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(54) **Title**: COMPOUNDS AND METHODS FOR IDENTIFYING AND/OR QUANTIFYING CARBONYLATED BIOMOLECULES

(57) **Abstract**: The subject invention provides compounds and methods for derivatizing, identifying and/or quantifying carbonylated bio molecules by using compounds of the invention. The invention provides compounds and a method of using such compounds for the analysis of oxidized bio molecular species (such as peptides, proteins, and lipids). This method advantageously allows simultaneous derivatization, quantification and enrichment of carbonylated samples and is fully amenable to analysis by mass spectrometry.

COMPOUNDS AND METHODS FOR IDENTIFYING AND/OR QUANTIFYING CARBONYLATED BIOMOLECULES

FIELD OF THE INVENTION

The subject invention provides compounds and methods for derivatizing, identifying and/or quantifying carbonylated biomolecules by using compounds of the invention.

BACKGROUND OF THE INVENTION

Oxidative stress is thought to be involved in many disorders ranging from neuronal to metabolic to chronic inflammatory diseases. It is widely accepted that reactive oxygen species (ROS) are responsible for damaging biomolecules, such as lipids, sugars, DNA and, in particular, proteins. ROS are also involved in *in vitro* processes in many other fields and processes, such as in the food industry, where oxidation of polyunsaturated fatty acids and proteins present problems in the production, storage and aging of foodstuffs. It is known that protein carbonylation arises from oxidative attack, and is therefore the major indicator of oxidative stress in biological samples. Carbonyl derivatives of proteins are formed by a direct attack of reactive oxygen species on specific amino acid side chains or can be the result of secondary reactions with reactive carbonyl compounds of advanced glycation or lipoxidation end products.

Determining the identity of proteins susceptible to carbonylation *in vitro* and *in vivo* can provide a new level of information that may be critical to understanding the specific pathophysiological consequences of oxidative stress-induced damage.

Oxidative stress is conventionally analyzed using various derivatization and detection methods, for example derivatization with 2,4-DNPH (i.e., 2,4-dinitrophenylhydrazine), and detection with antibodies directed against the DNP (i.e., dinitrophenyl) moiety. The major current derivatization method is with biotin hydrazide, and this has been recently employed in conjunction with detection by avidin-coupled techniques by Hensley *et al.*, 2009. Fluorescence based methods are also in effect. Carbonyl trapping mechanisms using a hydrazide moiety are known in the art, for example US 2001/0036538 and US 2005/0250152. All of the disclosed methods for detection are however limited in their usefulness and applicability due to the low specificity and system-limited nature of the markers used for detection.

The present invention, in contrast, provides a highly specific marker for the detection/measurement of carbonylated biomolecules, which can provide information that may be critical to understanding the specific pathophysiological consequences of oxidative stress-induced damage.

The method of the invention by using the presently disclosed compounds offers the advantage of simultaneous derivatization, quantification and enrichment of carbonylated biomolecules, which is fully compatible with analysis by mass spectrometry.

SUMMARY OF THE INVENTION

The invention provides compounds and methods for enriching, identifying and/or quantifying carbonylated biomolecules by using compounds of the invention.

The current invention thus provides compounds and a method of using such compounds for the analysis of carbonylated biomolecular species (such as peptides, proteins, and lipids). This method advantageously allows simultaneous derivatizing and enrichment of carbonylated samples and is fully amenable to analysis by mass spectrometry, in particular in MS and MS/MS mode. Mass spectrometry-based methods have in addition the potential to determine the localization of protein carbonylation.

In a preferred embodiment of the invention, proteins, or more generally biomolecules, that give rise to a reactive carbonyl upon oxidation are identified using the compounds and methods of the invention. As noted above, there appears to be a relationship between the number of protein carbonyl groups and oxidative stress and subsequent diseases or conditions associated with oxidative stress. Accordingly, one object of the invention is the identification and usage of protein carbonyl groups as biomarkers of oxidative stress.

In another aspect, the present invention relates to a method of the invention comprising the following steps: (i) biomolecules derivatization by the compound of the invention, (ii) identification of specifically carbonylated biomolecules, (iii) optional quantification of carbonylated species and/or of the rate of carbonylation.

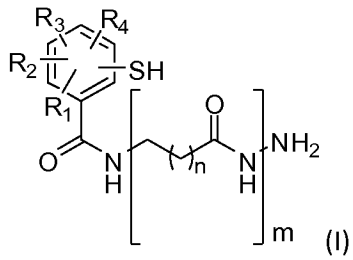
The term “derivatization” refers to the step where the carbonyl groups of the biomolecules are reacted with the compounds of the invention.

The term "identification" refers to the determination of the nature (for instance for lipids) and/or sequence (for instance for peptides, proteins, or the like) of the carbonylated biomolecules. The identification step may further include the localization of the carbonylation on the biomolecule. The identification step can be more specifically carried out by using spectrophotometry and/or mass spectrometry techniques.

The term "quantification" refers to the determination of the proportion of carbonylated biomolecules in the sample and/or of the number of carbonylations per biomolecule.

The terms "biomolecular species" and "biomolecule" refer to a single biomolecule or to a mixture of biomolecules, in particular peptides, proteins, lipids, and the like. These terms may also refer to a biological sample comprising at least one biomolecule susceptible to carbonylation.

In another aspect, the present invention relates to compounds having the general formula (I):

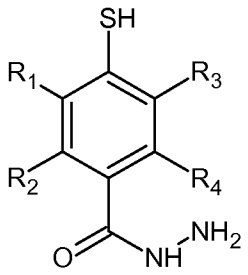


(I)

wherein m is 0 or 1, n is an integer from 0 to 6 (inclusive) and R_1, R_2, R_3, and R_4, identical or different, represent H, OH, NH_2, (C1-C8)alkyl, (C3-C8)cycloalkyl, (C6-C18)aryl, heteroaryl, heterocycle, halogen, or a silyl group, such as trialkylsilyl groups, dialkylarylsilyl groups, alkyldiarylsilyl groups, and triarylsilyl groups.

In an embodiment, m is 0 and at least one of R_1, R_2, R_3, and R_4 represents NH_2, (C2-C8)alkyl, (C3-C8)cycloalkyl, (C6-C18)aryl, heteroaryl, heterocycle, or a silyl group as defined above.

The present invention more specifically discloses compounds having the general formula 1, 2, 3, 4, 5 or 6:



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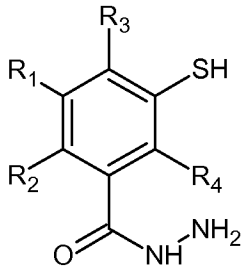

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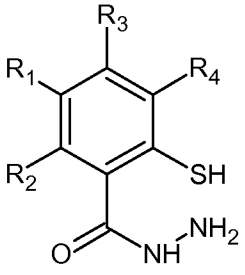

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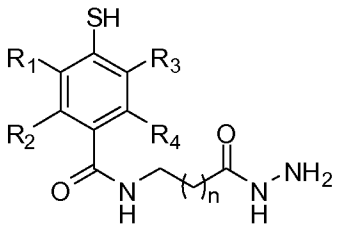

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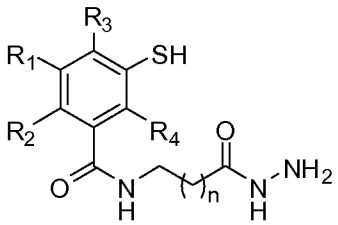

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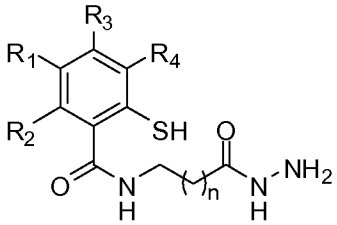

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wherein n, R_1, R_2, R_3, and R_4 are as defined above. m is 0 for compounds 1, 2 and 3. m is 1 for compounds 4, 5 and 6. A more specific compound of the invention is a compound of formula 4 wherein R_1, R_2, R_3, and R_4 are hydrogen atoms and n is 2.

Preferably, n is equal to 1, 2, 3, 4, 5, or 6. A highly preferred value for n is 4.

The term "alkyl" when used alone or in combination with other terms, comprises an optionally substituted straight or branched C_1-C_8 alkyl chain which refers to monovalent alkyl groups having 1 to 8, preferably 2 to 8, carbon atoms. This term is exemplified by groups such as methyl, ethyl, n-propyl, i-propyl, n-butyl, t-butyl, n-pentyl, 1-ethylpropyl, 2-methylbutyl, 3-methylbutyl, 2,2-dimethylpropyl, n-hexyl, 2-methylpentyl, 3-methylpentyl, 4-methylpentyl, n-hexyl, 2-methylhexyl, 3-methylhexyl, 4-methylhexyl, 5-methylhexyl, n-heptyl, n-octyl. According to a specific embodiment, the alkyl group has 1 to 4 carbon atoms.

The term "cycloalkyl" when used alone or in combination with other terms, refers to an optionally substituted saturated carbocycle of from 3 to 8 carbon atoms having a single ring (e.g cyclohexyl) or multiple condensed rings (e.g norbornyl).

The term "aryl" when used alone or in combination with other terms, refers to an optionally substituted unsaturated aromatic carbocyclic group of from 6 to 18 carbon atoms having a single ring (e.g phenyl) or multiple condensed rings (e.g indenyl, naphthyl). The term aryl

includes phenyl, naphthyl, anthryl, phenanthryl and the like. The aromatic group may be at least partially hydrogenated.

The term "heterocycle" refers to a cycloalkyl group as defined above, substituted in at least one position of the cycle with a heteroatom that may be an atom of N, O or S.

The term "heteroaryl" refers to an aryl group as defined above, substituted in at least one position of the cycle with a heteroatom that may be an atom of N, O or S.

The term "halogen" refers to any atom from group 17 of the "IUPAC" periodic table, comprising in particular chlorine, fluorine, bromine, or iodine.

The silyl groups are well known in the art and can be selected by anyone of ordinary skill in the art, for instance, among alkylsilyl groups, dialkylarylsilyl groups, alkyldiarylsilyl groups, and triarylsilyl groups.

The approach involves therefore the synthesis and use of compounds of formula (I), in particular of formulae (1) to (6) that are capable of providing these functions, having the generic name "PACa" (which stands for "Piège A Carbonyles"). The compounds of the invention are suitable for both *in vivo* and *in vitro* studies. They allow derivatization and identification of the carbonylated species by spectrophotometry and/or mass spectrometry and specific enrichment of the carbonylated species, for instance in complex samples.

The compounds of the invention are thus brought into contact with a biomolecule, such as a peptide or lipid, in the derivatization step. The compounds of the invention are highly specific to reaction with carbonyls only and form stable hydrazones. As the compounds of the invention are small molecules, they are susceptible to react with several carbonyl groups in the same biomolecule, contrary to biotin hydrazide that is classically used for detection of carbonylated biomolecules. Simultaneous reaction with several carbonylated groups of the same biomolecule affords the possibility to quantify the rate of carbonylation of said biomolecule, in other words the proportion of carbonylated aminoacids per biomolecule. Quantification of the rate of carbonylation of the biomolecule may be performed subsequently to contacting the biomolecule with a compound of the invention, as described below.

The compounds of the invention may be mounted on supports, either before or after derivatization with the biomolecule, more specifically via disulphide bonds through the thiol

groups on the compounds of the invention. The support on which the compounds of the invention may be mounted on gold supports, thio-silica magnetic beads, sepharose columns, and/or any other solid support susceptible to be reacted with the thiol function of the compounds of the invention, for instance susceptible to bear thiol groups that can be reacted with the thiol function of the compounds of the invention. The compounds mounted on a support facilitate analyses, in particular they allow an enrichment step where the concentration of carbonylated species can be amplified.

The term "enrichment" refers to increase the concentration of the carbonylated biomolecular species linked to the compound of the invention, with respect to the various other species in a sample. This may be associated with a separation process, for instance via disulfide bonds reduction, whereby only the carbonylated species are collected for further analysis.

