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Efficient and Selective Chemical Labeling of Electrochemically Generated Peptides Based on Spirolactone Chemistry

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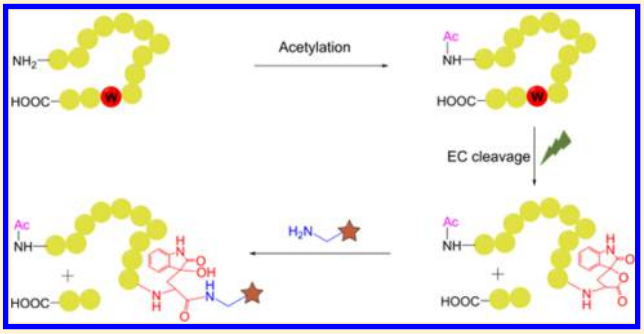
Supporting Information

ABSTRACT: Specific digestion of proteins is an essential step for mass spectrometry-based proteomics, and the chemical labeling of the resulting peptides is often used for peptide enrichment or the introduction of desirable tags. Cleavage of the peptide bond following electrochemical oxidation of Tyr or Trp results in a spirolactone moiety at the newly formed C-terminus offering a handle for chemical labeling. In this work, we developed a highly efficient and selective chemical labeling approach based on spirolactone chemistry. Electrochemically generated peptide-spirolactones readily undergo an intramolecular rearrangement yielding isomeric diketopiperazines precluding further chemical labeling. A strategy was established to prevent intramolecular arrangement by acetylating the N-terminal amino group prior to electrochemical oxidation and cleavage allowing the complete and selective chemical labeling of the tripeptide LWL and the decapeptide ACTH 1-10 with amine-containing reagents. As examples, we show the successful introduction of a fluorescent label and biotin for detection or affinity enrichment. Electrochemical digestion of peptides and proteins followed by efficient chemical labeling constitutes a new, powerful tool in protein chemistry and protein analysis.



Mass spectrometry-based proteomics plays a central role in protein identification and quantification in complex biological matrixes starting with protein digestion followed by analysis of the resulting peptides by reversed-phase liquid chromatography coupled to tandem mass spectrometry (RPLC–MS/MS).[1−3] Enzymatic digestion with proteases is the most widespread method for cleavage of proteins at specific peptide bonds, and a number of proteases with different specificities are available.[4−7] Chemical cleavage is sometimes used as an alternative to enzymatic digestion if specificity for a certain amino acid sequence is required for which no protease is known.[8−10] Electrochemical oxidation of peptides and proteins has been shown to lead to specific cleavage of the peptide bond C-terminal to Tyr and Trp, which makes electrochemistry (EC) a potential instrumental alternative to chemical and enzymatic peptide bond cleavage, since EC is fast, does not require the addition of reagents, and works under denaturing conditions.[11−15]

Chemical labeling with fluorescent dyes, affinity tags, or other groups was developed to enrich molecules from complex mixtures, notably from biological samples, to allow for their selective and sensitive detection or to generate internal standards for mass spectrometry through incorporation of stable isotopes.[3,16−18] An easy capture and labeling strategy for the peptides of interest based on specific reactions would further boost proteomics analysis efficiency after protein digestion. Ideally, the reaction for chemical labeling should have high selectivity and efficiency as well as produce a single chemical moiety without side reactions or multiplicity of labeling. However, most commonly used chemical labeling reactions rely on reactions of the side chains of natural amino acids and N-terminal or C-terminal residues and are thus limited by a lack of selectivity resulting in multiple reaction products that often require tedious purification steps to arrive at single compounds.[19−22] Thus, the lack of reactions with distinct and tunable reactivity and selectivity for efficient chemical labeling remains a challenge, which we address by electrochemically mediated cleavage of the peptide bond with subsequent chemical labeling.

An interesting aspect of electrochemically cleaved peptides is that the newly generated C-terminus is converted into a unique

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activated ester in the form of a spirolactone.[13] We have previously shown that this spirolactone is reactive toward amines and employed its reactivity for chemical labeling with hexylamine.[14] However, Tyr- and Trp-derived spirolactones show modest coupling yields even at a large molar excess of the amine under aqueous conditions at the slightly acidic pH values required to prevent spirolactone hydrolysis.[14,23−25] In this work, we developed a chemical labeling approach that overcomes these limitations and allows complete and selective peptide labeling after electrochemical cleavage.

