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(54) Title: MEANS AND METHODS FOR SITE-SPECIFIC LABELING OF PROTEINS.

(57) Abstract: The invention relates to protein chemistry and protein analysis, more specifically to means and methods for the site-specific labeling of polypeptides. Provided is a method for the site-specific labelling of a polypeptide, comprising providing a polypeptide comprising at its C-terminus a reactive spirolactone moiety and reacting said spirolactone moiety with an appropriate nucleophilic labeling reagent in the presence of Cu^{2+} ions to provide a C-terminally labelled polypeptide. Also provided is a kit comprising reagents for performing said labeling method.

Title: Means and methods for site-specific labeling of proteins.

The invention relates to protein chemistry and protein analysis. More specifically, it relates to means and methods for the site-specific labeling of a polypeptide, among others to incorporate a tag or stable isotope label allowing for their selective enrichment and/or sensitive detection, for example by mass-spectrometry.

Mass spectrometry-based proteomics plays a central role in protein identification and quantification in complex biological matrices 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 entity 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. For example, site-specific tagging of peptides and proteins is often difficult due to multiple reactive sites. Site-specific tagging at the peptide level is usually achieved through a combination of protecting group chemistry and peptide synthesis. Site-specific tagging of larger polypeptides, i.e. at the protein level, is usually achieved through genetic engineering to introduce non-natural amino acids that can be used for site-specific labelling.

The lack of reactions with distinct and tunable reactivity and selectivity for efficient chemical labeling has been addressed in the art 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 activated ester in the form of a spirolactone. [13] We have previously shown that this spirolactone is reactive towards amines and employed its reactivity for chemical labeling with hexylamine. [14] Unfortunately however, electrochemically generated spirolactones show modest coupling yields (typically up to maximally 50 %) even at a large molar excess of the amine under conditions required to prevent spirolactone hydrolysis or intramolecular rearrangement[14] and Zhang *et al.* Anal Chem 2016, 88, 6465-6471. Since incomplete labelling will require sophisticate purification of the

labelled products with concomitant losses and increase in cost, this has hampered wide scale applications of the prior art methods.
The present inventors therefore aimed at providing an improved approach for the site-specific chemical labeling of a polypeptide that overcomes the limitations of the prior art and allows for (nearly) complete and selective labeling in a facile and cost-effective manner.

It was surprisingly found that the yield of the labelling reaction of a lactone moiety with nucleophiles (e.g. primary amines) could be increased from less than 50% to close to 100% by performing the reaction in the presence of divalent metal cations, notably Cu^{2+}. Not wishing to be bound by theory, the divalent metal ions are thought to stabilize the active ester moiety of the lactone, thereby reducing or completely avoiding its hydrolysis into the corresponding carboxylic acid or rearrangement to the corresponding diketopiperazines, which are considered the main competing reactions. [14]

Accordingly, in one embodiment the invention relates to a method for the site-specific labelling of a polypeptide, comprising-providing a polypeptide comprising at its C-terminus a reactive lactone moiety and reacting said lactone moiety with an appropriate nucleophilic labeling reagent in the presence of Cu^{2+} ions to provide a C-terminally labelled polypeptide.

Lactones are cyclic esters of hydroxycarboxylic acids, containing a 1-oxacycloalkan-2-one structure, or analogues having unsaturation or heteroatoms replacing one or more carbon atoms of the ring. Lactones are formed by intramolecular esterification of the corresponding hydroxycarboxylic acids, which takes place spontaneously when the ring that is formed is five- or six-membered. In one embodiment, the polypeptide to be labelled comprises at its C-terminus a lactone moiety comprising a 5- or 6-membered ring. In a preferred embodiment, the lactone moiety is a

spirolactone moiety. In a specific aspect, the polypeptide comprising at its C-terminus a reactive lactone moiety is prepared by electrochemical cleavage of a polypeptide, preferably a Trp- or Tyr-containing polypeptide. Methods for electrochemical cleavage of a (Trp- or Tyr-containing) polypeptide are well known in the art. [13, 14] Since spirolactones may already hydrolyze or rearrange during electrochemical cleavage and further sample handling, especially when positioned C-terminal to Tyr, a method of the invention may comprise adding Cu^{2+} ions prior to electrochemical cleavage to prevent these side reactions. This refinement of the method may significantly increase the recovery of electrochemically cleaved peptides and facilitate subsequent (affinity) labelling e.g. biotinylation.

Since amines have basic dissociation constants, basic conditions are favorable. Accordingly, it is preferred to react under basic conditions, since the amine must be deprotonated to react.

A metal-catalyzed labelling method, as herein disclosed, typically comprises reacting the polypeptide comprising the lactone moiety in the presence of at least 500 nM Cu^{2+} ions. Preferably, the molar ratio between spirolactone and metal ion is in the range of 10:1 to 1:1. Typically, a concentration of 1 µM to 1 mM is used, preferably 2 to 500 µM, such as 5 to 300 µM. Sources of Cu^{2+} ions suitable for use in accordance with the present invention are known in the art and commercially available. Most convenient are salts of the divalent metal ions, for example, metal salts comprising a halide counter ion. Exemplary metal ion sources include $CuCl_2$, a $Cu(HCO_3)_2$ solution and Cu $(CH_3CO_2)_2$).

According to the invention, a polypeptide comprising at its C-terminus a reactive spirolactone moiety is reacted with an appropriate nucleophilic labeling reagent carrying the desired labelling moiety. For example, the

labeling reagent comprises a fluorescent dye, an affinity tag, a stable-isotope containing tag and/or an element inducing a mass "defect", like a halogen.

In one specific embodiment, the labeling reagent comprises an affinity tag. Affinity tags are appended to proteins or peptides so that they can be purified from their crude biological source using an affinity technique. For example, the labelling reagent comprises biotin, preferably biotin with a spacer and more preferably PEGylated biotin. Biotin binds to streptavidin and avidin with an extremely high affinity, fast on-rate, and high specificity, and these interactions are commonly exploited for purification and enrichment of various molecules of interest. Biotin-binding to streptavidin and avidin is resistant to extremes of heat and pH, making capture of biotinylated molecules possible in a wide variety of environments. For example, as demonstrated herein below, a labeling method of the present invention is advantageously used in proteomics e.g. to provide protein fragments resulting from electrochemical cleavage with an affinity tag followed by selective affinity enrichment and (MS/MS) analysis. Thus, the invention also provides a method for enriching electrochemically cleaved peptides from a mixture of electrochemical cleavage products that may serve as starting material for LC-MS/MS-based protein identification.

In another specific embodiment, the labeling reagent comprises a fluorescent dye, for example a dye for use in FRET analysis. Fluorescent labeling is widely used to modify peptides and proteins for sensitive and selective detection. Exemplary dyes include EDANS, FAM, TAMRA, ROX, Cy3 and Cy5 and the fluorescein products FITC, FAM, TET and HEX. Preferred dyes are those containing a primary amine, like EDANS, TAMRA cadaverine, FITC cadaverine and ROX cadaverine.

The labelling reagent also contains a nucleophile to allow for covalent polypeptide conjugation via the reactive spirolactone. Preferably, the labeling reagent comprises a primary amine which is capable of reacting

with the lactone moiety by ring-opening and the formation of a new amide bond under basic conditions.

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] Therefore, non-aqueous conditions are preferable for a labeling method of the invention, since hydrolysis of the (spiro)lactone is prevented, and the molar excess of amine can be reduced. Accordingly, in one embodiment a polypeptide comprising at its C-terminus a reactive spirolactone moiety is reacted with an appropriate nucleophilic labeling reagent in a non-aqueous solvent or solvent mixture.