Quantification of the carbonyl content of the derivatized biomolecules may be carried out for instance by using UV/Vis spectroscopy or mass spectrometry.

Detailed characterization studies of the biomolecules with carbonyls bonded to the compounds of the invention are then carried out, in particular with the aid of mass spectrometry, to provide an in depth insight into the carbonylation of the biomolecule that has taken place.

In particular, mass spectrometry may be used both to confirm the derivatization of the biomolecule by the compounds of the invention did take place, and to identify the biomolecule. Derivatization of the biomolecule by the compounds of the invention is assessed by the detection of a specific mass on the mass spectrometry spectra. This specific mass is that of a "reporter ion" and corresponds to a fragment of compounds of the invention. Detection of this ion is a proof of the derivatization of the biomolecule with the compound of the invention. For example, this specific mass is 137 Da for molecules of the formula 1, 2 or 3 wherein R_1 to R_4 are hydrogen atoms.

This reporter ion is an important asset of the compounds and methods of the invention because it is easily detected on a mass spectrum, even if the spectrum presents a great number of peaks due to the fragmentation of many biomolecules and biomolecule derivatives possibly present in the sample.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the MALDI TOF MS spectrum in positive mode of the commercial carbonylated peptide (PepCO) before and after derivatization with PACa.

Figure 2 shows the MALDI TOF MS spectrum in positive mode of the carbonylated A9 peptide after derivatization with PACa. The mass shift of 149.65 Da (1257.25-1107.62) corresponds to the specific interaction between the carbonylated lysine of the A9 peptide and PACa, and a second mass shift of 149.65 Da (1408-1258) corresponds to a second carbonylated lysine derivatized with PACa.

Figure 3 shows the MALDI TOF MS spectrum in positive mode of three phospholipids: DMPC (A), PGPC (B) and POVPC (C), before and after reaction with PACa. A and B DMPC and PGPC in the presence and absence of PACa doesn't make any difference. C, POVPC with and without PACa shows the total derivatization of the carbonylated compound with PACa.

Figure 4 shows MALDI MS spectra of (A), pepCO in solution before enrichment on the magnetic silica beads with thiol surface, and (B) after elution from these beads.

Figure 5 shows MALDI TOF MS spectra. Spectrum A: PACa enrichment of a mix of phospholipids (POVPC, DMPC, PGPC) with an equal ratio placed in the column. Spectrum B: the solution collected from the column (POVPC was held on the column). Panel C: elution with DTT 100mM and collecting only the carbonylated phospholipid.

Figure 6 shows the UV-visible spectrum from 250 to 500 nm of PACa and a carbonylated protein (human serum albumin), the shift of the maximum of absorbance of PACa from 310 nm when free to 340 nm when fixed to protein carbonyl can be noticed.

Figure 7 shows CID MALDI spectrum of the PepCO before (the left spectrum) and after PACa derivatization (the right spectrum). b fragments are the same whereas y fragments shifted by 150.6 Da. The PACa's reporter ion is at 137 Da.

Figure 8 shows CID MALDI spectrum of (A), the non modified peptide A9 and (B), the carbonylated lysine derivatized to PACa. Note the presence of the specific signature at 137

Da, the neutral loss of 168 Da (mass of PACa). b and y: non modified ions, b* and y*: PACa derivatized ions, §: internal ions, §*: internal derivatized ions.

Figure 9 shows MS/MS spectrum of POVPC-PACa on the upper spectrum and POVPC on the lower spectrum. Both of them give a fragment at 184.5 Da. The peak at 137 Da signs the presence of PACa.

Figure 10 shows MS spectra of carbonylated A9 derivatized with A, PACa, or B, biotin hydrazide. 2 PACa molecules could be fixed on a bicarbonylated peptide whereas only one biotin hydrazide was fixed on the same oxidized peptide.

Figure 11 shows MS spectra of the supernatants comprising the oxidized A9 peptide after enrichment on gold beads in presence (GNP+A9ox-PACa) and in absence (GNP + A9ox) of derivatization of the oxidized A9 peptide with PACa.

Figure 12 shows MS spectra of the protein digest and A9 oxidized peptide derivatized with PACa (lower spectrum) and of the non retained fraction on GNP (upper spectrum)(a), and MS spectra of the eluted fraction in presence (upper spectrum) and in absence (lower spectrum) of PACa derivatization (b).

Figure 13 shows MS spectra of Tau protein (lower spectrum) and of oxidized Tau protein (upper spectrum).

Figure 14 shows MS spectra of the_oxidized Tau eluted from the GNP without PACa derivatization (a), the non fixed fraction of oxidized tau-PACa on GNP (b) and the eluted fraction from GNP with oxidized Tau-PACa (c).

Figure 15 shows the chemical formulation of PACa with its specific functions (left side), and UV spectrum of PACa with the maximum of absorption at 310 nm (right side).

Figure 16 shows ms/ms spectrum of PACa acquired in positive mode by infusion in Q-Tof mass spectrometer. The spectrum is composed of the pseudo molecular parent ion ($M+H^+$) at m/z =169 and different fragments obtained by collision induced dissociation. A specific ion at m/z=137 in the spectra is used as signature of the PACa combined with carbonyl compounds.

Figure 17 shows the mass spectrum (a) and the MS/MS spectrum (b) of the second synthesized molecule.

DETAILED DESCRIPTION OF THE INVENTION

According to a preferred embodiment, compounds of the invention have general formula 1.

According to another embodiment, the compounds of the invention are of formulae 1-3 in which R_1, R_2, R_3, and R_4, identical or different, are hydrogen atoms or (C1-C8)alkyl groups, more specifically hydrogen atoms.

According to another embodiment, the compounds of the invention are of formulae 4-6 in which R_1, R_2, R_3, and R_4, identical or different, are hydrogen atoms or (C1-C8)alkyl groups, more specifically hydrogen atoms.

More specifically, the compound of the invention is of formula 1 in which R_1, R_2, R_3, and R_4 are hydrogen atoms or (C1-C8)alkyl groups (in particular (C1-C4)alkyl groups).

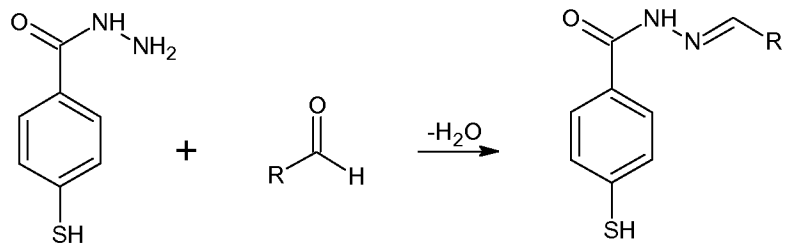
According to a particular embodiment, the compound of the invention is of formula 1 in which R_1, R_2, R_3, and R_4 are hydrogen atoms (i.e., 4-sulfanylbenzohydrazide).

According to another aspect, the invention relates to a method for the derivatization, enrichment, identification and/or quantification of carbonyl groups of biomolecules by using a compound as defined above.

One method of the invention comprises the following steps: (i) derivatization of biomolecules by the compound of the invention, (ii) identification of specifically carbonylated biomolecules, (iii) optional quantification of carbonylated species and/or of the rate of carbonylation.

Step (i): Derivatization of biomolecules

Derivatization of biomolecules is carried out to form the stable hydrazone. Said step can be represented as follows.



According to the present invention, biomolecules derivatization by the compound of the invention can be carried out by contacting at least one biomolecule or a sample comprising a mixture of molecules that include biomolecules of interest with at least one compound of the invention.

According to a particular embodiment, step (i) is performed by contacting a sample comprising a mixture of molecules that include biomolecules of interest with a compound of the invention mounted on a solid support. The compounds of the invention may be mounted on supports, such as thio-silica magnetic beads, gold nanoparticles, or a sepharose column, more specifically via bonds, in particular disulphide bonds, through the thiol groups on the compounds of the invention. The support may be any solid support susceptible to be reacted with the thiol function of the compounds of the invention, for instance any support susceptible to bear thiol groups that can be reacted with the thiol function of the compounds of the invention. Fixation of the compound of the invention on the solid support may also be performed after derivatization of the biomolecule of interest with the compound of the invention.

The use of compounds of the invention on a solid support in the method of the invention affords an enrichment of the derivatized biomolecules, thus easing their subsequent analysis. After derivatization, the solid support may be eluted and/or washed with any appropriate solvent or reagent in order to recover a sample comprising a high proportion of biomolecules derivatized with compounds of the invention.

Samples containing the biomolecules of interest can be obtained from any source including, but not limited to, any biological or environmental source. For example, the sample may be a biological material, such as fermentation fluid, soil, water, food, pharmaceutical, organ culture, tissue culture, cell culture; any plant tissue or extract including root, stem, leaf, or seed, exhaled breath, whole blood, blood plasma, urine, semen, saliva, lymph fluid, meningal fluid, amniotic fluid, glandular fluid, sputum, feces, sweat, mucous, cerebrospinal fluid, and

experimentally separated fractions of all of the preceding solutions or mixtures containing homogenized solid material, such as feces, organs, tissues, and biopsy samples.

Step (ii): Identification of specifically carbonylated biomolecules

Step (ii) may be performed by any characterization technique known in the art. In a specific embodiment, mass spectrometry techniques are used in step (ii). Preferably, MALDI techniques may be used, in particular MALDI-TOF.

In a specific embodiment, mass spectrometry, preferably MALDI, in particular MALDI-TOF, is the only characterization technique used in step (ii).

Step (ii) is eased by the chemical nature of compounds of the invention that triggers the presence of a reporter ion easily identifiable on mass spectra, as detailed above.

According to a particular embodiment, MS and MS/MS mass spectrometric techniques are subsequently used to characterize the carbonylated biomolecule fixed with the compounds of the invention.

In a preferred embodiment, the method of the invention comprises after step (ii) an additional step of using the results of step (ii) to identify biomolecules susceptible to carbonylation by using known methods. For example, the nature or the sequence of the biomolecules susceptible to oxidation may be determined (e.g., by mass spectrometry and other non-mass spectrometry based sequencing techniques). In a particular embodiment, the sequence may subsequently be searched on a sequence database to identify the protein.

According to a specific embodiment, the sample comprising proteins can be subjected to enzymatic digestion or chemical cleavage, such as with trypsin, resulting in peptides. Said enzymatic digestion or chemical cleavage can be implemented before step (i) or between steps (i) and (ii). In that embodiment, step (ii) leads to identification of the peptides, when the additional step described in the above paragraph leads to identification of the proteins.

Step (iii): Optional quantification of carbonylated species and/or of the rate of carbonylation

Two different parameters can be determined to quantify the carbonylation. The method of the invention allows determination of the number of carbonylated biomolecules out of the total number of biomolecules present in the sample. Alternatively, the method of the invention may

be used to determine the number of carbonylated aminoacids per biomolecule of the sample (rate of carbonylation). The aminoacids susceptible to carbonylation are lysine (K), arginine (R), proline (P) and threonine (T).

According to a specific embodiment, quantification is carried out using UV/Vis spectroscopy and more specifically via the calculation of the Beer Lambert Coefficient of the compound of the invention.

According to a particular embodiment, the method further comprises the step of (iv) identifying a disease, disorder, or condition associated with the identified biomolecule susceptible to oxidation as determined from step (ii).

Identifying a disease, disorder, or condition includes forecasting, detecting, diagnosing or monitoring a disease, disorder, or condition.