EXPERIMENTAL SECTION

Materials and Chemicals. The tripeptide LWL was obtained from Research Plus Inc. (Barnegat, NJ). LFL was purchased from Bachem (Weil am Rhein, Germany). Adrenocorticotropic hormone (ACTH) 1-10 (SYSMEHFRWG), formic acid (HCOOH, FA, 98%), triethylamine (TEA, 99%), trifluoroacetic acid (TFA, 99%), hexylamine (99%), acetic acid anhydride (99%), dimethyl sulfoxide (DMSO, anhydrous, 99.8%), 5-((2-aminoethyl)amino)-naphthalene-1-sulfonic acid, sodium salt (EDANS), and dimethyl sulfoxide-d_6 (100%, 99.96 atom % D) were obtained from Sigma-Aldrich (Steinheim, Germany). EZ-Link amine-PEG_2-biotin was purchased from Pierce Biotechnology (Rockford). Acetonitrile (HPLC SupraGradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands). Water was purified by a Millipore system (conductivity 18.2 MΩ cm, Millipore Corp., Billerica, MA).

N-Terminal Acetylation of Peptides and Ac-Peptide Purification. A volume of 100 μL of LWL and ACTH 1-10 were prepared in DMSO at a concentration of 10 mM and 100 μL of acetic acid anhydride (15 mM in DMSO) were added. Acetylation was performed at room temperature with shaking at 900 rpm in an Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany) for 2 h. The mixture was diluted with 99/1 (v/v) water/formic acid to a final concentration of 1 mM and the reaction products purified by HPLC. The HPLC system (Shimadzu, Kyoto, Japan) was equipped with a SIL-20AC autosampler, an LC-20AT pump, an SPD-20A absorbance detector, and an FRC-10A fraction collector. The 100 μL samples were injected and purified on a Vydac RP-C18 column (250 mm × 4.6 mm i.d., 5 μm particles, 300 Å pore size, Grace Vydac, Lokeren, Belgium) with a 40 min gradient of 5−40% acetonitrile in water/0.1% formic acid at a flow rate of 500 μL/min. Fractions were collected and evaporated under nitrogen and dried in an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany) at 30 °C.

Electrochemical Cleavage. Stock solutions of LWL, Ac-LWL, ACTH 1-10, and Ac-ACTH 1-10 were prepared in 89/10/1 (v/v/v) water/acetonitrile/formic acid at a concentration of 1 mM shortly before electrochemical oxidation and cleavage. Stock solutions of LWL and Ac-LWL were diluted to 10 μM and 20 μM in 89/10/1 (v/v/v) water/acetonitrile/formic acid, respectively, and oxidized at a flow-rate of 10 μL/min (syringe pump, KD Scientific Inc., Holliston, MA) with a Coulochem 5021 conditioning cell (ESA Inc., Bedford, MA) containing a porous graphite flow-through working electrode, a palladium auxiliary electrode and a palladium reference electrode. ATCH 1-10 and Ac-ACTH 1-10 were oxidized and cleaved at a concentration of 50 μM and a flow-rate of 5 μL/min, following the same procedure. Online EC-MS experiments of peptides were done by linearly ramping the cell potential from 300 to 1500 mV at a scan rate of 2 mV/s with a homemade potentiostat controlled by a MacLab system (AD Instruments, Castle Hill, NSW, Australia) and EChem software (eDAQ, Denistone East, NSW, Australia) to determine the optimal oxidation/cleavage potentials. LWL, Ac-LWL, ACTH 1-10, and Ac-ACTH 1-10 were oxidized at 700 mV, 710 mV, 750 mV, and 750 mV vs Pd/H_2, respectively, for LC−MS analysis. Currents recorded at the working electrode were 3.0, 3.2, 3.5, and 3.2 μA, respectively. The reaction product mixtures (EC-LWL, EC-Ac-LWL, EC-ACTH 1-10, and EC-Ac-ACTH 1-10, respectively) were collected for further reactions and analyses at different time points to assess the stability of the cleavage products.

Intramolecular Rearrangement of Spirolactone-Containing Peptides. A volume of 2 mL of 10 μM EC-LWL were concentrated by evaporation under nitrogen (2 h) and dried in an Eppendorf Concentrator at 30 °C. EC-LWL conversions were performed by dissolving the dried sample in DMSO at a concentration of 1 mM by pipetting for 30 s. Reactions were performed at 25 °C with shaking at 900 rpm in an Eppendorf Thermomixer. Intramolecular rearrangement was monitored at different time points, (0, 1, 2, 3, 4, and 6 h) by subjecting 40 μL of the reaction mixtures to LC−MS analysis after dilution to 2.5 μM with 99/1 (v/v) water/formic acid.

Conversions of EC-Ac-LWL and EC-Ac-ACTH 1-10 were done at a concentration of 1 mM in DMSO after drying following the same procedure. The 40 μL samples (10 μM) at time points 0 and 6 h were prepared by dilution with 99/1 (v/v) water/formic acid and analyzed by LC−MS.

Intramolecular rearrangements under acidic and basic conditions were studied by drying 2 mL of 10 μM EC-LWL as described above. Dried samples were dissolved in DMSO/TFA (99.5/0.5) or DMSO/TEA (99.5/0.5) at a final concentration of 1 mM by pipetting for 30 s. The samples were incubated at 25 °C with shaking at 900 rpm, and the conversion reaction was monitored at different time points by LC−MS after dilution with 99/1 (v/v) water/formic acid.

