TEMED, was separated using multiple injections on the HPLC using water−acetonitrile−trifloroacetic acid (95:5:0.1, v/v/v), which after 5 min linearly changed to acetonitrile−trifloroacetic

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acid (100:0.1, v/v) in 25 min with a flow rate of 1 mL/min, and the fractions that contained methylated Trp-Lys were collected and, after freeze-drying of the pooled fractions, analyzed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra were recorded on a Bruker DRX-500 spectrometer operating at 500 MHz using DMSO-d_6 as the solvent; the DMSO peak at 2.52 ppm was used as the reference line. The NMR peak assignments are based on ChemDraw calculation software.

■ ASSOCIATED CONTENT

Supporting Information

UPLC chromatogram of TP5, native and exposed to APS/TEMED, and all the tandem mass spectra of dipeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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