Examples of diseases, disorders and conditions that can be forecasted or detected or diagnosed or monitored in a patient using the PACa compounds and methods of the subject invention include, but are not limited to, neurological and neurodegenerative diseases and conditions such as age-associated dementia, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, peripheral neuropathy, stroke, traumatic injury, and various neurological and other degenerative consequences of neurological and chest surgeries, schizophrenia, epilepsy, Down's Syndrome, and Turner's Syndrome; degenerative conditions associated with AIDS; various bone disorders including osteoporosis, osteomyelitis, ischemic bone disease, fibrous dysplasia, rickets, Cushing's syndrome and osteoarthritis; other types of arthritis and conditions of connective tissue and cartilage degeneration including rheumatoid, psoriatic and infectious arthritis; various infectious diseases; muscle wasting disorders such as muscular dystrophy; skin disorders such as dermatitis, eczema, psoriasis and skin aging; degenerative disorders of the eye including macular degeneration and retinal degeneration; disorders of the ear such as otosclerosis; impaired wound healing; various cardiovascular diseases and conditions including stroke, cardiac ischemia, myocardial infarction, chronic or acute heart failure, cardiac dysrhymias, artrial fibrillation, paroxysmal tachycardia, ventricular fibrillation and congestive heart failure; circulatory disorders including atherosclerosis, arterial sclerosis and peripheral vascular disease, Type I or Type II diabetes ; cancers, including glioblastomas, (promyelocytary) leukemias, cancers of the prostate, the ovaries, the lungs, the breasts, the

digestive tract, in particular of the liver, of the pancreas, of the head and of the neck, of the colon, of the bladder, non-Hodgkin lymphomas and melanomas ; various diseases of the lung including lung cancer, pneumonia, chronic obstructive lung disease (bronchitis, emphysemia, asthma); disorders of the gastrointestinal tract such as ulcers and hernia; dental conditions such as periodontitis; liver diseases including hepatitis and cirrhosis; pancreatic ailments including acute pancreatitis; kidney diseases such as acute renal failure and glomerulonepritis; and various blood disorders such as vascular amyloidosis, aneurysms, anemia, hemorrhage, sickle cell anemia, autoimmune disease, red blood cell fragmentation syndrome, neutropenia, leukopenia, bone marrow aphasia, pancytopenia, thrombocytopenia, and hemophilia. The preceding list of diseases and conditions which can be identified according to the subject invention is not intended to be exhaustive or limiting but presented as examples of such degenerative diseases and conditions.

The term "patient," as used herein, describes an animal, including mammals, for which biomarkers of oxidative stress can be identified using compounds and methods of the present invention. Mammalian species that can benefit from the disclosed methods of the invention include, and are not limited to, humans, apes, chimpanzees, orangutans, monkeys; and domesticated animals such as mice, rats, dogs, cats, guinea pigs, and hamsters.

In plants, all stress phenomena are accompanied by an increased production of reactive oxygen species (ROS) and this can lead to damage to proteins, lipids and DNA. In that respect, the method may further comprise the step of (iv) identifying a plant pathological situation associated with the identified biomolecule susceptible to oxidation as determined from step (ii). Accordingly, the present invention provides compounds and methods for identifying biomolecules susceptible to, and thus biomarkers of, oxidative stress that may be used to forecast plant pathological situations earlier than the actual manifestation of symptoms.

This invention offers a simple, unexpensive method for the analysis of oxidative damage to biomolecules *in vitro* and *in vivo*. The PACa compounds react specifically with carbonyls on proteins, peptides as well as lipids to form stable hydrazones. The novel compounds share three important features which make them suitable for the analysis of oxidized biomolecules using mass spectrometry.

The PACa compounds have application in the research field for diseases such as Alzheimer's disease and cancer, as specified above, but also in the food industry for the testing the carbonyl content of food. They can be a valuable tool for use in proteomic research, since compounds of the invention can be used to characterize *in vivo* and *in vitro* carbonyl contents.

In accordance with another aspect, the present invention provides a composition comprising at least one compound of the invention and a carrier.

In still another aspect, the composition is contained within a kit. PACa compounds can be used in kits in the form of a pre-loaded solid support, such as a column or beads, preferably nanobeads, developed for the enrichment of biological molecules having low carbonyl content.

A "carrier" refers to, for example, a diluent, adjuvant, preservative (e.g., benzyl alcohol), anti-oxidant (e.g., ascorbic acid, sodium metabisulfite), solubilizer (e.g., Tween 80, Polysorbate 80), emulsifier, buffer (e.g., Tris HCl, acetate, phosphate), water, bulking substance (e.g., lactose, mannitol), excipient, or any auxiliary agent.

In another aspect, the present invention provides an article of manufacture where the functionality of a method of the invention is embedded on a computer-readable medium, such as, but not limited to, a floppy disk, a hard disk, an optical disk, a magnetic tape, a PROM, an EPROM, CD-ROM, DVD-ROM, or resident in computer or processor memory. The functionality of the method can be embedded on the computer-readable medium in any number of computer readable instructions, or languages such as, for example: FORTRAN, PASCAL, C, C++, BASIC and, assembly language. Further, the computer-readable instructions can, for example, be written in a, script, macro, or functionally embedded in commercially available software, (e.g. EXCEL or VISUAL BASIC).

Further aspects and advantages of this invention will be disclosed in the following examples, which should be regarded as illustrative and not limiting the scope of this application.

EXAMPLES

Materials and Methods

Peptides

A carbonylated commercial peptide [AcN-methyl-YVAD-aldehyde] called PepCO was purchased from Bachem (GMBH, Ge). Another peptide [NH_2NKPPKKGPANG-OH] called A9 was synthesized.

Phospholipids

Three phospholipids were purchased from Avanti Polar Lipids:

- DMPC (1,2-dimyristoyl-*sn*glycero-3-phosphocholine)
- PCPC (1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine)
- POVPC (1-palmitoyl-2-(5'-oxo-valeroyl)-*sn*-glycero-3-phosphocholine)

Other Reagents: Sodium hypochlorite purchased from Across Organics, Bovine Serum Albumin (BSA) purchased from Sigma Aldrich. $FeCl_3$, DNPH, HCL, TCA, thiopropylsepharose 6B purchased from GE Healthcare (Sweden). Gold nanoparticles can be purchased from Sigma Aldrich.

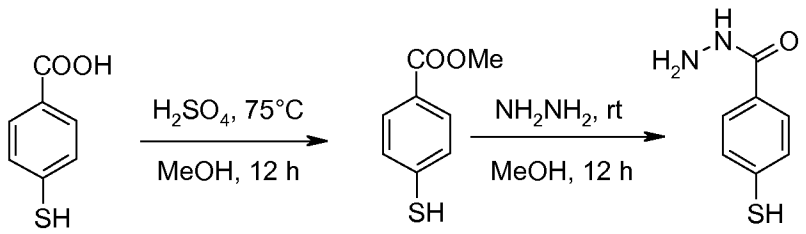
Instrument: MALDI TOF Axima Performance - Shimadzu®, Q-Tof mass spectrometer – Bruker®, spectrophotometer-Lambda 800 UV/Vis.

1. Synthesis of the compounds of the invention

In the examples and figures of the present invention, the compound noted PACa is 4-sulfanylbenzohydrazide, *ie* the compound of formula (1) with R_1 to R_4 being hydrogen atoms.

First molecule: 4-sulfanylbenzohydrazide

The compound of the invention was synthesized in the following manner:



Sulfuric acid (1.6mL) was added drop wise to a mixture comprising 4-sulfanylbenzoic acid (1.6g, 10.4 mmol), methanol (2.52 mL, 62.2 mmol) and chloroform (6.4mL). The mixture was stirred overnight at 75°C, and the solution was extracted with chloroform. The organic layer was washed with aqueous saturated $NaHCO_3$, dried over $MgSO_4$, and the solvent was evaporated to afford methyl 4-sulfanyl benzoate compound as a yellow solid in 86% yield.

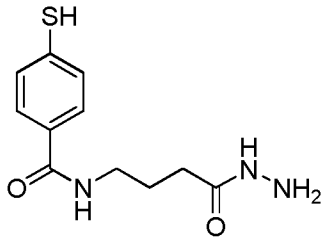
The compounds were confirmed using ^1H and ^{13}C NMR with the following spectral information:

^1H NMR (CD_3OD): δ = 7.96-7.93 (m, 2H), 7.52-7.49 (m, 2H), 3.88 (s, 3H), 3.17 (s, 1H);
^{13}C (CD_3OD): δ = 165.44, 144.12, 129.27, 127.77, 124.97, 51.19

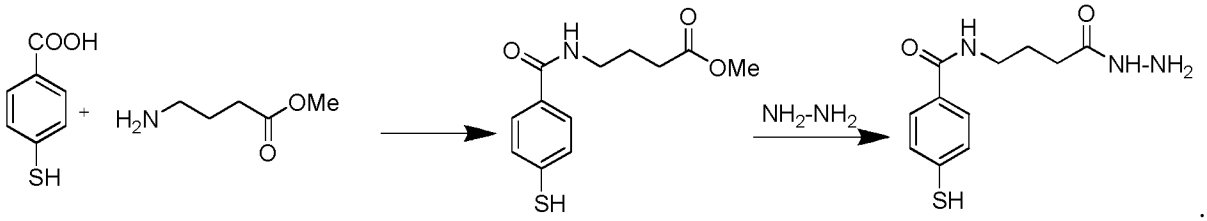
1g of methyl 4-sulfanyl benzoate compound in 10 mL of methanol was added to a round necked flask, along with 7mL hydrazine solution (50% in water) and the mixture was refluxed overnight. The solvents were evaporated and the crude residue was purified by silicagel chromatography using methanol as eluent affording 4-sulfanylbenzohydrazide in 72% yield as a white solid. The compound was confirmed by NMR with the following spectral information.

^1H NMR (CD_3OD): δ = 7.63-7.61 (m, 2H), 7.48-7.46 (m, 2H), 6.01 (s, 3H), 3.18 (s, 1H);
^{13}C (CD_3OD): δ = 169.96, 145.32, 130.32, 130.13, 123.08

Second molecule:



was synthesized according to a process similar to that described above for 4-sulfanylbenzohydrazide, according to the following scheme:



The mass spectrum (MH^+ 253.1 Da) and the MS/MS spectrum (signature at m/z=137) of the second molecule are provided in figure 17.

2. Derivatization of the biomolecular species

Peptides

A9 peptide was oxidized using NaOCl 0.5mM for 20 minutes at 37°C which induced carbonyls. The carbonylated commercial peptide and the oxidized A9 peptide were derivatized with the compound of the invention or conventional biotin hydrazide (10 mM) in HCl 2N for 60 minutes at room temperature, and were then purified and concentrated using a C18 stage tips column.

Phospholipids

Phospholipids - DMPC, PGPC and POOVPC - were dissolved in methanol at 2.5 µg/µl (4.2mM) and treated with the compound of the invention (10 mM in methanol) volume ratio 1:1 for 1 hour at room temperature. Samples were then spotted on the MALDI TOF plate 0.5 µl (1 nmol) with 0.5 µl of the DHB matrix (40 mg/ml in 97% ethanol).

The sensitivity of the derivatization was estimated by successive dilutions of the POVPC (0.25 µg/µl, 0.025 µg/µl, 0.005 µg/µl and 0.0025 µg/µl) before the reaction of the phospholipid with the compound of the invention (10 mM in methanol) volume ratio 1:1 for

one hour at room temperature. Samples were spotted on a MALDI TOF plate corresponding respectively to 2.1 nmol, 0.1 nmol, 0.01 nmol, 2 pmol, and 1 pmol on the target.

Protein

Tau protein was oxidized with NaOCl 1 mM for 15 min at 37°C. The pH switched from around 6 to around 7 after oxidation. Figure 13 presents the MS spectra of Tau protein (lower spectrum) and of oxidized Tau protein (upper spectrum).

Oxidized Tau protein was derivatized with PACa (10 mM in water) at a volume ratio of 1:1, for 1 hour, at room temperature.

3. UV quantification of a carbonylated biomolecule

Human serum Albumin HSA (10 mg/ml) was oxidized with NaOCl 5 mM for 20 minutes at 37°C. Oxidation was stopped by addition of 1 mM EDTA. Carbonylated HSA was derivatized with conventional DNPH or the compound of the invention (10 mM) in HCl 2N for 1 hour at room temperature. HSA was precipitated with TCA to remove excess DNPH or compound of the invention. Binding to BSA carbonyl groups was spectrophotometrically quantified using a LAMbda 800 UV/Vis spectrophotometer at 340 nm and compared to the DNPH binding to carbonylated BSA at 370 nm. The same procedure was carried out for non oxidized HSA.