Analysis of Intramolecular Rearrangements Product by LC−MS Analysis. Liquid chromatography was performed on an Ultimate plus system (Dionex-LC Packings, Amsterdam, The Netherlands) equipped with a gradient pump and a Famos autosampler. A Vydac RP-C18 column (150 mm × 2.1 mm i.d., 5 μm particles, 300 Å pore size, Grace Vydac, Lokeren, Belgium) was used for chromatographic separation at a flow rate of 300 μL/min. Mobile phase A consisted of ultrapure water with 0.1% formic acid. Mobile phase B was acetonitrile with 0.1% formic acid.

A volume of 40 μL of reaction mixtures (EC-LWL, 2.5 μM; EC-Ac-LWL, 10 μM; and Ac-ACTH 1-10, 10 μM) were injected and separation was achieved with a gradient of 5−40% B at 1%/min. The column was directly coupled to an API 365 triple quadrupole mass spectrometer (PE-Sciex) with an EP10+ upgrade (Ionics) for product detection in positive ion mode. All experiments were performed in triplicate and peak areas obtained by LC−MS analyses were normalized to the peak area of 100 nM LFL, which was added as internal standard prior to LC/MS analysis.

Isomer Preparation and NMR Analysis. A volume of 300 mL of EC-LWL were collected from a Coulochem 5021 conditioning cell to prepare approximately 200 μg of spirolactone-containing peptide isomers for NMR analysis. EC-LWL solutions were evaporated under nitrogen and dried in an Eppendorf Concentrator at 30 °C. Intramolecular rearrangements were performed at a final concentration of 30

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mM by adding 100 μL of DMSO/TEA (99.5/0.5) to dissolve the dried EC-LWL and incubating it for 24 h at 25 °C (Eppendorf Thermomixer at 900 rpm). After reaction, the solution was diluted with 900 μL of 99/1 (v/v) water/formic acid prior to purification.

The isomers were purified on the Shimadzu chromatographic system described above with a 210 min gradient of 5−15% acetonitrile in water/0.1% formic acid at a flow rate of 200 μL/min. Fractions were collected, evaporated under nitrogen, and dried in an Eppendorf Concentrator at 30 °C. The samples were stored at −20 °C. Purity of the isolated isomers was checked by LC−MS immediately prior to NMR analysis.

All NMR experiments were performed at 298 K on a Varian UnityINOVA spectrometer operating at 800 MHz (^{1}H resonance frequency) equipped with a cryo-cooled probe. Each isomer was dissolved in 700 μL of perdeuterated DMSO. 1D ^{1}H and 2D total correlation spectroscopy (TOCSY) experiments were acquired for both samples with identical parameter settings. A total of 128 transients were acquired for the 1D experiments using a 30-deg excitation pulse, an acquisition time of 1.5 s, and a preparation period of 8.5 s.

The 2D TOCSY data matrix consisted of 400 × 5000 complex points, with spectral widths of 9 000 and 10 000 Hz in the indirect (t1) and acquisition (t2) dimension, respectively. A MLEV17 sequence of 40 ms was employed for TOCSY mixing. For each increment, 16 transients were collected using a recycle delay of 2.5 s. All 2D data sets were processed with NMRPipe[26] using 72° shifted squared sine-bell apodization in both dimensions, prior to zero filling to 2048 (t1) × 16384 (t2) complex points, and Fourier transformation. The software iNMR (http://www.inmr.net) was used for 1D processing and analysis of all spectra.

Chemical Coupling of Electrochemically Cleaved Peptides with Amine-Containing Reagents. Chemical coupling of the LW+14, Ac-LW+14, and Ac-SYSMEHFRW +14 with hexylamine in DMSO/TEA (99.5/0.5) was performed according to Roeser et al.[14] with slight modification. A volume of 1 mL of EC-LWL (10 μM), EC-Ac-LWL (20 μM), and EC-Ac-ACTH 1-10 (50 μM) solutions were dried following the same procedure and the coupling with hexylamine was performed at a concentration of 500 μM by adding 20, 40, and 100 μL of a mixture of DMSO, TEA, and hexylamine (95:1:4), respectively. Chemical coupling of the peptide-spirolactones Ac-LW+14 and Ac-SYSMEHFRW+14 with EDANS and amine-PEG_2-biotin were performed with the same procedure expect that the added mixtures were composed of DMSO, TEA, and amine-containing reagent (59:1:40).

The 20 μL reaction mixtures (5 μM of EC-LWL, 10 μM EC-Ac-LWL, and 25 μM EC-Ac-ACTH 1-10) at time points 0 and 6 h were prepared by dilution with 99/1 (v/v) water/formic acid and analyzed by LC−MS. The LC−MS analysis was performed on an HPLC system equipped with a 2.0 mm i.d. × 50 mm Shim-pack XR-ODS column (Shimadzu, Kyoto, Japan) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The separation of the hexylamine reaction mixture was achieved with a 40 min gradient of 2−60% acetonitrile in water/0.1% formic acid at a flow rate of 300 μL/min while a 65 min gradient was employed for the separation of the EDANS and amine-PEG_2-biotin reaction mixtures.