To that end, an aqueous peptide solution comprising a polypeptide having at its C-terminus a reactive spirolactone moiety and further comprising a Cu^{2+} ion source can be dried under a stream of nitrogen, and thereafter a labelling mixture comprising the labelling reagent and a non-aqueous solvent is added to initiate the site-specific labeling reaction. For example, $CuCl_2$ is added at a 2-500 μM final concentration to a solution containing 500 μM of spirolactone-activated polypeptide. After drying, a labelling mixture comprising the labelling reagent is added and the reaction is allowed to proceed to obtain the desired labelling yield, typically after several hours, at room temperature. In a specific aspect, the labelling mixture comprises a nonaqueous solvent, a tertiary or sterically hindered amine that cannot react and an amine-containing labelling reagent (tag). Non-aqueous solvents used for chemical labeling include DMSO, acetonitrile, DMF, formamide, N-methylacetamide and THF while TEA, DBTU, TBD, Pyridine and DBU can be used as tertiary or sterically hindered amines, for example in a ratio of 199:1 (v/v).

In a specific embodiment, the kit comprises a source of Cu^{2+} ions, like a $CuCl_2$ solution, and a labeling reagent comprising a primary amine. Furthermore, it may contain a solvent for peptide reconstitution.

In a typical exemplary embodiment of the invention, the method can be split into 3 consecutive parts.

Part 1: Preparation of spirolactone-containing peptides by electrochemical cleavage (EC).

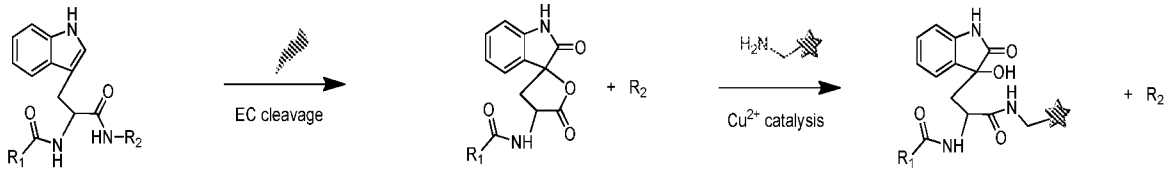
Peptide is prepared in 89/10/1 (v/v/v) water/acetonitrile/formic acid, and oxidized at a flow-rate of 10 µL/min in an electrochemical cell. The reaction product mixture is collected and dried for chemical labeling.

Part 2: The EC reaction mixture is dissolved in the reaction solvent A. Reaction solvent A with catalyst, Cu^{2+} (5-500 µM) in a solution containing one of following non-aqueous solvents and one of following tertiary or sterically hindered amines, preferably in a ratio of 199:1 (v/v).
Catalyst / source: Cu^{2+} ions / $CuCl_2$ solution, $Cu(HCO_3)_2$ solution or $Cu(CH_3CO_2)_2$ solution.
Organic solvents: DMSO, acetonitrile, DMF, formamide, N-methylacetamide and THF
Non-nucleophilic amine (non-reactive tertiary or sterically hindered amine): TEA, DBTU, TBD, Pyridine and DBU

Part 3: The labeling mixture is added to the reaction solvent A, and mixed for e.g. 6 hours at room temperature. The labeling products can be used for further applications.

Chemical labeling of electrochemically cleaved peptides with amine-containing tags with Cu $^{2+}$ as a catalyst.

Scheme 1 shows chemical labeling of electrochemically cleaved peptides via their spirolactone moieties with amine-containing tags under basic non-aqueous conditions with Cu^{2+} (also herein referred to as copper II or Cu(II)) as a catalyst.



Scheme 1: Chemical labeling scheme of electrochemically cleaved peptides via their spirolactone moieties with amine-containing tags under basic conditions with copper II as a catalyst. R_1 and R_2 are the parts of the peptide N-terminal and C-terminal to the Trp residue, respectively. Amine-containing tags are indicated with a star.

The person skilled in the art will appreciate that a labeling method of the invention has a wide range of applications. For example, provided is a method for protein or peptide identification and/or quantification, comprising providing a site-specifically labelled polypeptide according to the invention in the presence of a divalent metal ion, and subjecting said labelled polypeptide to an analytical procedure. The analytical procedure may be aimed at detection, identification, characterization and/or quantitation. In a specific aspect, the analytical procedure comprises mass spectrometry (MS), preferably tandem mass spectrometry (MS/MS), more preferably RPLC-MS/MS, HILIC-MS/MS, CE-MS/MS, and fluorescence detection (FLD), preferably RPLC-FLD, HILIC-FLD and CE-FLD.

A method of the invention is also suitably used in synthetic processes, such as the preparation of protein or peptide polymers, for example, dendrimers.

A further aspect of the invention relates to a kit-of-parts for the site-specific labelling of a polypeptide. The kit comprises a source of Cu^{2+} ions and a nucleophilic labeling reagent. Preferred Cu^{2+} ion sources, labeling reagents and labelling mixtures are mentioned herein above. For example, the labeling reagent comprises a fluorescent dye, an affinity tag, like biotin, a stable-isotope-containing tag and/or a tag containing an element inducing a mass defect, like a halogen. The reagents can be in solution or they may be supplied as dried powders to be reconstituted prior to use.

LEGEND TO THE FIGURE

Figure 1. Stability of the electrochemical cleavage product LW+14 in the absence of Cu (II) (A) and in the presence of Cu (II) (B) after incubation for 6 h in DMSO/TEA (99.9/0.1) at room temperature. For clarity, the 6 h traces in both (A) and (B) were offset by 1 min on the x-axis and by 5×105 cps on the y-axis.

Figure 2. Chemical labeling of LW+14 with a 50-fold molar excess of hexylamine in the absence (A and B) and in the presence (C and D) of Cu (II). Extracted ion chromatograms of the electrochemically cleaved tripeptide LWL (LW+14, m/z 332.150) (A) before reaction, and of the chemical labeling product LW-hexylamine (433.256) (B) after reaction in DMSO containing 0.1% TEA for 6 h at room temperature in the absence of Cu(II). In the same order, panels C and D show extracted ion chromatograms of LW+14 (C) and the chemical labeling products LW-hexylamine (D) after reaction under the same conditions in the presence of

Cu (II). Peaks marked with * depict the ion of LWL containing one ^{13}C atom, which has the same mass as LW-hexylamine.

Figure 3. Biotinylation of LW+14 with amine-PEG2-biotin in the absence (A and B) or in the presence (C and D) of Cu (II). Extracted ion chromatograms of the electrochemically cleaved tripeptide LWL (LW+14, m/z 332.16) (A) before reaction, and the biotinylated product Biotin-LW (m/z 706.37) (B) after reaction with amine-PEG2-biotin in DMSO containing 0.1% TEA for 16 h at room temperature in the absence of Cu (II). In the same order, panels C and D show extracted ion chromatograms of LW+14 (m/z 332.16) (C) and the biotinylated products Biotin-LW (m/z 706.37) (D) after reaction under the same conditions in the presence of 2 μM Cu (II). LW+14* symbolizes the intramolecular rearrangement products (diketopiperazines).

Figure 4. (A) Scheme for enriching electrochemically generated peptide fragments after electrochemical cleavage of peptides and proteins via Cu (II)-mediated biotinylation of the spirolactone-containing peptide fragments and selective affinity enrichment. (B) Scheme showing the chemical conversion of a Trp-containing peptide from electrochemical cleavage to a biotinylated residue using amine-PEG2-biotin. R1 and R2 represent the peptide chains N-terminal and C-terminal to the Trp residue, respectively. The dashed line indicates the main MS/MS fragmentation site producing the characteristic fragment ion at m/z = 375.21 (see Figure 5).