4. Supports/enrichment

- Fixation of PACa on a sepharose column and use for enrichment of a biomolecule

Supports of thiopropyl sepharose 6B which contain reactive 2-thiopyridyl disulphide groups attached to sepharose through a chemically stable ether linkage react with thiol containing solutes under mild reaction conditions to form mixed disulphides. These were used to fix the compounds of the invention through disulphide bonds in a chromatographic technique. The powder form of the thiopropyl sepharose 6B was suspended in water to allow swelling and then drawn into the column and equilibrated by PBS. The compound of the invention (20mM in PBS) was added into the column and the column was left for one hour at room temperature to incubate before being washed with three volumes of the buffer prior to the addition of the carbonylated biomolecule. The biomolecule was then added and allowed to incubate for one

hour. A washing step with buffer was subsequently carried out before elution with DTT 100mM.

The same chromatographic system was used for the carbonylated pospholipid enrichment but the buffer was replaced by methanol.

- Enrichment of oxidized A9 peptide derivatized with PACa on gold nanoparticles

A solution of Oxidized A9 peptide derivatized with PACa as described above was added togold nanoparticules (GNP), prealably washed once with water. The reaction mixture was incubated during 1 hour at room temperature, then centrifugated to recover the GNP in the centrifugation pellet. The recovered GNP were redispersed in water by sonication once for washing, and washed GNP were obtained by centrifugation.

Elution of the trapped peptides from GNP was performed by reaction with 25 mM Dithiothreitol (DTT) as a reducing agent for 30 min at room temperature. The reaction mixture was then centrifugated, and the supernatant was recovered and analyzed.

Control experiment was performed in parallel with oxidized A9 peptide and GNP, without derivatization of the peptide with PACa.

The supernatants comprising the oxidized peptide were analyzed by MALDI analysis, by putting 1µl of the eluted samples with 1 µl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix. The obtained spectra are presented on figure 11.

- The same experiment was performed with a mixture comprising a protein digest and the oxidized A9 peptide (instead of the oxidized A9 peptide alone). The oxidized A9 peptide is the only carbonylated compound in said mixture. Results are presented on figure 12. Enrichment of oxidized Tau protein derivatized with PACa on gold nanoparticles

A solution of oxidized Tau protein derivatized with PACa as described above was contacted with gold nanoparticules (GNP), prealably washed once with water, for 1 hour at 25°C under agitation. The reaction mixture was centrifugated to recover the GNP in the centrifugation

pellet. The recovered GNP were redispersed in water by sonication once for washing, and washed GNP were obtained by centrifugation.

Elution of the trapped proteins from GNP was performed by reaction with 25 mM Dithiothreitol (DTT) as a reducing agent for 30 min at 25°C. The reaction mixture was then centrifugated, and the supernatant was recovered and analyzed.

Control experiment was performed in parallel with oxidized Tau protein and GNP, without derivatization of the protein with PACa.

The supernatants comprising the oxidized Tau protein were analyzed by MALDI analysis. The obtained spectra are presented on figure 14.

Results

The whole process from steps 1 to 4 as described above can be monitored by mass spectrometry techniques, as is demonstrated in this section. The PACa product obtained by chemical synthesis was 4-sulfanylbenzohydrazide, ($C_7H_8O_1N_2S_1$ with a molecular mass 168.2174 g mol^{-1}) and is a white powder which is soluble in water up to 13 mM, in methanol up to 49 mM, and shows higher solubility in DMSO.

The "trapping" step of the invention involves the reaction of carbonyl groups with the PACa molecules to form marked carbonyl groups that are later counted in the "quantification" step. This reaction is known as derivatization and is here described with reference to figures 1-3.

Figure 1 relates to the derivatization with PepCO. It shows the MALDI TOF MS spectrum in positive mode of the commercial carbonylated peptide (PepCO). Three main species were measured at 507.5 Da, 528.5 Da and 544.5 Da, corresponding to MH^+, $M+Na^+$, and $M+K^+$ respectively. The reaction with the compound of the invention induced a mass shift of 150.6 Da for all these species that were therefore detected at MH^+ = 658.1 Da (507.5 + 168.6 -18).

Figure 2 relates to the derivatization with peptide A9. NaOCl carbonylation of the A9 peptide on one lysine induces a mass loss of 1.03 Da, the net mass shift with PACa derivatization is then 149.57 Da. Figure 2 shows the MALDI TOF MS spectrum in positive mode of the

carbonylated A9 peptide. We measured species at 1107.62 Da, 1129.62Da, 1145.62 Da corresponding to MH^+, $M+Na^+$ and $M+K^+$ respectively. The PACa fixed to the A9 carbonylated peptide gave species at 1257.25 Da and 1406.89 Da corresponding to one and two sites of carbonylated lysines derivatized with PACa.

Figure 3 relates to the derivatization with phospholipids. Three phospholipids: DMPC (A), PGPC (B) and POVPC (C) were analyzed by MALDI-Tof MS before and after reaction with PACa. POVPC is a carbonylated phospholipid and therefore should be the only one to react with PACa. Figure 3 clearly showed that the derivatization worked only with the carbonylated compound POVPC and not with the others. The peak at mass 594.3 Da disappeared and reappeared at 744.8 Da, corresponding to the PACa's fixation on the POVPC. A sensitivity study gave a limit of detection for PACa labeling with POVPC at 2 pmol. For the 1 pmol both m/z = 594 and 744 were absent from the spectrum (data not shown).

Figure 4 demonstrates PACa's enrichment of the PepCO. A support of thio-silica magnetic beads was used to test the enrichment capacity of PACa to the PepCO. Firstly, the beads were contacted with PACa to form disulfide bonds. PepCO was then mixed with the beads for 90 minutes. Beads were subsequently washed, then eluted with DTT (see Fig. 4, B), the expected peaks were present at masses: 679.55 Da, 695.55 and 717.55; corresponding to PACa-PepCO+Na^+, PACa-PepCO+K^+ and PACa-PepCO+Na^++K^+.

Figure 5 shows PACa's enrichment of phospholipids. Figure 5A shows a MALDI spectrum in the positive mode for a mix of three phospholipids with an equal ratio in methanol and DHB matrix: a carbonylated lipid POVPC (594.3 and 616.3 Da for MH^+ and $M+Na^+$), as well as the other lipids PGPC (610.4, 632.3 and 654.3 Da for MH^+, $M+Na^+$ and $M+2Na^+$) and DMPC (678.5, 700.4 and 716.4 Da for MH^+, $M+Na^+$ and $M+K^+$). This mixture was passed through a PACa-sepharose column prepared in house to enrich and isolate specifically the POVPC. Figure 5 B shows the remainder of species from the mixture after passing through the PACa-column. One can note the absence of POVPC which means that it was the only species that fixed PACa. The elution with DTT 100mM after a washing step provided only POVPC fixed with PACa at MH^+ 744.6 Da (see Fig. 5 C).

The quantification of protein carbonyls was carried out using UV/Vis spectroscopy. The PACa's molar extinction coefficient was calculated according to the Beer Lambert Law and was found to be ε = 13 000 L. cm^{-1}.mol^{-1} at 310 nm. This maximum was shifted to a

wavelength of 340 nm which allowed quantification of carbonylated protein. The results for quantification of carbonyl contents were similar for both DNPH and PACa (see Fig.6).

Figure 7 demonstrates the MALDI TOF TOF characterization of the PepCO fixed to PACa. It is the CID MALDI spectrum of the PepCO before and after reaction with PACa. Because of the location of the carbonyl at the C-terminus end, solely 'y' fragments were shifted by 150.6 Da. One can observe a specific daughter ion signature for PACa at 137 Da.

Characterization of the A9 peptide fixed to PACa was carried out using MALDI TOF TOF mass spectroscopy. Figure 8 shows the CID MALDI spectrum of the A9 peptide on the upper spectrum and the derivatized carbonylated lysine with PACa on the lower spectrum. The 'b' and 'y' fragments correspond to the non modified ions and 'b*' and 'y*' fragments correspond to the PACa's derivatized carbonylated lysine, we also note the signature at 137 Da and a peak at m/z = 1089 Da corresponding to the neutral loss of PACa (M-168 Da).

MS/MS characterization of the carbonylated phospholipid fixed to PACa is demonstrated in figure 9 and shows the MS/MS spectrum of the POVPC with and without PACa's derivatization.

Figure 10 compares the reactivity of PACa and biotin hydrazide to carbonyl using the same oxidized A9 peptide.

Figure 11 presents the MALDI analysis of the supernatants comprising the oxidized A9 peptide after enrichment on gold beads in presence (GNP+A9ox-PACa) and in absence (GNP + A9ox) of derivatization of the oxidized A9 peptide with PACa. The control spectrum in absence of derivatization with PACa comprises only background noise. The spectrum in presence of PACa derivatization presents peaks at m/z 1214.20 and 1257.21 that are characteristic of carbonylated A9 peptide. Fixation of PACa on gold beads thus allowed specific enrichment of oxidized A9 peptide.

The presence of the m/z 1257.27 peak in the lower spectrum of the MS spectra of figure 12 a) and its absence in the upper spectrum shows that the oxidized A9 peptide derivatized with PACa is preferentially retained on the GNP when compared to the rest of the (protein digest + oxidized A9 peptide) mixture.

Similarly, the presence of the m/z 1214.2 and 1257.27 peaks in the upper spectrum of the MS spectra of figure 12 b) shows an efficient enrichment of the carbonylated A9 peptide derivatized with PACa was obtained on the GNP. The m/z 1046.5 and 1672.9 peaks correspond to the signature of a peptide mix used for calibration.

Spectrum c) of figure 14 shows that an average of 5 PACa molecules per protein were fixed.

The PACa scaffold is composed of three main components, (see Fig. 15):

- a hydrazide function that reacts specifically with carbonyls to form a stable hydrazone at acidic pH.
- an aromatic ring allowing a UV quantification with a molar coefficient extinction of 11500 $cm^{-1}.M^{-1}$ at 310 nm
- a thiol function for the enrichment of carbonyls by disulfide linkage on thiol activated chromatographic support.

Characterization of PACa was concluded by MS/MS mass spectrometry. Figure 16 shows the MS/MS spectra of PACa fragmentation. Many ion fragments were observed; the most intense being at 137 Da, corresponding to the non-implicated region of PACa with the carbonyl. This was subsequently used as a signature for the presence of the fixed PACa on a carbonyl. Furthermore, the mass 137 does not match with any charged amino acid, making it an unambiguous marker ion of PACa in protein carbonyl identification.

PACa compounds are able to specifically react with more than one modified amino acid on a peptide sequence and are far less limited than conventional derivatizing agents with respect to steric hindrance. In the embodiment where PACa is 4-sulfanylhydrazide, studies on the carbonylated A9 peptide sample at the same concentration for each derivatizing agent; PACa and biotin hydrazide, demonstrated that two molecules of PACa could be fixed onto the peptide whereas only one molecule of biotin hydrazide could be fixed (Fig. 10). According to this specific embodiment, PACa bound to a carbonyl gives rise to a specific MS/MS signature at 137 Da, which is highly practical because this mass neither matches any charged amino acid mass fragment, nor any immonium ion fragment. This is of particular interest for the analysis of MS/MS modified amino acids.

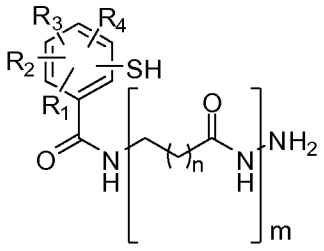
Easy characterization and quantification of modified substances is possible with UV testing due to the specific absorbance at 310 nm in water and buffers.

The PACa compounds have excellent solubility characteristics which allow the use of organic solvents such as methanol and DMSO, as well as with pure water or biological buffers. The solubility is highly compatible with *in vivo* cellular analyses. The solubility is excellent in comparison with other previously engineered compounds, for example, biotin hydrazide.