High-Resolution MS/MS Analysis. High-resolution MS/MS experiments of the spirolactone-containing cleavage products including LW+14 (LWL), Ac-LW+14 (Ac-LWL), SYSMEHFRW+14 (ACTH 1-10, SYSMEHFRWG), Ac-SYSMEHFRW+14 (Ac-ACTH 1-10, Ac-SYSMEHFRWG) and the rearrangement product LW+14* (LW+14) were performed on a Q Exactive Plus hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Separation was performed with an HPLC system equipped with a 2.0 mm i.d. × 50 mm Shim-pack XR-ODS column (Shimadzu, Kyoto, Japan). A 30 min gradient of 5−40% acetonitrile in water/0.1% formic acid was used at a flow rate of 300 μL/min.

Isomers were prepared by intramolecular rearrangements of 100 μM EC-LWL in DMSO for 6 h. A volume of 10 μL of 2.5 μM EC-LWL and 10 μM EC-Ac-LWL, EC-ACTH 1-10, and EC-Ac-ACTH 1-10 were prepared by dilution with 99/1 (v/v) water/formic acid and injected for LC−MS/MS analysis. MS scans from m/z 200 to 800 (LW+14, LW+14*, and Ac-LW +14) and MS scans from m/z 200 to 1400 (SYSMEHFRW+14 and Ac-SYSMEHFRW+14) were recorded at a resolution of 72 000 and MS/MS spectra were recorded at a resolution of 17 500 after collision-induced fragmentation in the HCD cell. The normalized collision energy was set at 10 V (LW+14 and LW +14*) and 35 V (Ac-LW+14, SYSMEHFRW+14 and Ac-SYSMEHFRW+14), respectively.

High-resolution MS/MS experiments of the spirolactone-containing cleavage products (Ac-LW+14 and Ac-SYSMEHFRW+14) and the coupling products (Ac-LW+hexylamine and Ac-SYSMEHFRW+hexylamine) were performed on an LTQ-Orbitrap XL mass spectrometer. MS scans from m/z 100 to 2000 were recorded at a resolution of 75 000 and MS/MS spectra were recorded at a resolution of 17 500 with a normalized collision energy at 35 V. All data was acquired in profile mode using positive polarity.

RESULTS AND DISCUSSION

Molecular Rearrangement of LW+14 and Formation of Isomers. Reactions of peptide-spirolactones with amines under aqueous conditions have been reported to require a basic pH and a large molar excess while reaching only limited labeling efficiencies.[14] Nonaqueous conditions are preferable for spirolactone labeling, since hydrolysis of the spirolactone is prevented and the molar excess of amine can be reduced. DMSO was selected in this work due to the good solubility of most peptides in this solvent and hexylamine was employed to study the spirolactone chemistry in more detail.[27−29] The reaction between the spirolactone-containing EC-cleavage product of the tripeptide LWL (LW+14) and hexylamine resulted in two chromatographically separated products (Figure S1A,B) which have identical MS and MS/MS spectra, as previously reported by Roeser et al.[14] However, a competing reaction led to the formation of a pair of isomers (LW+14*) with the same mass as LW+14 (Figure S1C).

The rearrangement reaction proceeded to 78% completion within 6 h in the absence of hexylamine indicating that it is independent of the actual labeling reaction (Figure 1). The newly generated LW+14* products lost reactivity toward primary amines suggesting that the spirolactone participated in the rearrangement. We hypothesized that LW+14* was formed by an intramolecular reaction between the N-terminal primary amino group and the spirolactone. Since this reaction requires a nonprotonated amino group, we investigated the conversion of LW+14 under basic and acidic conditions in DMSO. Figure S2 shows that conversion does not take place under acidic conditions (0.5% trifluoroacetic acid (TFA)) while

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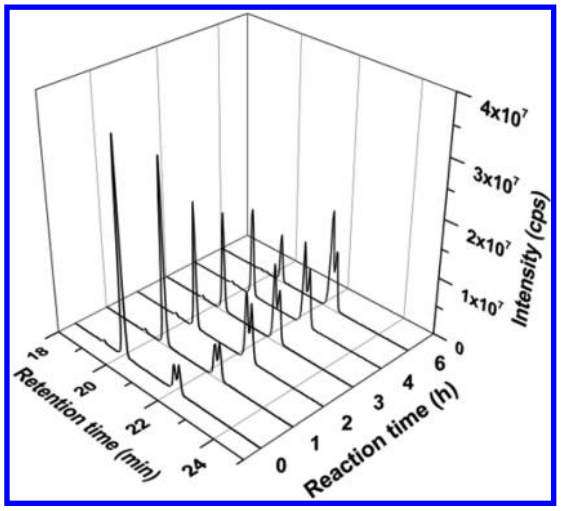