Figure 5. LC-MS analysis of the electrochemical cleavage products of LWL (EC-LWL) (A), the reaction mixture after biotinylation and solid-phase extraction (SPE) (B) and the biotinylated LW+14 (Biotin-LW) after enrichment on monomeric avidin agarose (C). (A) Combined extracted ion chromatograms of the electrochemical cleavage products of LWL. The

asterisk * indicates unoxidized LWL (m/z 431.26). The symbol # represents uncleaved isomeric oxidation products LWL+32 (m/z 463.26) next to LW+14 (m/z 332.16). (B) LC-MS analysis of the biotinylation products of EC-LWL after SPE purification to remove excess amine-PEG2-biotin showing complete biotinylation of LW+14 (m/z 332.16) to LW-amine-PEG2-biotin (Biotin-LW, m/z 706.37) (mass increment of 374.21 Da). The more hydrophobic, later-eluting isomer of LWL+32 was also removed during SPE. (C) LC-MS analysis of biotinylation products of EC-LWL after enrichment on monomeric avidin agarose.

Figure 6. MS/MS spectrum of the biotinylated peptide (54GILQINSRW62+388.18) resulting from electrochemical cleavage of chicken egg lysozyme. The peptide was enriched by monomeric avidin agarose after Cu (II)-mediated biotinylation. The fragment at m/z 375.2062 comprises the entire amine-PEG2-biotin tag, whereas Fragments 1 to 4 are parts thereof (see the proposed structures and m/z values of the fragments in Figure 7). The y and b fragment ions match the amino acid sequence.

Figure 7. Proposed structures of the MS/MS fragment ions that are related to the amine-PEG2 biotin tag.

EXPERIMENTAL SECTION

Materials. Formic acid (HCOOH, FA, 98%), dimethyl sulfoxide (DMSO, anhydrous, 99.8%), acetic acid anhydride (99%), chicken egg white lysozyme, iodoacetamide (IAM), dithiothreitol (DTT), ammonium bicarbonate (99.5%), Cu (II) chloride dihydrate (99.9%), hexylamine (99%), sodium phosphate (96%), sodium chloride (99.0%), pyridine (99.8%), 2-hydroxypyridine (97.0%), sodium cyanide (97.0%), Cu (I) chloride (99.99%), nickel (II) chloride hexahydrate (98%), zinc acetate dihydrate (99.99%), iron (II) perchlorate

hydrate (98%), iron (III) perchlorate hydrate (crystalline) and D-biotin (analytical standard) were purchased from Sigma Aldrich (Steinheim, Germany). LWL was obtained from Research Plus Inc. (Barnegat, NJ, USA). Amine-PEG2-biotin and monomeric avidin agarose were obtained from Pierce Biotechnology (Rockford, USA). Acetonitrile (HPLC SupraGradient grade) was acquired from Biosolve (Valkenswaard, The Netherlands). Ultrapure water was obtained from a Milli-Q Advantage A10 water purification system at a resistivity of 18.2 MΩ cm (Millipore Corporation, Billerica, MA, USA).

Peptide and protein preparation. LWL at a concentration of 1 mM was prepared in 89/10/1 (v/v/v) water/acetonitrile/formic acid as stock solution. A stock solution of reduced and alkylated lysozyme was prepared in 89/10/1 (v/v/v) water/acetonitrile/formic acid at a protein concentration of 100 µM. For reduction and alkylation, lysozyme (100 µM), was prepared in 100 mM ammonium bicarbonate buffer (pH 8). Two mM DTT in 100 mM ammonium bicarbonate buffer were added, and the mixture was incubated with shaking at 500 rpm at 60 °C for 30 min. IAM was added at a concentration of 20 mM after cooling and reacted at room temperature in a dark environment for 40 min. After alkylation, 8 mM DTT was added to quench the alkylation reaction for 30 min. Lysozyme precipitated upon reduction and alkylation. The reaction mixture was centrifuged at 13000 rpm and the supernatant was removed. Water/acetonitrile/formic acid 89/10/1 (v/v/v) was added to dissolve the precipitated lysozyme and prepare a 100 µM stock solution of reduced and alkylated lysozyme. LWL was diluted to a final concentration of 10 µM (LWL) and lysozyme to 5 µM prior to electrochemical cleavage. To prevent formylation or acid hydrolysis, the formic acid content of lysozyme was increased to 5 % just before the electrochemistry experiments.

Electrochemical cleavage. Electrochemical oxidation and cleavage was performed in a µ-PrepCell electrochemical cell (thin-layer cell, Antec,

Zoeterwoude, NL) with a boron-doped diamond (BDD, 12 × 30 mm × 1 mm) working electrode (Antec), a titanium counter electrode and a palladium reference electrode (Pd/H_2). A flow rate of 10 µL/min was employed to introduce analyte solutions via a syringe pump (KD Scientific Inc., Holliston, MA, USA). The electrochemical potentials were controlled with a ROXY potentiostat (Antec) operating in Scan and DC mode. Cathodic pretreatment of BDD electrodes at a negative potential of -3000 mV was used to regenerate the electrode surface prior to all experiments by pumping 0.5 M nitric acid in water at a flow rate of 50 µL/min for 1 h. Prior to use, the cell was flushed with electrolyte solution at a potential of 2000 mV for 1 h.

The optimal cleavage potentials of peptides and proteins were first determined via on-line EC-MS experiments by ramping the cell potential from 0 to 3000 mV linearly at a scan rate of 10 mV/s. The detection of electrochemical cleavage products in on-line EC-MS was achieved in an API 365 triple quadrupole mass spectrometer (PE-Sciex, Concord, Ontario, Canada) with an EP10+ upgrade (Ionics, Bolton, Ontario, Canada) in positive ion mode. LWL and lysozyme were oxidized at 1000 mV and 2000 mV vs Pd/H_2, and the product mixtures (EC-LWL and EC-lysozyme) were collected for LC-MS analysis and further reactions.

Effect of Cu (II) on stability of cleavage products and chemical tagging. Cu (II) chloride dehydrate at a concentration of 100 µM was prepared in 89/10/1 (v/v/v) water/acetonitrile/formic acid as stock solution. EC-LWL with Cu (II) was prepared by adding 4 µL Cu (II) chloride dehydrate (100 µM) into 2 mL solution and dried. EC-LWL without Cu (II) was dried following the same procedure as a control.

To study the effect of Cu (II) on the stability of the spirolactone in peptides, 2 mL EC-LWL with or without Cu (II) were concentrated by

evaporation under nitrogen (2 h) and dried in an Eppendorf Concentrator at 30 °C. EC-LWL was prepared at a concentration of 400 µM by dissolving the dried sample in 50 µL DMSO/TEA (99.9/0.1) by pipetting for 30 s. Reactions were performed at 25 °C with shaking at 900 rpm in an Eppendorf Thermomixer. Ten µL of reaction product mixtures at time point 0 h and 6 h were prepared at a concentration of 5 µM by dilution with 390 µL 99/1 (v/v) water/formic acid, and 40 µL EC-LWL were subjected to LC-MS analysis. LC-MS analyses were performed on an Ultimate plus system (Dionex-LC Packings, Amsterdam, The Netherlands) coupled to an API 365 triple quadrupole mass spectrometer (PE-Sciex) with an EP10+ upgrade (Ionics). The separation of the reaction mixtures was achieved on a Vydac RP-C18 column (150 mm × 2.1 mm i.d., 5 µm particles, 300 Å pore size, Grace Vydac, Lokeren, Belgium) with a 35 min gradient of 2-50% acetonitrile in water/0.1% formic acid at a flow rate of 250 µL/min.