Although the PACa compounds show similar specificity to the conventional derivatizing agent DNPH, the latter does not allow enrichment of the carbonylated biomolecular species. Elution following biotin hydrazide coupling and enrichment via Streptavidin beads was attempted and was unsuccessful due the strong affinity between the two partners that prevented efficient release. By contrast, the enrichment with PACa compounds is easy, and the elution from thio binding materials occurs via simple pH steps.

CLAIMS

1. A method for derivatizing, identifying and/or quantifying carbonylated biomolecules by using a compound having the general formula I:



(I),

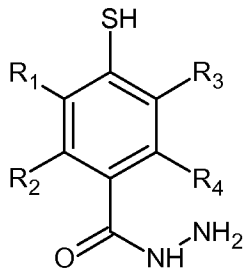
wherein:

- m is 0 or 1,

- n is an integer from 0 to 6 (inclusive), and

- R_1, R_2, R_3, and R_4, identical or different, represent H, OH, NH_2, (C1-C8)alkyl, (C3-C8)cycloalkyl, (C6-C18)aryl, heteroaryl, heterocycle, halogen, or a silyl group.

2. A method according to claim 1, wherein the compound has the general formula 1, 2 or 3:



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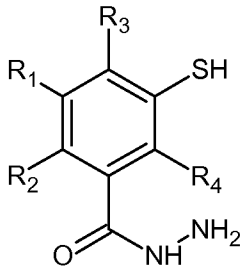

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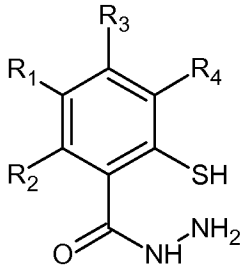
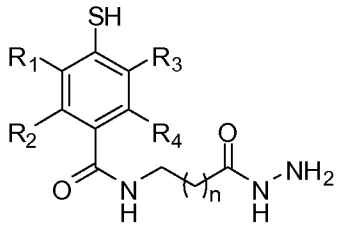


3

wherein R_1, R_2, R_3, and R_4 are as defined in claim 1.

3. A method according to claim 1, wherein the compound has the general formula 4, 5 or 6:



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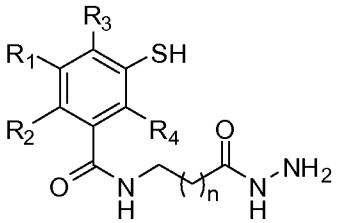

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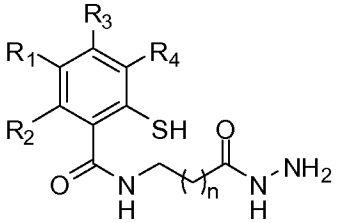


6

,

wherein n, R_1, R_2, R_3, and R_4 are as defined in claim 1.

4. The method according to claim 2, wherein the compound has the general formula 1, and preferably R_1, R_2, R_3, and R_4 are hydrogen atoms.

5. The method according to any of claims 1 to 4, wherein R_1, R_2, R_3, and R_4, identical or different, are hydrogen atoms or (C1-C8)alkyl groups, more specifically hydrogen atoms.

6. The method according to anyone of claims 1 to 5, wherein it comprises the following steps:

   (i) biomolecules derivatization by the compound as described in anyone of the previous claims,
   (ii) identification of specifically carbonylated biomolecules, and
   (iii) optional quantification of carbonylated species and/or of the rate of carbonylation.

7. The method according to anyone of claims 1 to 6, where the biomolecules are peptides, proteins, or lipids.

8. The method according to anyone of claims 1 to 7, where the compound is mounted on a support, such as thio-silica magnetic beads or gold beads.

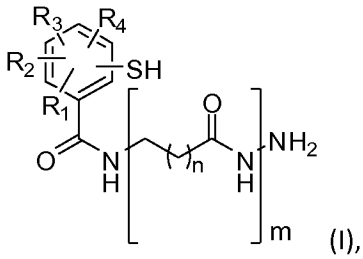
9. The method according to anyone of claims 1 to 8, wherein mass spectrometry, preferably MALDI, in particular MALDI-TOF, is the only characterization technique used in step (ii).

10. The method according to anyone of claims 1 to 9, where the method of the invention comprises after step (ii) an additional step of using the results of step (ii) to identify biomolecules susceptible to carbonylation.

11. The method according to anyone of claims 1 to 10, where the quantification step is carried out using UV/Vis spectroscopy.

12. The method according to anyone of claims 1 to 11, which further comprises the step of (iv) identifying a disease, disorder, or condition or a plant pathological situation associated with the identified biomolecule susceptible to oxidation as determined from step (ii).

13. A compound having the general formula I:



(I),

wherein:

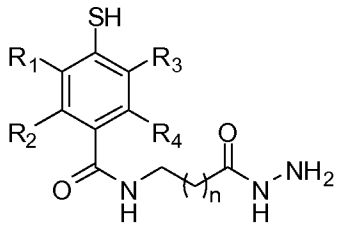
- m is 0 or 1,

- n is an integer from 0 to 6 (inclusive),

- R_1, R_2, R_3, and R_4, identical or different, represent H, OH, NH_2, (C1-C8)alkyl, (C3-C8)cycloalkyl, (C6-C18)aryl, heteroaryl, heterocycle, halogen, or a silyl group, and

- when m is 0, at least one of R_1, R_2, R_3, and R_4 represents NH_2, (C2-C8)alkyl, (C3-C8)cycloalkyl, (C6-C18)aryl, heteroaryl, heterocycle, or a silyl group.

14. The compound according to claim 13, wherein the compound has the general formula 4, 5 or 6:



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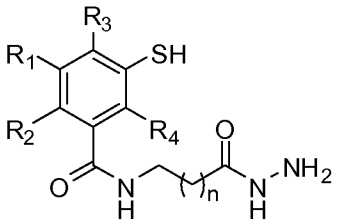
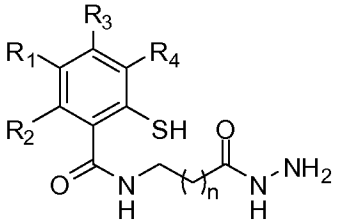


5



6

,

wherein n, R_1, R_2, R_3, and R_4 are as defined in claim 14.

15. A composition comprising at least one compound as defined in claim 13 or 14 and a carrier.

Figure 1

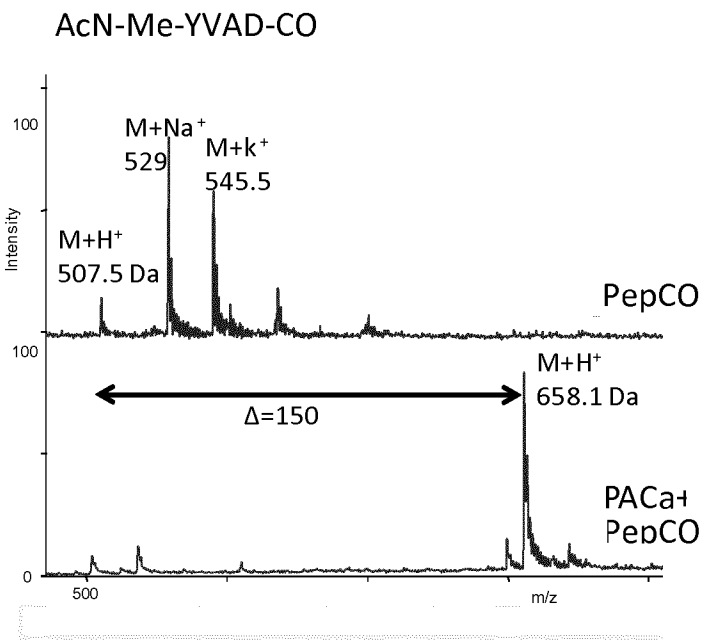


Figure 2

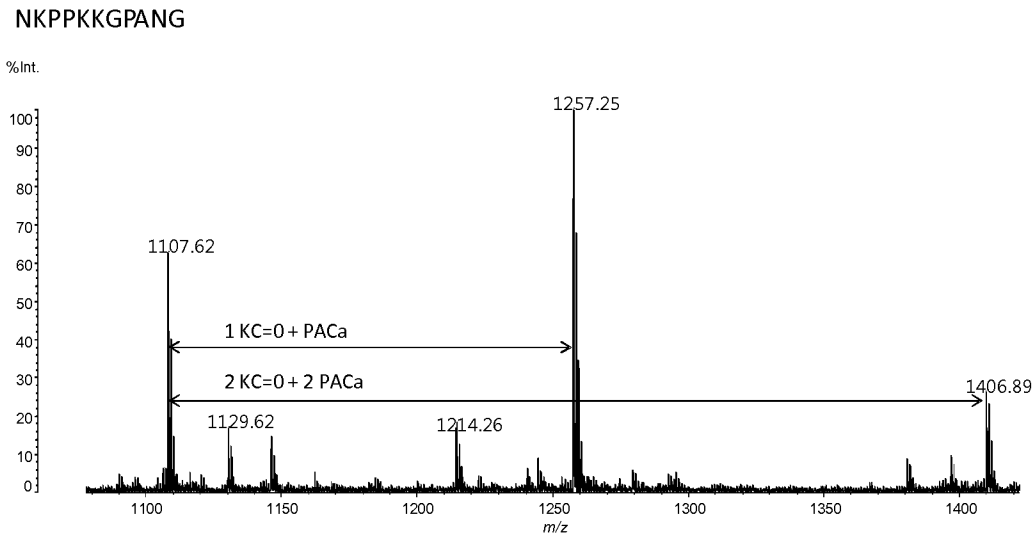


Figure 3

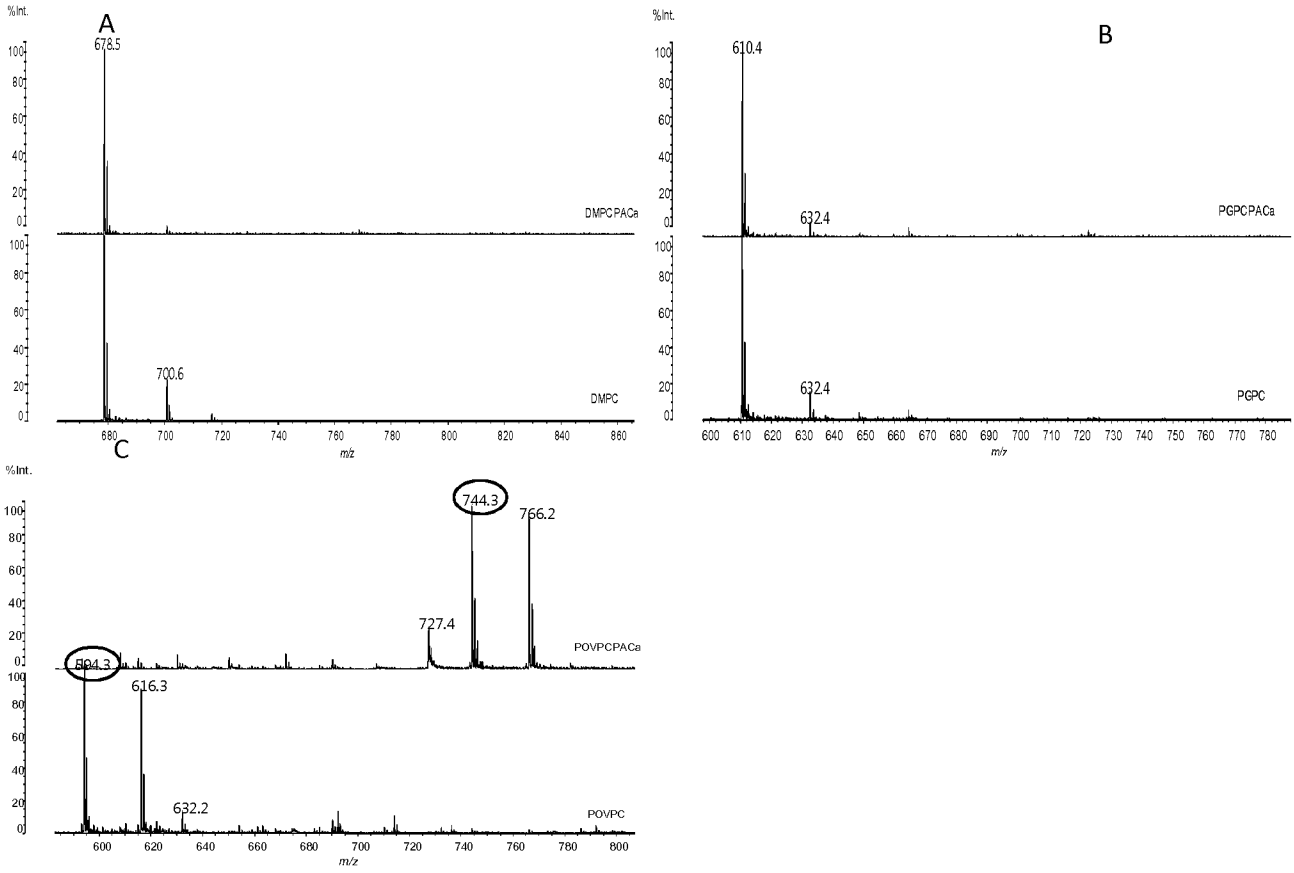

A
%Int.
678.5
DMPCPACa
700.6
DMPC
m/z
B
%Int.
610.4
PGPCPACa
632.4
632.4
PGPC
m/z
C
%Int.
744.3
766.2
727.4
POVPCPACa
804.3
616.3
632.2
POVPC
m/z