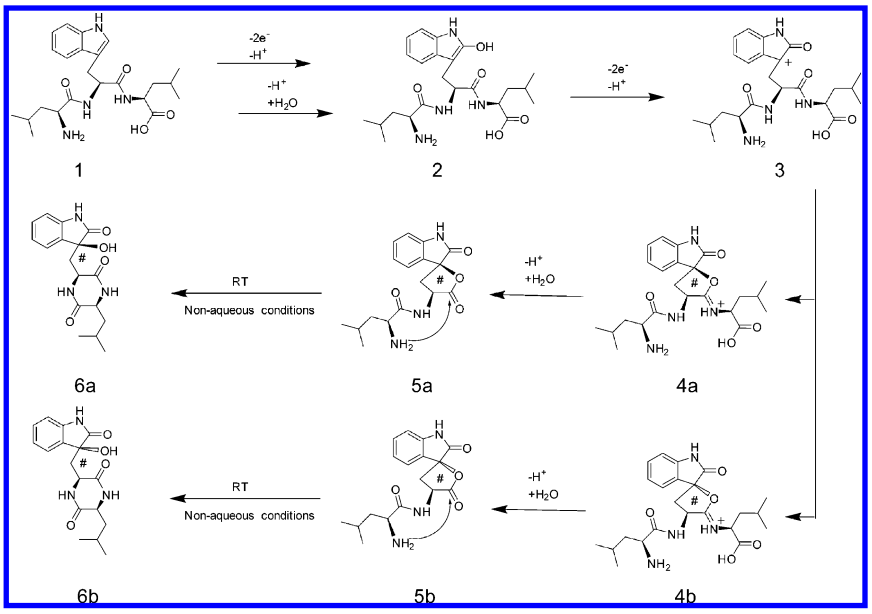
Figure 1. LC−MS analysis of the rearrangement of the spirolactone-containing LW+14 (retention time 19.5 min) in pure DMSO to a pair of isomers (LW+14*, retention times 23.6 and 23.8 min) that no longer react with primary amines. Some LW+14* was already observed after sample preparation (0 h time point).

it proceeds readily under basic conditions (0.5% triethylamine (TEA)), supporting the hypothesis of an intramolecular rearrangement involving the N-terminal amino group.

On the basis of the intramolecular aminolysis involving the primary amino group in peptide chemistry,[30−32] we assumed that a similar rearrangement of LW+14 might occur under neutral or basic conditions. The proposed mechanism (Scheme 1) suggests that the single bond attached to the tertiary carbocation can rotate freely to form isomers **4a** and **4b** from **3** due to formation of a new chiral center. The peptide bond in **4a** and **4b** is subsequently cleaved to form spirolactones **5a** and **5b** (LW+14) under acidic, aqueous conditions. While **5a** and **5b** are stable under acidic conditions, they undergo molecular rearrangement to the corresponding diketopiperazines **6a** and **6b** (LW+14*) under neutral or basic conditions in DMSO.

To confirm the structure of LW+14* in support of our mechanistic hypothesis, structural studies on the early eluting and late-eluting LW+14* isomers were carried out with the aid of high-resolution LC−MS/MS, 1D ^{1}H NMR, and 2D TOCSY. High-resolution LC−MS/MS of LW+14 and LW+14* was performed on a sample obtained after reaction for 1 h at a concentration of 400 μM in DMSO containing both LW+14 and the two LW+14* isomers in comparable amounts (Figure 2A). The MS/MS spectrum of LW+14 was similar to the one reported by Roeser et al.[13] Noteworthy is the high intensity of the immonium ion of Leu (m/z 86.097) indicating the presence of an unmodified Leu residue at the N-terminus (Figure 2B). This peak was greatly reduced after intramolecular rearrangement in LW+14*, since the Leu residue becomes part of the diketopiperazine ring structure, which is difficult to fragment (Figure 2C,D). The two LW+14* isomers produced identical MS/MS spectra. Fragments were assigned based on ion fragmentation rules for peptides in combination with the elemental compositions derived from accurate mass measurements. The easy loss of water to form the fragment at m/z 314.149 even at the low collision energy of 10 V is consistent with the presence of a tertiary hydroxyl group.[33,34] The fragment at m/z 286.154 was generated from the loss of CO from the fragment at m/z 314.149 followed by a further loss of ammonia to yield the fragment at m/z 269.128. The fragment at m/z 297.123 was generated from loss of ammonia due to ring opening of the diketopiperazine. None of these fragments was detectable in the LW+14 spectrum. The intense peak at m/z 146.060 in LW+14* corresponds to a Trp-related fragment ion, which was also observed in LW+14 but at a much lower intensity. These results are consistent with the proposed tertiary hydroxyl group and diketopiperazine structure as a result of the molecular rearrangement proposed in Scheme 1. The obtained ^{1}H NMR spectra of the LW+14* isomers in DMSO-d_6 as well as 2D-TOCSY spectra results were in