Chemical tagging with hexylamine was performed at a concentration of 500 µM by adding 20 µL of a mixture of DMSO, TEA and hexylamine (99.65:0.1:0.25) to 1 mL dried EC-LWL mixture in presence or absence of Cu (II), respectively, with a 50-fold molar excess of hexylamine. The reaction mixtures were incubated with shaking at 900 rpm at 25 °C for 6 h and analyzed by LC-MS. Biotinylation of EC-LWL was performed at a concentration of 500 µM for peptides in the presence or absence of Cu (II). Prior to use, 2 mg amine-PEG2-biotin was prepared in 1 mL water and purified on an Oasis HLB extraction cartridge (1cc, 30mg, Waters Corporation, Milford, Massachusetts, USA) by elution with 15 mL 10% ACN. After evaporating under nitrogen and drying in an Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany), amine-PEG2-biotin was dissolved in 50 µL DMSO/TEA (99.9/0.1). 20 µL amine-PEG2-biotin in solution was added to the sample in the presence or absence of Cu (II), and shaken at 900 rpm at 25 °C for 16 h, with a 200-fold molar excess of

hexylamine. Biotinylated EC-LWL (Bio-EC-LWL) was diluted to 5 μM by adding 99/1 (v/v) water/formic acid, and 40 μL of the reaction mixtures were analyzed by LC-MS. Liquid chromatography was performed on a Waters UPLC I-class system (Waters Corporation, Milford, USA) with a Waters Acquity Peptide BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particles, 300 Å pore size, Waters Corporation, Milford, USA) at 400 μL/min using a linear gradient from 5-40% acetonitrile in water/0.1% formic acid in 25 min. For mass spectrometry, a Maxis plus quadrupole time-of-flight mass spectrometer (QTOF, Bruker, Bremen, Germany) in positive electrospray ionization mode was used.

Biotinylation of EC-lysozyme with amine-PEG2-biotin. For biotinylation of EC-lysozyme with amine-PEG2-biotin, a reaction was performed in the presence of Cu (II). EC-lysozyme with Cu (II) was prepared by adding 4 μL Cu (II) chloride dehydrate (100 μM) into 2 mL EC-lysozyme (5 μM) and dried following the same procedure as described in our previous work. 20 Two mg amine-PEG2-biotin was purified, dried and dissolved in 50 μL DMSO/TEA (99.9/0.1) as described above. Twenty μL of amine-PEG2-biotin in DMSO/TEA (99.9/0.1) were added to the dried EC-lysozyme followed by pipetting for 30 s, and the mixture was incubated for 16 h with shaking at 900 rpm at 25 °C.

Removal of excess biotin by SPE after biotinylation. The biotinylation reaction mixture was diluted with 1 mL water/formic acid (99.9/0.1) and loaded on a Strata C18-E cartridge (55 μm, 70 Å, Phenomenex, Utrecht, The Netherlands). Twenty mL of 10% ACN and 3 mL 15% ACN in H2O/FA (99.9/0.1) were used to remove the excess of amine-PEG2-biotin (200-fold) followed by elution of the biotinylated peptide by adding 3 mL of 50% ACN to the cartridge. Elution fractions were collected and concentrated by evaporation under nitrogen (2 h, 15 μL) at 30 °C. Bio-EC-LWL and bio-EC-lysozyme were diluted with 100 mM phosphate-buffered saline (PBS, 100

mM sodium phosphate, 150 mM sodium chloride, pH 7) to a final volume of 500 µL prior to affinity enrichment.

Affinity enrichment of biotinylated peptides. One mL monomeric avidin agarose was packed and prepared in a disposable column according to the supplier's instructions. The biotinylated electrochemically cleaved peptides were captured with 1 mL monomeric avidin agarose in the column by incubation for 30 min at room temperature. The immobilized monomeric avidin agarose column was washed with 2 mL of PBS. Biotinylated peptides were eluted with 2 mL of D-biotin at a concentration of 2 mM in 100 mM PBS and concentrated by evaporation using nitrogen at 30 °C for 2 h. The elution fractions of bio-EC-LWL and bio-EC-lysozyme were prepared to a final volume of 5 mL and 2 mL, respectively, by adding 99/1 (v/v) water/formic acid prior to LC-MS/MS analysis.

Analysis by LC-MS/MS. LC-MS/MS analyses of the EC-cleaved peptide mixture, the biotinylation reaction mixture and the affinity-enriched biotinylated peptides were performed on a UPLC I-Class system (Waters) coupled to a quadrupole time-of-flight mass spectrometer (Maxis plus, Bruker) as described above. MS scans from m/z 200 to 1000 were recorded in profile mode using positive polarity.

The LC-MS/MS analyses of complex peptide mixtures obtained after avidin purification of EC-lysozyme were performed on a Dionex Ultimate 3000 nano-LC system equipped with an Acclaim Pepmap column (75 µm i.d. × 150 mm, Thermo Scientific, Bremen, Germany) coupled to a Q-Exactive Plus quadrupole-Orbitrap mass spectrometer (Thermo Scientific). To separate peptides, a linear gradient of 2-60 % acetonitrile in water/0.1 % formic acid in 50 min at a flow rate of 300 nL/min was employed. MS scans from m/z 200 to 1750 were recorded at a resolution of 75000. MS/MS spectra were recorded at a resolution of 17500 with a normalized collision energy of 35 V

in data-dependent mode. The top 10 highest intensity peaks were chosen for MS/MS.

Data analysis and database searching. The database search engine PEAKS (version 8.0, Bioinformatics Solutions Inc.) was used to analyze LC-MS/MS data using the chicken UniProt protein sequence database (Gallus gallus, updated 06-12-2016, SwissProt reviewed entries only) containing 2601 proteins. The search parameters were as follows: Parent Mass Error Tolerance: 10.0 ppm; Fragment Mass Error Tolerance: 0.05 Da; Enzyme: EC (custom-defined, digestion after Y or W); Max Missed Cleavages: 5; Non-specific Cleavage: one; Variable Modifications: Oxidation (on MFWHYC): +15.99, Carbamidomethylation (C): +57.02; EC-Y-2 (custom-defined: on C-terminal Y): -2.02; EC-W+14 (custom-defined: on C-terminal W): +13.98; EC-Y+372.16 (custom-defined: on C-terminal Y): +372.16; EC-W+388.18 (custom-defined: on C-terminal W): +388.18; Maximum variable post-translational modifications per peptide: 5.

EXAMPLE 1: Stabilization of peptide-spirolactones against intramolecular rearrangement in the presence of Cu (II) ions. Specific cleavage after Tyr and Trp in peptides and proteins upon electrochemical oxidation yields a complex mixture of peptides with a reactive spirolactone moiety at the C-terminus of the N-terminal fragment in addition to other peptide and protein modifications.

To investigate a range of catalysts that have been described to facilitate the reaction between an activated ester and a primary amine, we isolated the peptide-spirolactone LW+14 resulting from the electrochemical cleavage of the tripeptide LWL and screened the nucleophilic catalysts pyridine, 2-hydroxypyridine and NaCN as well as the following transition metal ions: Ni (II), Cu (I), Cu (II), Zn (II), Fe (II) and Fe (III). To study the effect of the

selected catalysts, we followed the stability of LW+14 in DMSO containing 0.1% TEA as well as the reaction of LW+14 with a 50-fold molar excess of hexylamine.

The presence of 0.5% pyridine or 2-hydroxypyridine in the reaction mixture had no effect on the chemical coupling yield and accelerated the undesirable intramolecular rearrangement (data not shown). NaCN and most of the transition metal ions had no effect on either coupling yield or the stability of the spirolactone-containing peptides with respect to intramolecular rearrangement. Out of all tested reagents, we found that only addition of Cu (II) ions prevented the intramolecular rearrangement reaction and significantly increased coupling yields with hexylamine.