Figure 4

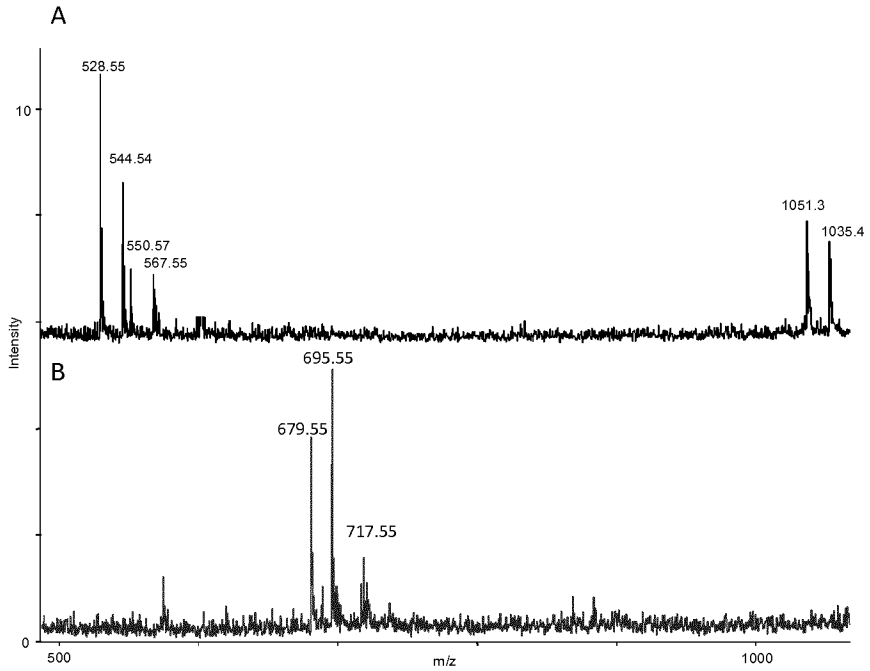


Figure 5

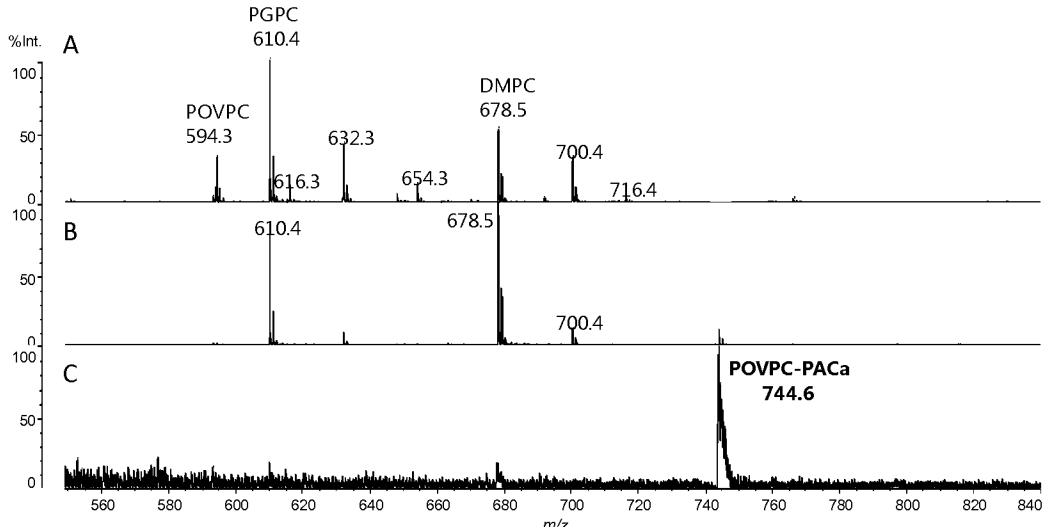


Figure 6

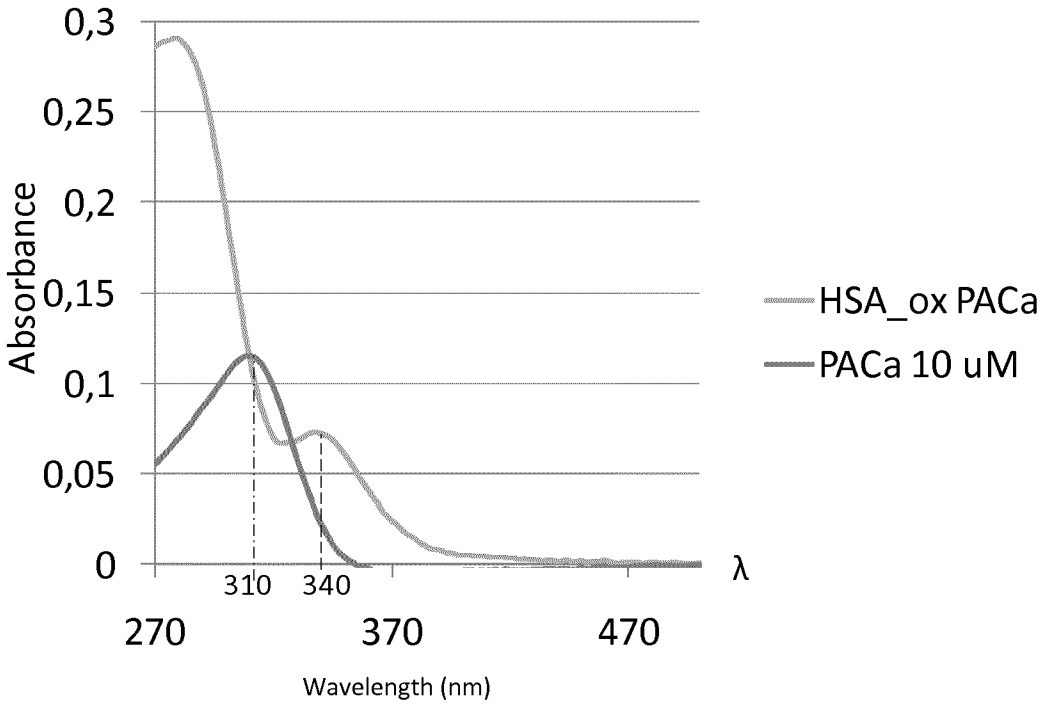

0,3
0,25
0,2
0,15
0,1
0,05
0
Absorbance
310
340
λ
270
370
470
Wavelength (nm)
HSA_ox PACa
PACa 10 uM