Scheme 1. Proposed Electrochemical Oxidation and Cleavage Pathway of the Trp-Containing Peptide LWL and the Ensuing Intramolecular Rearrangement to the Corresponding Diketopiperazines Containing a Chiral Tertiary Alcohol Indicated with Numbers 6a and 6b (Partially Adapted from Roeser et al.[13] Copyright 2010 American Chemical Society)



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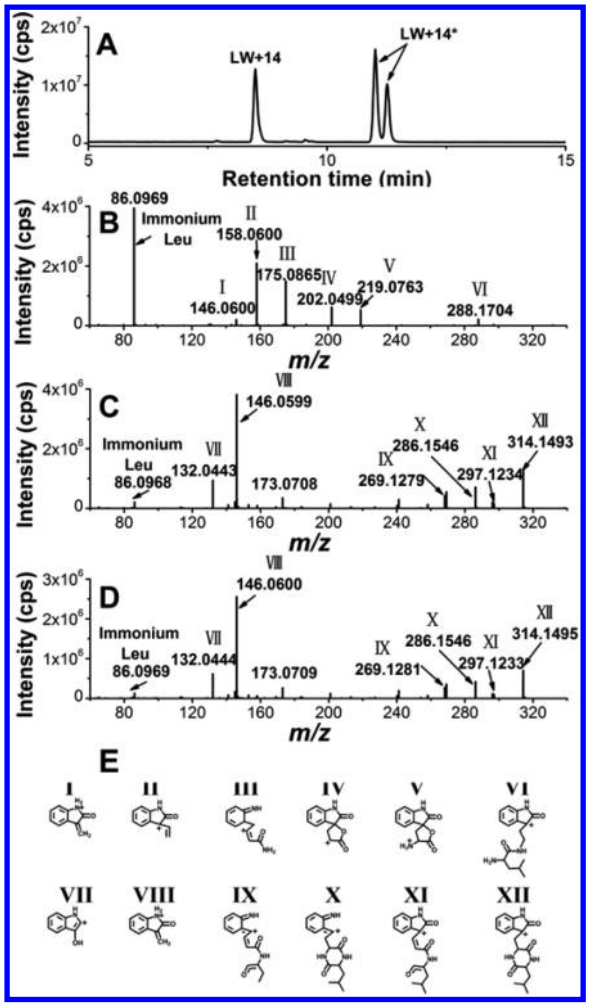

Figure 2. Extracted ion chromatogram of m/z 332.160 showing LW +14, the early eluting LW+14* isomer, and the late-eluting LW+14* isomer (A). MS/MS spectra of the electrochemical cleavage products of LW+14 (B), the early eluting LW+14* isomer (C), and the late-eluting LW+14* isomer (D). Proposed structures of the fragment ions of LW+14 and LW+14* (E).

agreement with the proposed structures of the LW+14* isomers supporting the proposed mechanism of rearrangement (see the Supporting Information, Figures S3–S6, for details).

Stabilization of Peptide-Spirolactones against Intramolecular Rearrangement. The proposed reaction mechanism (Scheme 1) implies that a free, nonprotonated N-terminal amino group is needed for the intramolecular rearrangement. Acetylation of the N-terminus should thus prevent rearrangement and stabilize the spirolactone-containing reaction products to prevent the side reaction that leads to the formation of the diketopiperazines **6a** and **6b**. The tripeptide LWL and the decapeptide adrenocorticotropic hormone (ACTH) fragment 1-10 (SYSMEHFRWG) were acetylated to Ac-LWL and Ac-ACTH 1-10, respectively, prior to electrochemical oxidation and cleavage. As expected, Ac-LW+14 and Ac-SYSMEHFRW+14 were stable and did not undergo intramolecular rearrangement even after incubation at room temperature for 6 h in DMSO containing 0.5% TEA (Figure 3A,B). In both cases, Ac-LW+14 and Ac-SYSMEHFRW+14 produced a pair of isomers in analogy to **5a** and **5b** (see Scheme 1) with the same MS/MS fragmentation patterns