Figure 1A shows that the spirolactone-containing peptide LW+14 rearranges to a pair of isomeric diketopiperazines (LW+14*) in the absence of Cu (II), as previously described, 20 while this was prevented by the addition of Cu (II). Rearrangement of LW+14 proceeded to 75% completion within 6 h in the absence of Cu (II), while LW+14 was stable in the presence of Cu (II) even under basic conditions in DMSO containing 0.1% TEA (Figure 1B). The small amount of diketopiperazines observed in the chromatogram was formed during the electrochemical cleavage reaction prior to adding Cu (II) ions.

EXAMPLE 2: Cu (II)-mediated spirolactone chemical tagging.

As shown in Example 1, Cu (II) stabilized the spirolactone-containing peptide and prevented rearrangement to diketopiperazines without having to resort to acetylation. Consequently, the chemical coupling of LW+14 to hexylamine was studied in the absence and in the presence of Cu (II) in DMSO containing 0.1% TEA. Efficient chemical tagging was only observed in the presence of Cu (II) at a 50-fold molar excess of hexylamine (Figure 2).

Reaction of LW+14 with amine-PEG2-biotin at a 200-fold molar excess was investigated next. Figures 3A and B show that only trace amounts of biotinylated LW+14 (Biotin-LW) were observed in the absence of Cu (II) ions since LW+14 underwent intramolecular rearrangement as the main reaction. Addition of Cu (II) prevented this side reaction resulting in complete biotinylation of LW+14 with amine-PEG2-biotin under otherwise identical conditions (Figures 3C and D). These results confirmed that addition of Cu (II) is critical for efficient chemical tagging of spirolactone-containing peptides.

EXAMPLE 3: Affinity enrichment of biotinylated peptides.

Since electrochemical peptide bond cleavage does not only generate the spirolactone-containing peptides, we investigated whether Cu (II)-mediated biotinylation can be used to enrich cleavage products from more complex mixtures. To this end, Cu (II)-mediated biotinylation according to the invention was combined with selective affinity enrichment on monomeric avidin beads as shown in Figure 4A. Figure 4B shows the conversion of a Trp-containing peptide from electrochemical cleavage to biotinylation of the C-terminus of the N-terminal fragment.

The approach was first tested on the tripeptide LWL. LWL was electrochemically cleaved at 1100 mV in an electrochemical cell with a BDD working electrode, yielding a mixture containing LWL, uncleaved oxidation products (LWL+32) and the spirolactone-containing cleavage product LW+14 (Figure 5A). LW+14 was completely biotinylated to LW-amine-PEG2-biotin (Biotin-LW) in DMSO containing 0.1% TEA in the presence of Cu (II) resulting in a mass increment of 374.21 Da (Figure 5B). Excess amine-PEG2-biotin was removed by SPE on a C18 cartridge, and the biotinylated peptide was captured on monomeric avidin agarose. After washing, biotinylated peptides were eluted with 2 mL D-biotin (2 mM) in

PBS. Figure 5C shows that biotinylated LW+14 (Biotin-LW) was effectively enriched from the complex mixture.

EXAMPLE 4: Affinity enrichment of biotinylated spirolactone-containing peptides from EC-cleaved lysozyme.

This example demonstrates that Cu (II)-mediated biotinylation after electrochemical peptide bond cleavage can be used to enrich spirolactone-containing peptides from proteins. Lysozyme (chicken egg), a protein of 14.6 kDa, was electrochemically cleaved at 2000 mV and the complex peptide mixture was biotinylated, enriched and subjected to nanoLC-MS/MS. Data were subjected to searching the chicken UniProt protein sequence database (*Gallus gallus*, updated 06-12-2016, SwissProt reviewed entries only) containing 2601 proteins. Modifications such as biotinylation (mass increment of 374.2062 Da) at the C-termini of predicted Tyr and Trp cleavage sites (resulting in an EC-Y+372.16 and an EC-W+388.18) were taken into account.

The biotinylated peptide ^{54}GILQINSRW^{62}+388.18, which derives from the spirolactone-containing peptide ^{54}GILQINSRW^{62}+14, was identified as a unique sequence of lysozyme. The MS/MS spectrum of the doubly-charged precursor ion (m/z at 737.8960) shows the characteristic, abundant fragment ion of the coupled biotin tag at m/z 375.2062 together with Fragments 1 to 4, which are smaller fragments of the amine-PEG2-biotin tag providing a signature of a biotin-tagged compound (Figure 6).

This result provides proof-of-principle that it is possible to identify a protein by combining electrochemical peptide bond cleavage followed by Cu (II)-mediated biotinylation and selective affinity enrichment on monomeric avidin agarose. This approach is suitably applied to more complex protein mixtures, with important advantages in proteomics applications.

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Claims

1. A method for the site-specific labelling of a polypeptide, comprising providing a polypeptide comprising at its C-terminus a reactive spirolactone moiety and reacting said spirolactone moiety with an appropriate nucleophilic labeling reagent, preferably under basic conditions, in the presence of Cu^{2+} ions to provide a C-terminally labelled polypeptide.

2. Method according to claim 1, wherein said polypeptide has a size up to about 20 kDa, preferably up to about 15 kDa.

3. Method according to claim 2, wherein said polypeptide is an oligopeptide comprising 2 to 100 amino acids, preferably 2 to 50 amino acids.

4. Method according to any one of the preceeding claims, wherein providing said polypeptide comprising at its C-terminus a reactive spirolactone moiety comprises electrochemical cleavage of a polypeptide, preferably a Trp- or Tyr-containing polypeptide.

5. Method according to claim 4, wherein said electrochemical cleavage is performed in the presence of Cu^{2+} ions.

6. Method according to any one of the preceding claims, comprising reacting said spirolactone moiety in the presence of at least 1 μM, preferably 5 to 500 μM Cu^{2+} ions.

7. Method according to any one of the preceding claims, wherein said labeling reagent comprises a primary amine.

8. Method according to any one of the preceding claims, wherein said labeling reagent comprises a fluorescent dye, an affinity tag, a stable-isotope-containing tag and/or a tag containing an element inducing a mass defect, like a halogen.

9. Method according to claim 8, wherein said affinity tag comprises biotin, preferably biotin provided with a spacer, more preferably PEGylated biotin.

10. Method according to any one of the preceding claims, comprising reacting a reactive spirolactone moiety with a labelling mixture comprising a non-aqueous solvent, a non-reactive tertiary or sterically hindered amine, and an amine-containing labeling reagent.

11. Method according to claim 10, wherein the non-aqueous solvent is DMSO, acetonitrile, DMF, formamide, N-methylacetamide or THF and/or wherein the tertiary or sterically hindered amine is TEA, DBTU, TBD, Pyridine or DBU.

12. Method according to claim 10 or 11, wherein said labelling mixture comprises DMSO, preferably DMSO and TEA.

13. A method for protein or peptide identification and/or quantification, comprising providing a site-specifically labelled polypeptide according to a method of any one of claims 1-12 and subjecting said labelled polypeptide to an analytical procedure.

14. Method according to claim 13, wherein said analytical procedure comprises mass spectrometry (MS), preferably tandem mass spectrometry (MS/MS), more preferably RPLC-MS/MS.