Figure 7

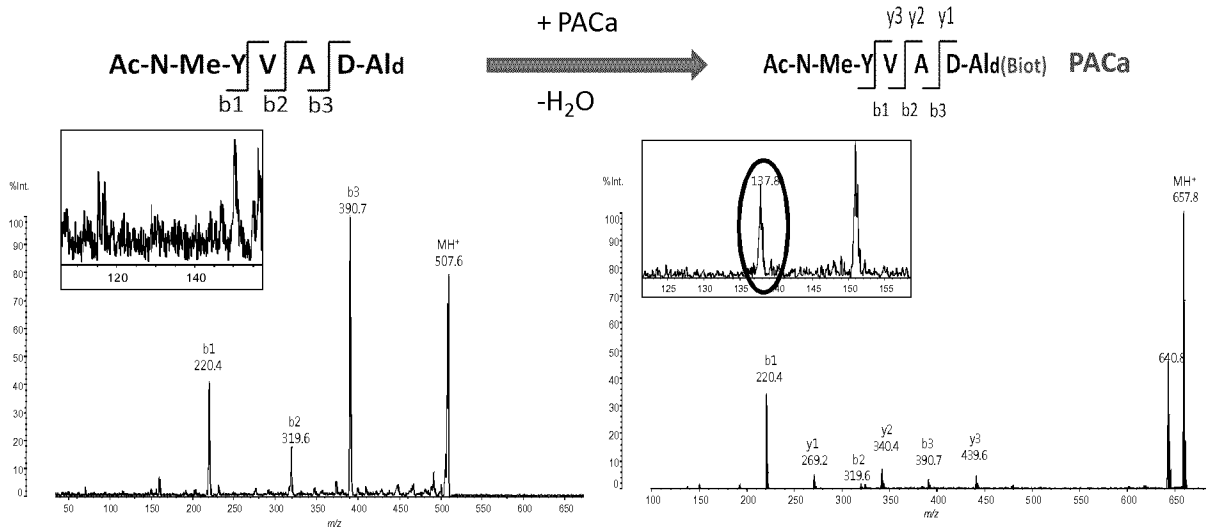


Figure 8

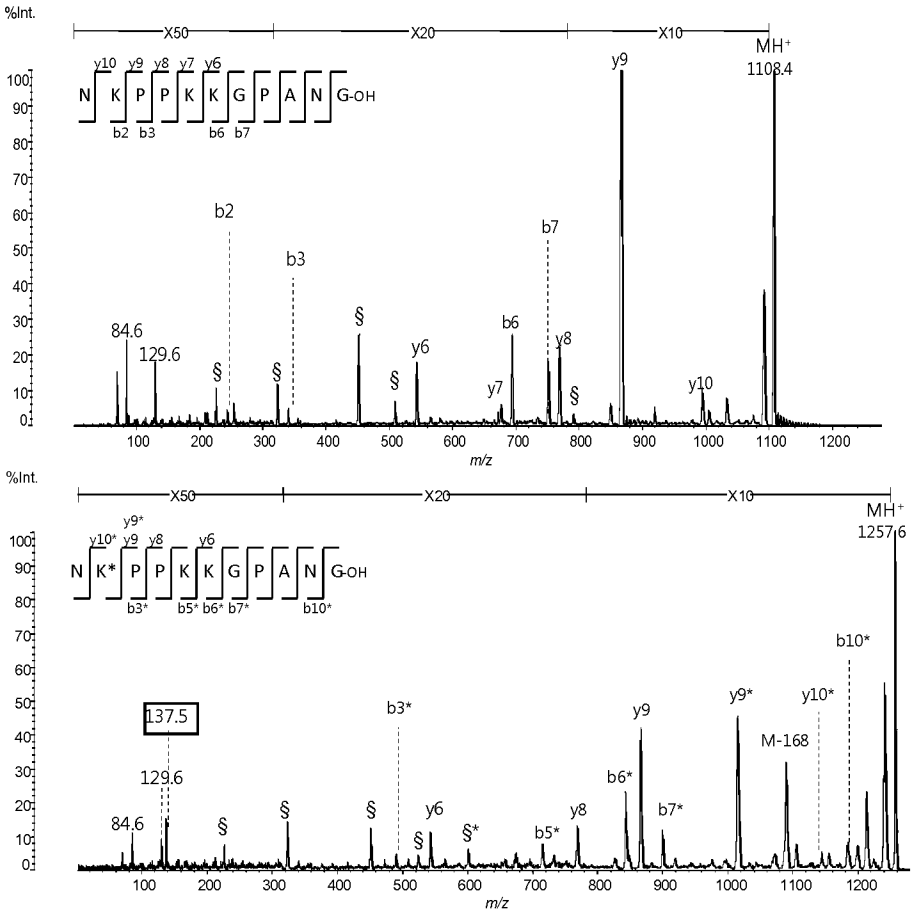
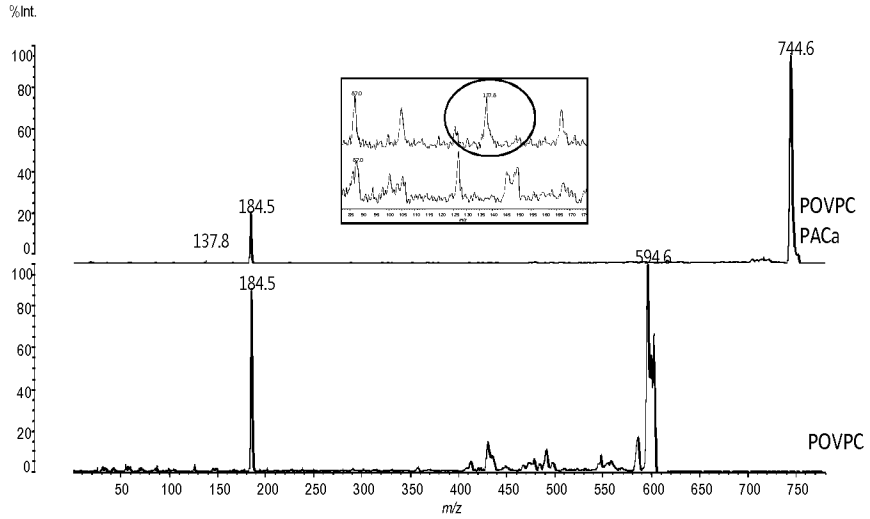


Figure 9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100
80
60
40
20
0
744.6
184.5
137.8
POVPC
PACa
594.6
184.5
POVPC
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
m/z

Figure 10

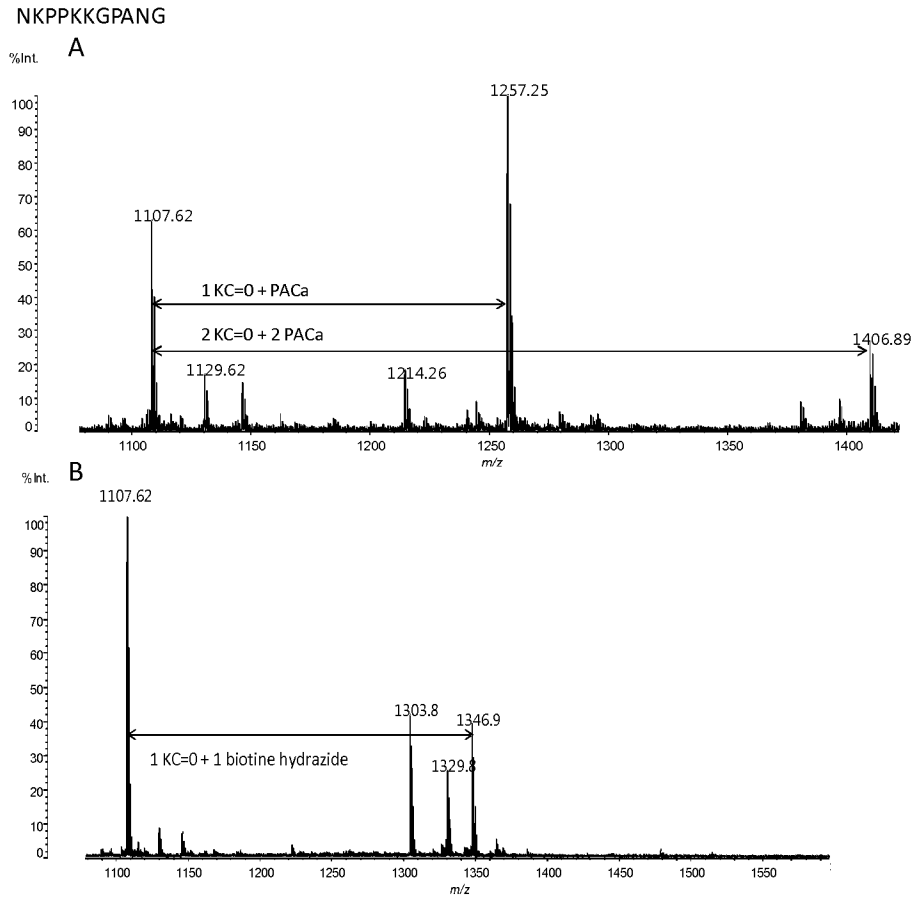


Figure 11

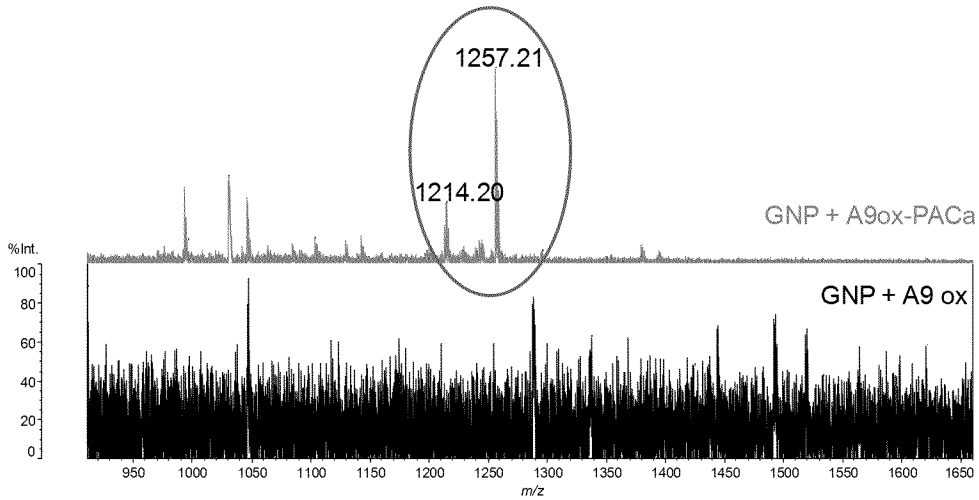


Figure 12

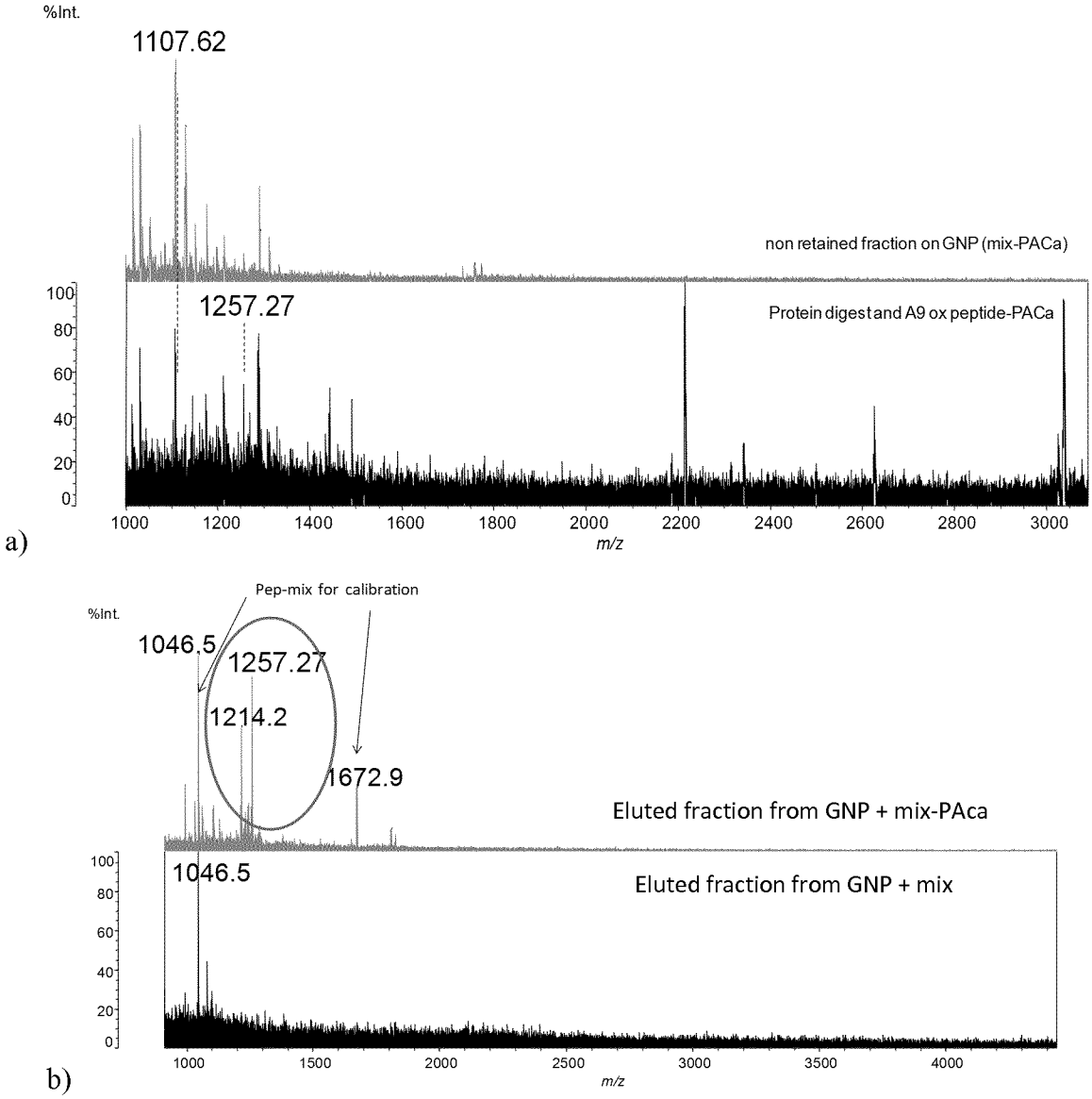

%Int.
1107.62
non retained fraction on GNP (mix-PACa)
1257.27
Protein digest and A9 ox peptide-PACa
100
80
60
40
20
0
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
3000
m/z
a)
Pep-mix for calibration
%Int.
1046.5
1257.27
1214.2
1672.9
Eluted fraction from GNP + mix-PAca
1046.5
Eluted fraction from GNP + mix
100
80
60
40
20
0
1000
1500
2000
2500
3000
3500
4000
m/z
b)