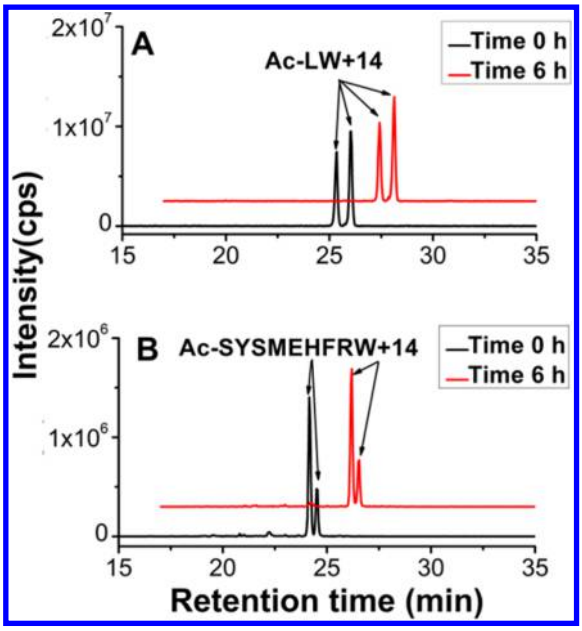

Figure 3. Stability of the spirolactone-containing cleavage products Ac-LW+14 (A) and Ac-SYSMEHFRW+14 (B) in DMSO containing 0.5% TEA after incubation for 6 h at room temperature. For clarity, the 6 h trace in part A was offset by 2 min and 3×10^6 cps and in part B by 2 min and 3×10^5 cps.

(Supporting Information, Figures S7 and S8), which further supports the proposed mechanism.

Chemical Labeling of Electrochemically Generated Peptide-Spirolactones. On the basis of these results, we established a three-step strategy resulting in the efficient labeling of peptide-spirolactones (Scheme 2). Peptides were first acetylated with acetic acid anhydride followed by electrochemical cleavage and labeling of the spirolactone. LWL and ACTH 1-10 were used to assess the feasibility of this strategy with hexylamine (see the Supporting Information, Figures S9–S11) followed by reaction with the amine-containing tags amine-PEG_2-biotin and the fluorescent dye EDANS (Figure 4).

Biotinylation of peptides and proteins is commonly exploited for purification and enrichment based on its affinity for avidin.[3,35−37] Fluorescent labeling is widely used to modify peptides and proteins for sensitive and selective detection.[38−41]

Figure 5 shows biotinylation of Ac-LW+14 to Ac-LW-amine-PEG_2-biotin (Figure 5A,B) and of Ac-SYSMEHFRW+14 to Ac-SYSMEHFRW-amine-PEG_2-biotin (Figure 5C,D) after reaction of the electrochemical cleavage products Ac-LW+14 (m/z 374.17) and Ac-SYSMEHFW+14 (m/z 649.77 (2+)) in DMSO containing 0.5% TEA at room temperature for 6 h. Both cleavage products were completely biotinylated to Ac-LW-amine-PEG_2-biotin (m/z 748.37) and Ac-SYSMEHFRW-amine-PEG_2-biotin (m/z 836.87 (2+)) with a mass increment of 374.20 Da. Complete coupling of Ac-LW+14 and Ac-SYSMEHFRW+14 with the amine-containing fluorescent dye EDANS was also achieved under the same conditions (see Figure S12) resulting in a mass increment of 266.04 Da (Ac-LW-EDANS (m/z 640.24) and Ac-SYSMEHFRW-EDANS (m/z 782.80 (2+)).

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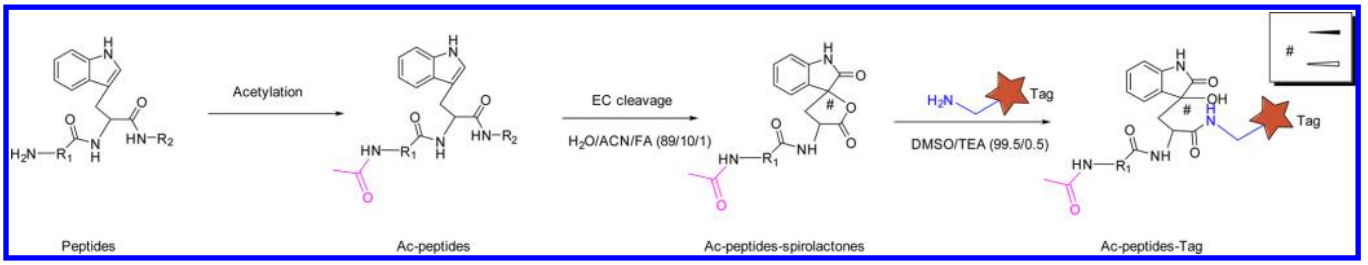
Scheme 2. Three-Step Labeling Scheme of Acetylated, Electrochemically Cleaved Peptides via Their Spirolactone Moieties with Amine-Containing Tags under Nonaqueous Conditions



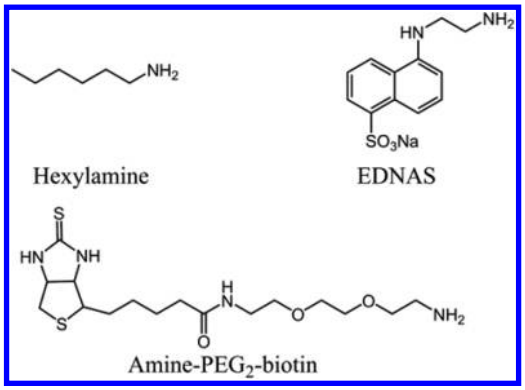


Figure 4. Structure of amine-containing reagents used for chemical labeling of spirolactones.