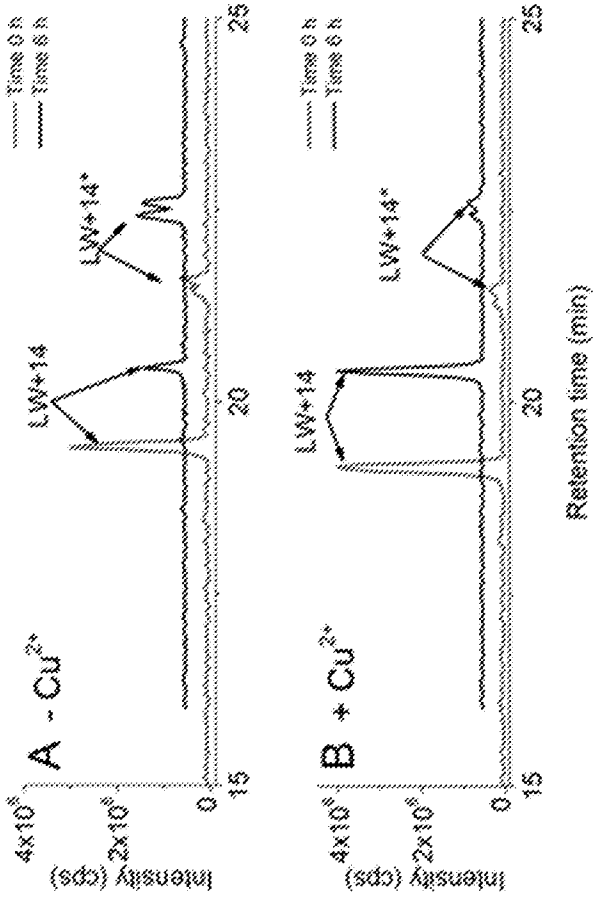
15. Kit-of-parts for the site-specific labelling of a polypeptide, comprising a source of Cu^{2+} ions, and a nucleophilic labeling reagent.

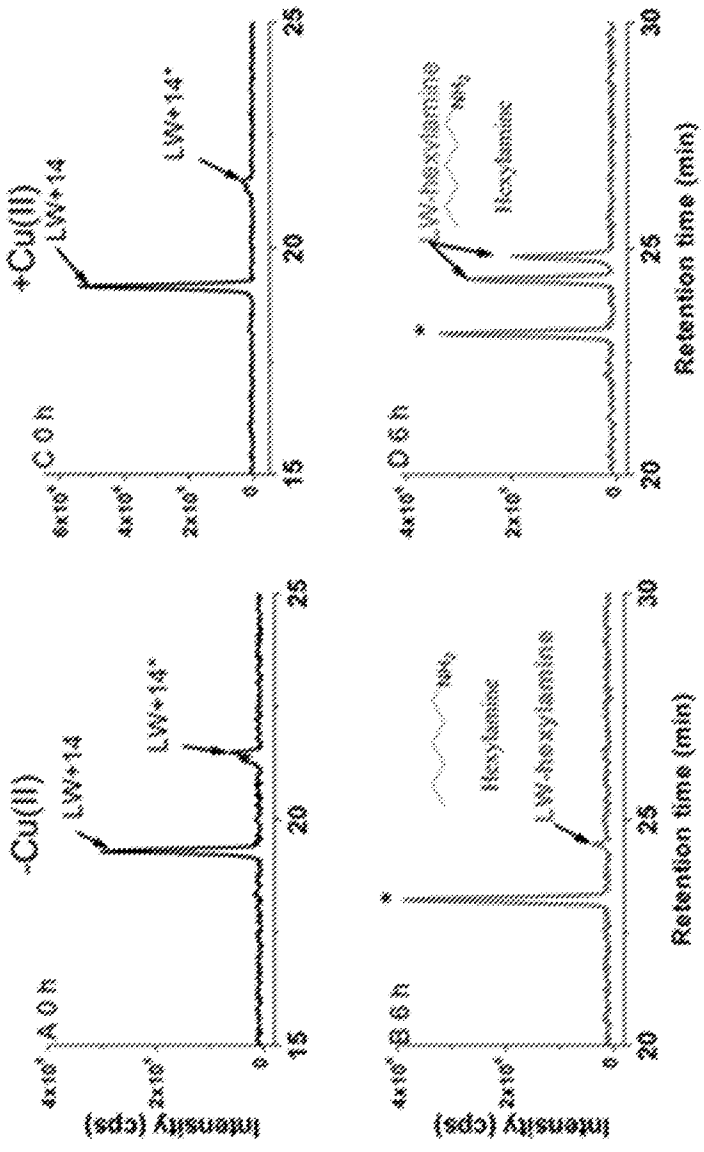
16. Kit according to claim 15, wherein said labeling reagent comprises a primary amine.

17. Kit according to claim 15 or 16, wherein said labeling reagent comprises a fluorescent dye, an affinity tag, a stable-isotope-containing tag and/or an element inducing a mass defect, like a halogen.

P111320PC00

Figure 1

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LW+14
LW+14'
A 0 h
+Cu(II)
LW+14
LW+14'
C 0 h
Hexylamine
LW-hexylamine
B 6 h
Hexylamine
LW-hexylamine
D 6 h
Intensity (cps)
Retention time (min)

Figure 2

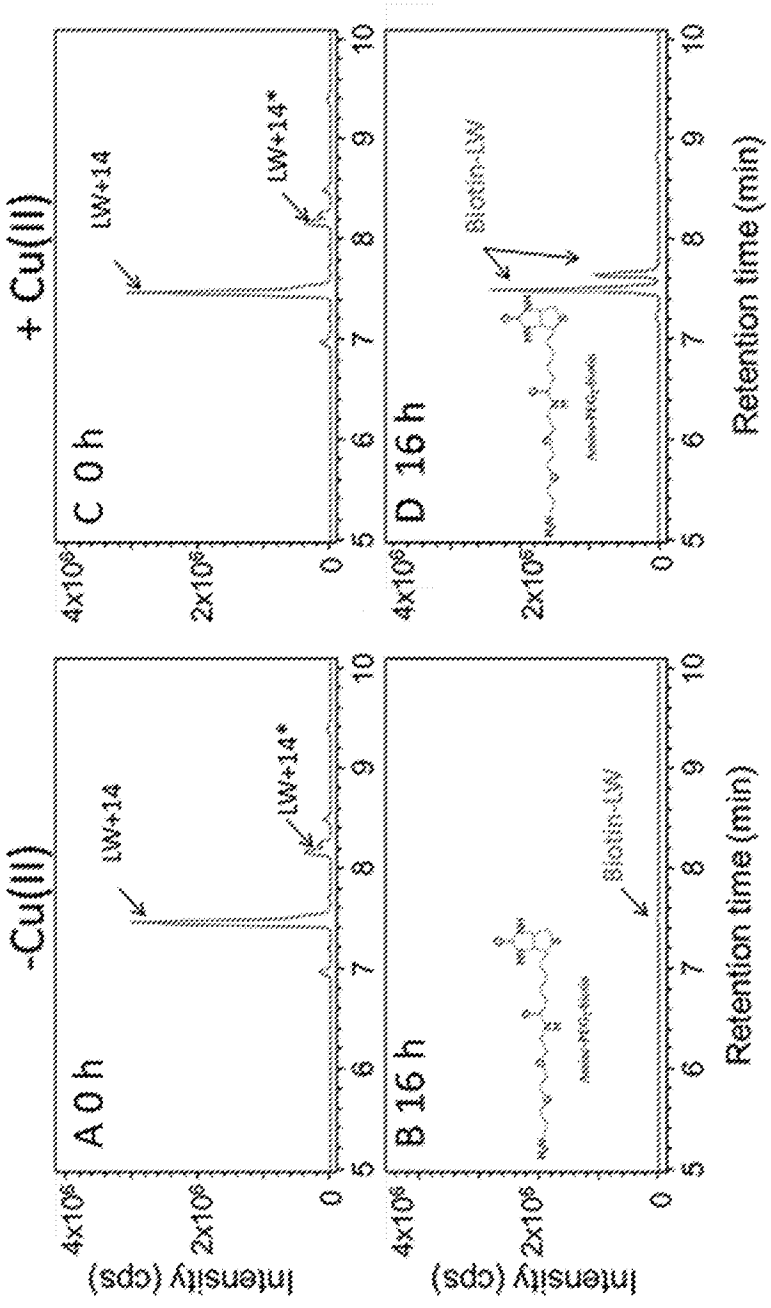

-Cu(III)
+ Cu(III)
A 0 h
B 16 h
C 0 h
D 16 h
LW+14
LW+14*
Biotin-LW
Intensity (cps)
Retention time (min)
4x10⁶
2x10⁶
0
5
6
7
8
9
10