Figure 13

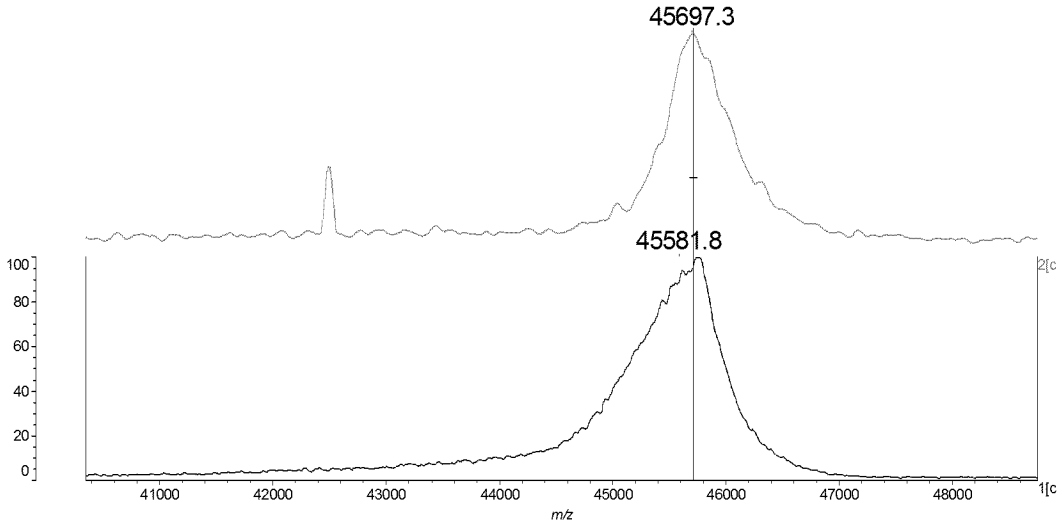


Figure 14

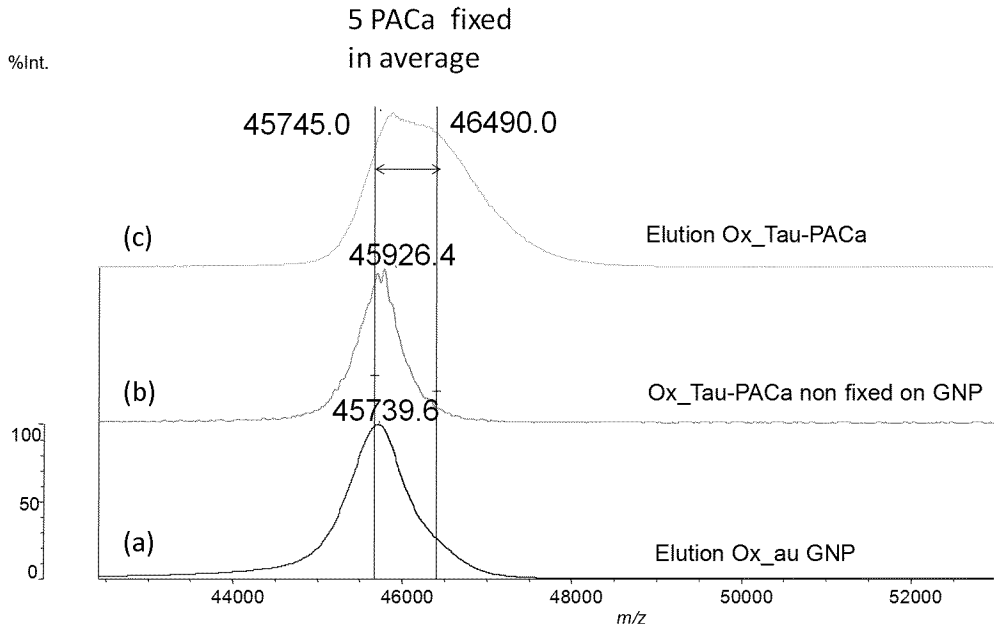


Figure 15

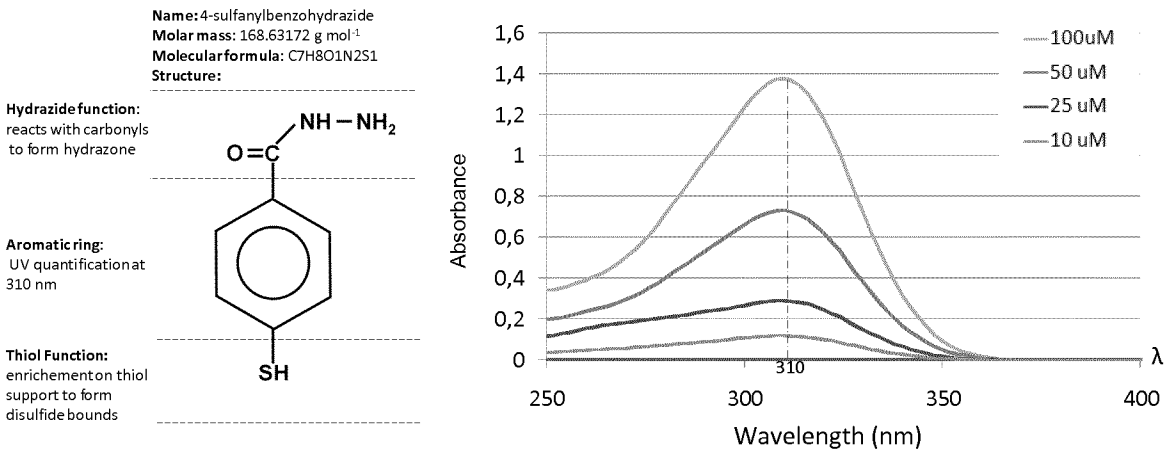


Figure 16

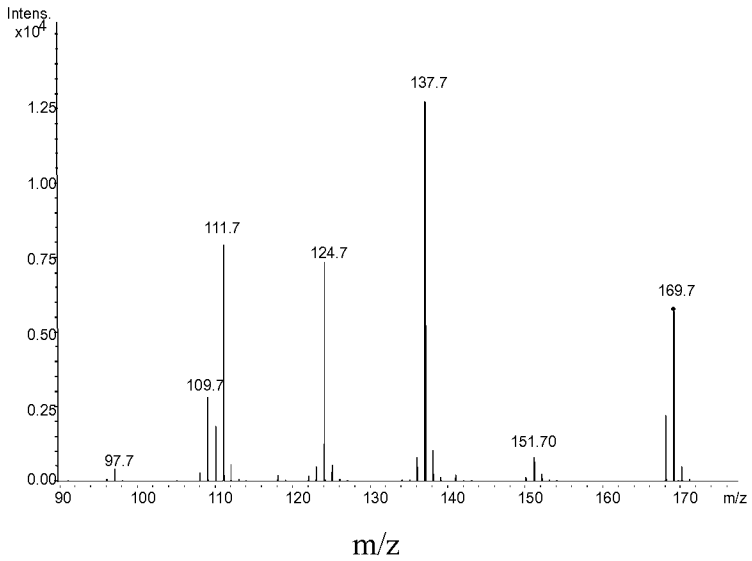
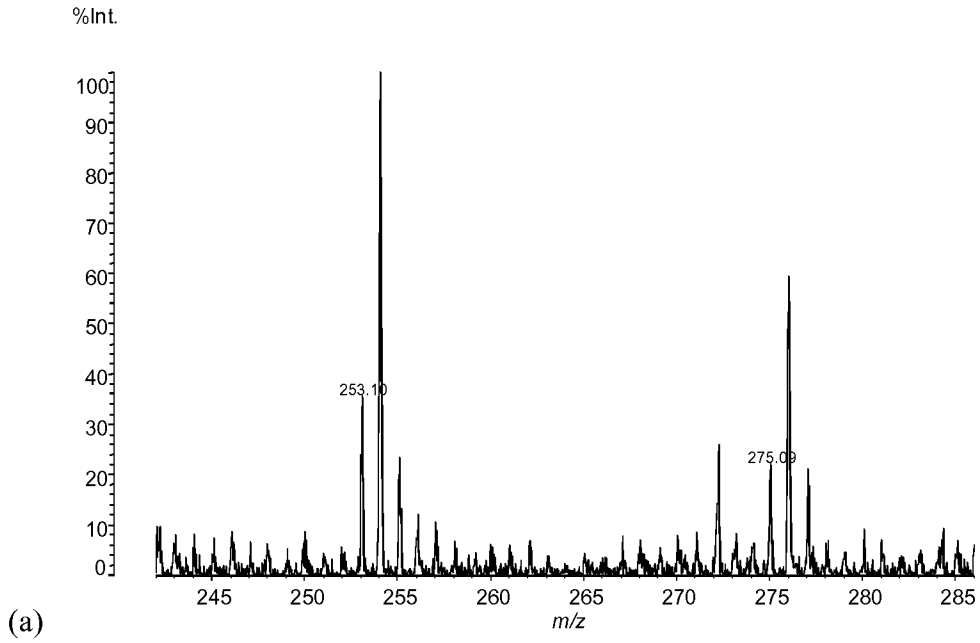


Figure 17


%Int.
100
90
80
70
60
50
40
30
20
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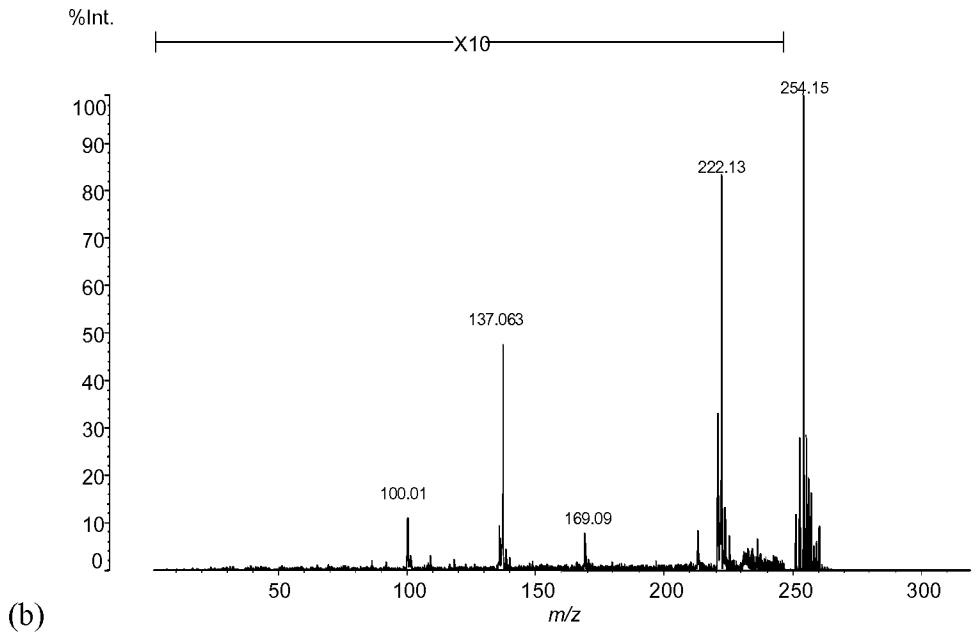
0
253.10
275.09
245
250
255
260
265
270
275
280
285
m/z

(a)


%Int.
X10
254.15
222.13
137.063
100.01
169.09
100
90
80
70
60
50
40
30
20
10
0
50
100
150
200
250
300
m/z

(b)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/058844

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07C321/26
ADD.

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)
C07C

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 767 027 A (SCHENLEY IND INC) 30 January 1957 (1957-01-30) Page 8, lines 12-14,20; page 9, lines 10-16;; example 1 -----	13-15
A	DATABASE REAXYS [Online] Elsevier Properties SA; XP002657311, Database accession no. 3247531, 3246837, 3249807, 3249806 abstract & JOURNAL OF ORGANIC CHEMISTRY, vol. 18, 1953, pages 1380-1400, ----- -/--	13-15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 September 2012	17/09/2012

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	Cooper, Simon

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE REAXYS [Online] Elsevier Properties SA; XP002657312, Database accession no. 4937771, 2717574 abstract & JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 64, 1975, pages 1425-1427, -----	13-15
A	DATABASE REAXYS [Online] Elsevier Properties SA; XP002657313, Database accession no. 3249803 abstract & ACAD. REPUB. POP. ROM., FIL. CLUJ, STUD. CERCET. CHIM., vol. 4, no. 3, 1959, pages 313-316, -----	13-15
A	WO 2007/047796 A2 (INST SYSTEMS BIOLOGY [US]; ZHANG HUI [US]; AEBERSOLD RUDOLF H [CH]) 26 April 2007 (2007-04-26) page 42, line 22 - page 44, line 23 -----	1-15
A	US 2002/137068 A1 (HAUGLAND RICHARD P [US] ET AL) 26 September 2002 (2002-09-26) the whole document -----	1-15

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/058844

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2007047796 A2	26-04-2007	AU 2006304605 A1	26-04-2007
		CA 2625403 A1	26-04-2007
		EP 1938104 A2	02-07-2008
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		WO 2007047796 A2	26-04-2007
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		US 2011117659 A1	19-05-2011
		WO 0228841 A2	11-04-2002