CONCLUSIONS

The electrochemical, oxidative cleavage of peptide bonds at Trp and Tyr opens possibilities for specific chemical labeling by introducing a reactive spirolactone moiety at the C-terminus of the cleavage products. To make full use of this reaction for peptide and protein analysis, it is crucial to prevent side reactions that inactivate the spirolactone. To advance along these lines, we studied the intramolecular rearrangement of spirolactone-containing peptides and characterized the observed isomeric products. The proposed reaction mechanism (Scheme 1) explains the existence of a pair of spirolactone-containing isomers and their subsequent rearrangement to nonreactive diketopiperazines. Acetylation of the N-terminal amino group prevents this rearrangement and stabilizes the spirolactone for further reaction with amine-containing tags (Scheme 2). Selective chemical coupling of acetylated, electrochemically cleaved spirolactone-containing peptides to the amine-containing reagents amine-PEG_2-biotin and the fluorescent dye EDANS in DMSO containing TEA went to completion without any competing side reactions. These results open new possibilities of chemical peptide and protein labeling via electrochemically generated spirolactones with desirable tags as tools in protein chemistry and protein analysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b01154.

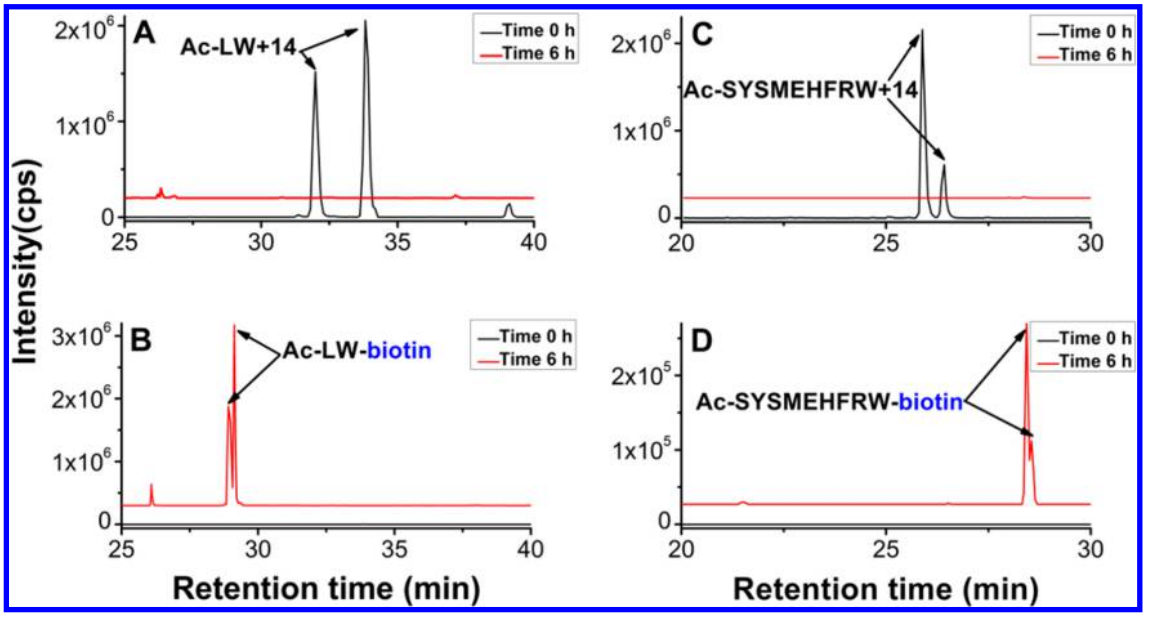


Figure 5. Chemical labeling of Ac-LW+14 and Ac-SYSMEHFRW+14 with amine-PEG_2-biotin. Extracted ion chromatogram of Ac-LW+14 (A) (m/z 374.171), Ac-SYSMEHFRW+14 (C) (m/z 649.767, (2+)) and the chemical labeling products Ac-LW-amine-PEG_2-biotin (B) (m/z 748.373) and Ac-SYSMEHFRW-amine-PEG_2-biotin (D) (m/z 836.871, (2+)) before and after reaction with a 4000-fold molar excess of amine-PEG_2-biotin in DMSO containing 0.5% TEA for 6 h at room temperature. For clarity, the y axis of 6 h trace in part A was offset by 2×10^5 cps, in part B by 3×10^5 cps, in part C by 3×10^5 cps, and in part D by 3×10^4 cps.

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Supplementary Figures S1−S12: intramolecular rearrangement, isomer formation and characterization of the isomers by NMR; MS/MS of cleavage products before and after acetylation; chemical labeling with hexylamine and with EDANS (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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