Figure 3

Figure 4

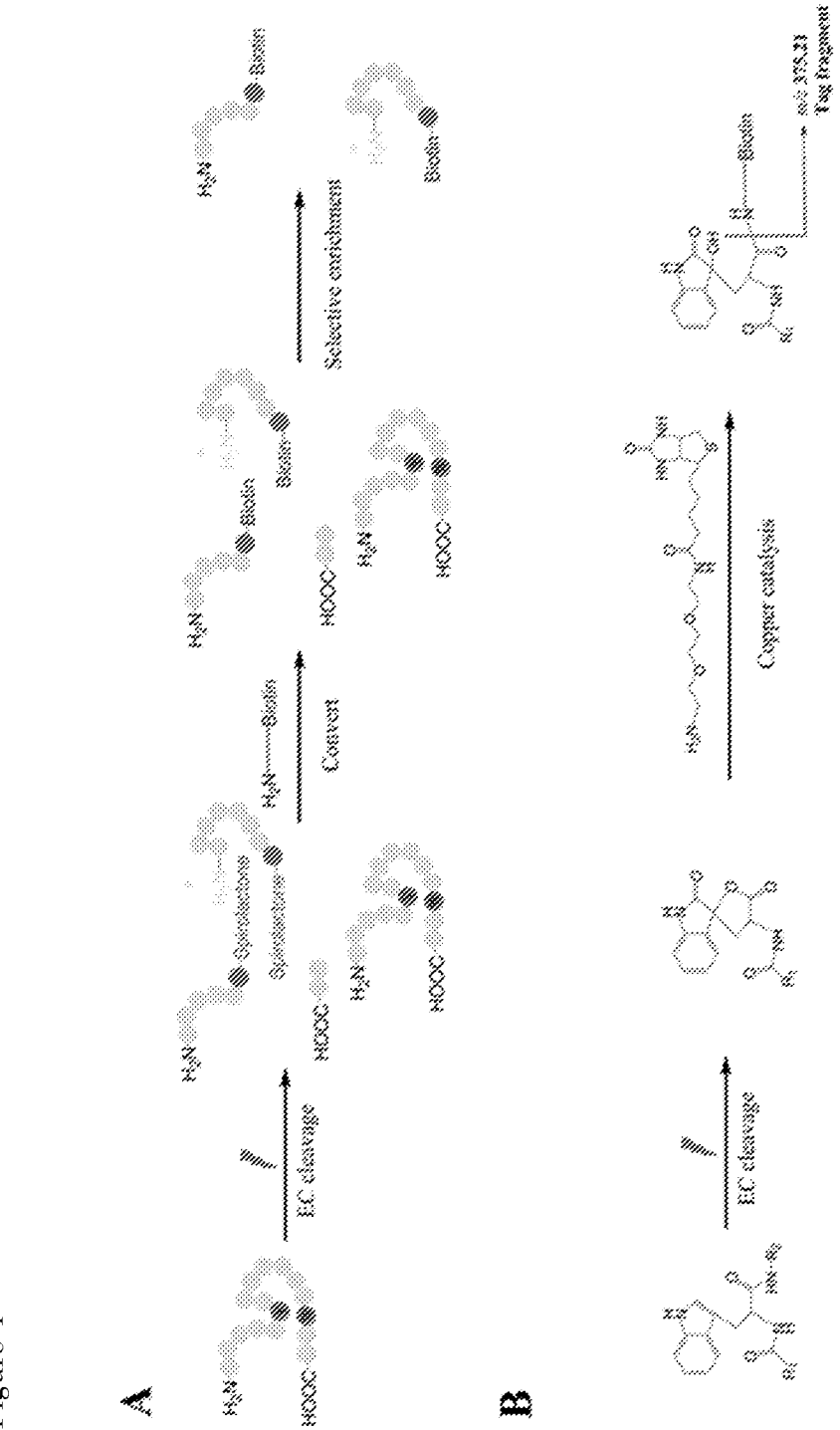

A
E.C. cleavage
Convert
Biotin
Selective enrichment
B
E.C. cleavage
Copper catalysis
Biotin
Tag Fragment

Figure 5

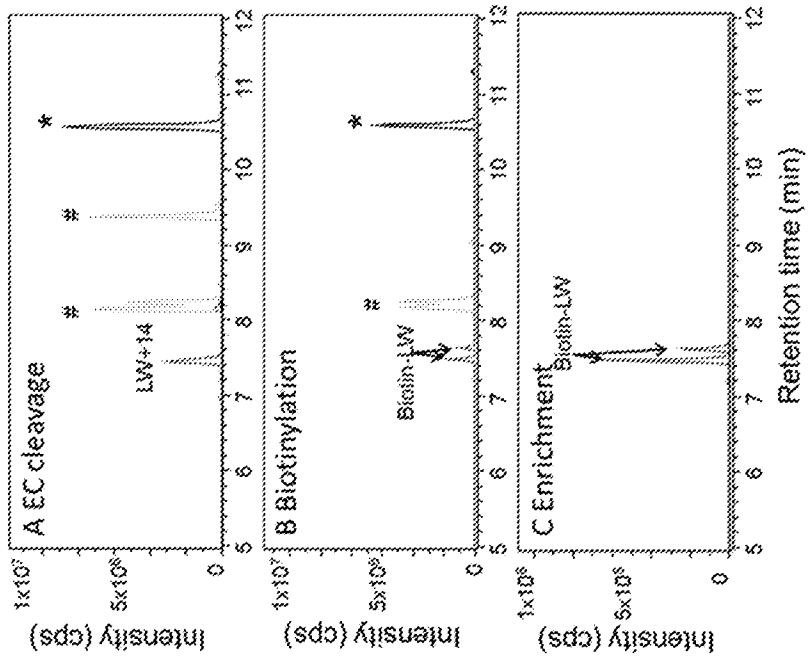

A EC cleavage
LW+14
B Biotinylation
Biotin-LW
C Enrichment
Biotin-LW
Intensity (cps)
Retention time (min)

Figure 6

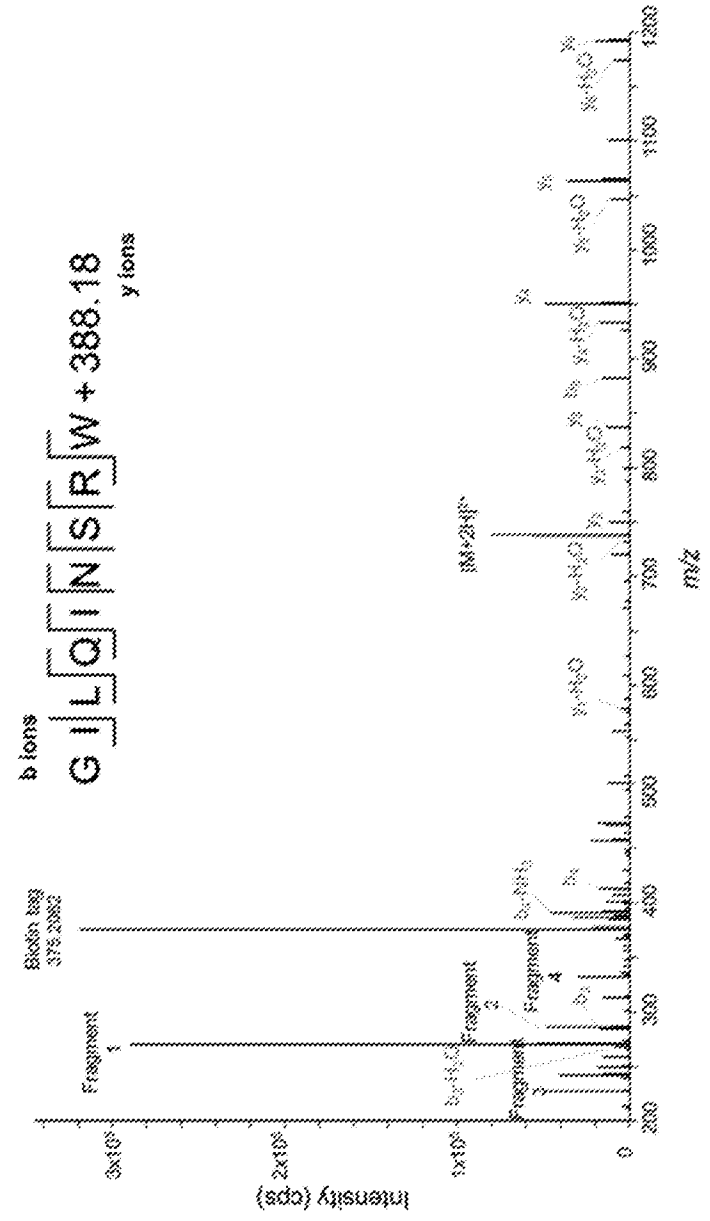
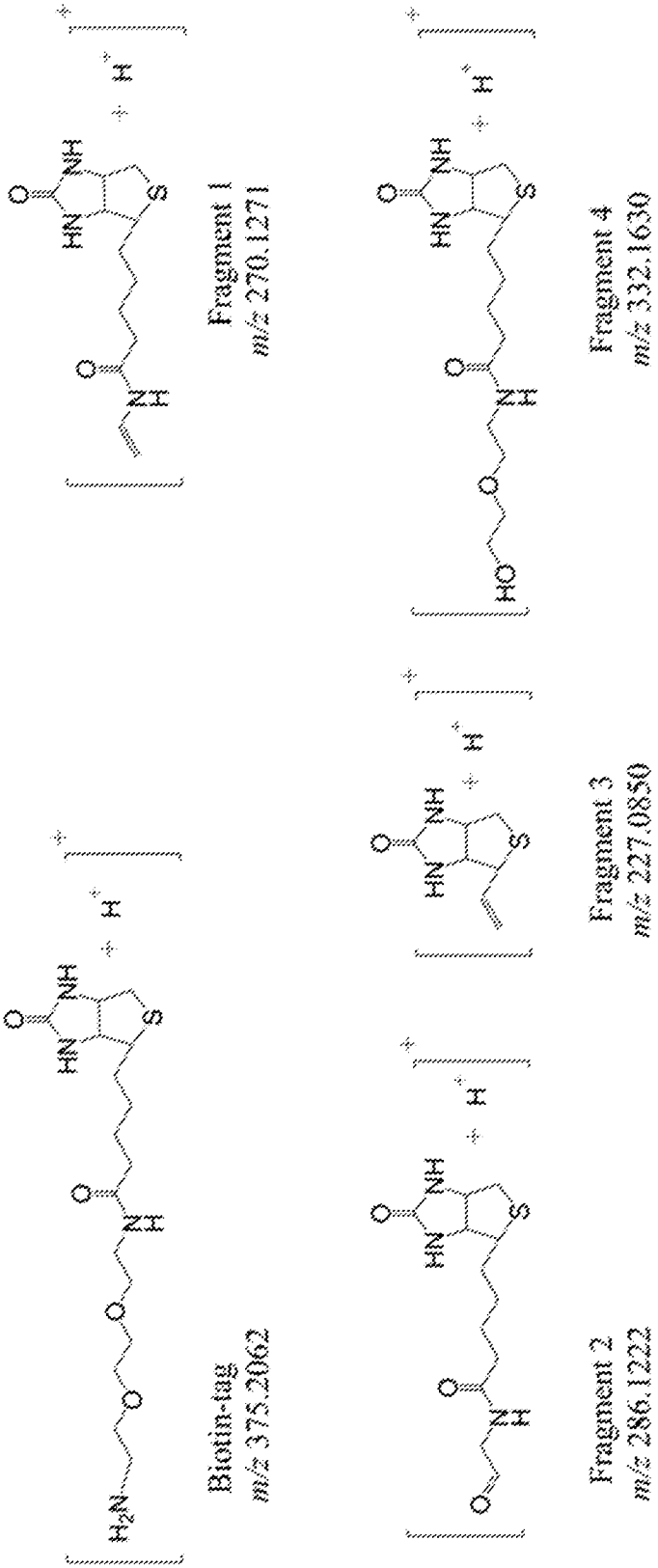

b ions
G I L Q I N S R W + 388.18
y ions
Biotin tag
372.2082
Fragment
1
Fragment
2
Fragment
3
Intensity (cps)
m/z
0
200
400
600
800
1000
1200

Figure 7


Biotin-tag
m/z 375.2062
Fragment 1
m/z 270.1271
Fragment 2
m/z 286.1222
Fragment 3
m/z 227.0850
Fragment 4
m/z 332.1630

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2017/050305

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K1/13
ADD. C07K1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JULIEN ROESER ET AL: "Chemical labeling of electrochemically cleaved peptides", RAPID COMMUNICATIONS IN MASS SPECTROMETRY., vol. 27, no. 4, 28 February 2013 (2013-02-28), pages 546-552, XP055317725, GB ISSN: 0951-4198, DOI: 10.1002/rcm.6479 cited in the application the whole document ----- -/--	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 September 2017	19/10/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schleifenbaum, A

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INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2017/050305

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZDZISLAW PARYZEK ET AL: "SYNTHESIS AND CLEAVAGE OF LACTONES AND THIOLACTONES. APPLICATIONS IN ORGANIC SYNTHESIS. A REVIEW", ORGANIC PREPARATIONS AND PROCEDURES INTERNATIONAL: THE NEW JOURNAL FOR ORGANIC SYNTHESIS, vol. 39, no. 3, 1 June 2007 (2007-06-01), pages 203-296, XP055317801, US ISSN: 0030-4948, DOI: 10.1080/00304940709356015 chapter II.2	1-14

X	SUBHASH CHANDRA GHOSH ET AL: "Copper-Catalyzed Oxidative Amidation of Aldehydes with Amine Salts: Synthesis of Primary, Secondary, and Tertiary Amides", THE JOURNAL OF ORGANIC CHEMISTRY, vol. 77, no. 18, 16 August 2012 (2012-08-16), pages 8007-8015, XP055101861, US ISSN: 0022-3263, DOI: 10.1021/jo301252c tables 1,2	15-17

X	SACHITANAND M. MALI ET AL: "Copper(ii) mediated facile and ultra fast peptide synthesis in methanol", CHEMICAL COMMUNICATIONS - CHEMCOM., vol. 48, no. 56, 1 January 2012 (2012-01-01), page 7085, XP055318434, ISSN: 1359-7345, DOI: 10.1039/c2cc32581k table 1	15-17

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