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PROVISIONAL SPECIFICATION

COMPOSITIONS AND METHODS FOR THE TREATMENT OF DEGENERATIVE CONDITIONS OF THE CENTRAL NERVOUS SYSTEM

We, **PACIFIC BRANDS INC., a New Zealand company, of 14 Twin Wharf Road, Herald Island, Auckland 0618, New Zealand** do hereby declare this invention to be described in the following statement:

COMPOSITIONS AND METHODS FOR THE TREATMENT OF DEGENERATIVE CONDITIONS OF THE CENTRAL NERVOUS SYSTEM

Field of Invention

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The present invention relates to methods of treating chronic degenerative conditions of the central nervous system related to oxidative stress. More particularly, the invention relates to the use of AOS protein found in rubber plant *Parthenium argentatum* or a functionally equivalent variant thereof in the treatment of such conditions.

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Background

Oxidative stress has been described as excess accumulation of free radicals such as nitrogen oxide (NO') and reactive oxygen species (ROS). Accumulation can occur when there is a
15 mismatch between the generation and elimination of free radicals. Oxidative stress in the central nervous system can lead to oxidative cell injury, for example neuronal and vascular damage can occur. Cell injury can lead to apoptosis (programmed cell death) and inflammation.

Allene oxide synthase (AOS) from the guayule rubber plant (GenBank CAA55025.2) also
20 known at the guayule rubber particle protein (RPP), was purified and cloned from the guayule rubber plant, *Parthenium argentatum* (US 5,633,433 and US 6,132,711).

This AOS has previously been shown to be a potent anti-oxidant. For example, it has been shown to be of benefit in the treatment of ischemic injury in a subject (WO 2004/010954).

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It is an object of the invention to provide methods of treating chronic degenerative conditions of the central nervous system. It is an alternative objective of the invention to provide medicaments for the treatment of such conditions.

30 Alternatively, it is an object of the invention to at least provide a useful choice to the public.

Summary of the Invention

According to a first aspect of the invention, there is provided a method of treating chronic
35 degenerative conditions of the central nervous system related to oxidative stress comprising

the step of administering a *Parthenium argentatum* AOS protein or a functionally equivalent variant thereof to a subject.

According to a second aspect of the invention, there is provided the use of *Parthenium argentatum* AOS protein or a functionally equivalent variant thereof in the manufacture of a medicament for the treatment of chronic degenerative conditions of the central nervous system related to oxidative stress.

According to a third aspect of the invention, there is provided a pharmaceutical composition for treating chronic degenerative conditions of the central nervous system related to oxidative stress, which comprises *Parthenium argentatum* AOS protein or a functionally equivalent variant thereof.

According to a fourth aspect of the invention, there is provided *Parthenium argentatum* AOS protein or a functionally equivalent variant thereof for use in treating chronic degenerative conditions of the central nervous system related to oxidative stress.

According to a fifth aspect of the invention, there is provided *Parthenium argentatum* AOS protein or a functionally equivalent variant thereof for use in treating chronic degenerative conditions of the central nervous system.

Preferred embodiments for all of the above aspects include:

Preferably the AOS is an AOS having the amino acid sequence of SEQ ID No. 1 or a functionally equivalent variant thereof.

Preferably the conditions of the central nervous system related to oxidative stress are neuronal-degenerative conditions.

Preferably the conditions of the central nervous system related to oxidative stress are vascular-degenerative conditions

Preferably the conditions of the central nervous system related to oxidative stress are glial cell degenerative conditions

Preferably the chronic degenerative conditions of the central nervous system are selected from any one or more of the following: dementia, aging, HIV-associated dementia, Alzheimer's disease, Parkinson's disease, Motor neurone disease, amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), Huntington's disease. More preferably aging.

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Preferably the AOS is administered prophylactically.

Preferably the AOS is administered orally, rectally or by injection, such as cutaneous, subcutaneous, or intravenous injection

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Preferably the injection is formulated for controlled slow release.

Further aspects of the invention, which should be considered in all its novel aspects, will become apparent to those skilled in the art upon reading of the following description which

15 provides at least one example of a practical application of the invention.

Brief Description of the Drawings

Embodiments of the invention will now be described, by way of example only, with

20 reference to the accompanying drawings in which:

Figure 1 illustrates the repair mechanism of AOS in relation to lipid peroxide/hydroperoxide formation during oxidative stress.

25 Figure 2 illustrates SEQ ID No. 1.

Detailed Description of Preferred Embodiments

In general terms, the invention relates to therapies for treatment of chronic degenerative

30 conditions of the central nervous system related to oxidative stress. The invention also includes pharmaceutical compositions of use to treat such conditions and methods for preparing suitable medicaments.

The inventors have found administration of AOS protein found in rubber plant (*Parthenium*

35 *argentatum*) or a functionally equivalent variant thereof (referred to hereinafter as "AOS" or

"AOS protein") is beneficial in the treatment of chronic degenerative conditions of the central nervous system related to oxidative stress. This AOS either improves the conditions, retards the progression of the conditions, or ceases progression of the conditions.

5 When referring to AOS (or *Parthenium argentatum* AOS) it should be taken to include not only AOS isolated from *Parthenium argentatum*, but functionally equivalent peptides and proteins including AOS obtained for example by chemical synthesis and/or gene expression techniques.

The AOS of use in the invention and its functionally equivalent variant(s) may be referred to 10 herein collectively as AOS. Accordingly, where not specifically mentioned, references to AOS of use in the invention herein should be taken to include reference to functionally equivalent variants thereof.

Without wishing to be bound to any particular hypothesis, it is believed AOS has an anti-oxidant 15 effect which acts to mitigate the effects of oxidative stress.

Many chronic degenerative conditions of the central nervous system are associated with oxidative stress. Examples of such conditions include dementia, aging, HIV-associated dementia, Alzheimer's disease, Parkinson's disease, Motor neurone disease, amyotrophic 20 lateral sclerosis (ALS, Lou Gehrig's disease), Huntington's disease. Treatment of these conditions by administration of AOS is to be considered an aspect of the current invention.

One of the detrimental effects of oxidative stress in the central nervous system is oxidative cell injury, for example neuronal, glial cell and vascular damage. Cell injury due to oxidative stress 25 can lead to apoptosis (programmed cell death) and inflammation.

One of the mechanisms known to lead to cell damage is the formation of lipid peroxides/hydroperxoides by free radicals such as nitrogen oxide and other reactive oxygen species (ROS) associated with oxidative stress. Lipid peroxides/hydroperxoides can 30 spontaneously generate additional peroxides by chain propagation reactions. The accumulation of these lipid peroxides in the central nervous system can lead to severe oxidative cell injury.

While not wishing to be bound by theory, it is believed the action of AOS breaks the chain propagation of peroxides and therefore acts as a form of repair mechanism (see Figure 1). It is

believed AOS converts lipid peroxides/hydroperoxides to lipid epoxides. The lipid epoxides are then thought to undergo decomposition to ketols, therefore breaking the chain reaction.

If left untreated lipid peroxides/hydroperoxides are thought to trigger apoptosis (programmed cell death). A further aspect of the invention should therefore be considered to be a method of treating chronic degenerative conditions of the central nervous system related to apoptosis comprising the step of administering AOS protein found in rubber plant *Parthenium argentatum* or a functionally equivalent variant thereof to a subject.

Lipid peroxides/hydroperxoides, apoptosis and oxidative stress have also been associated with inflammation. Accordingly a still further aspect of the invention should be considered to be a method of treating chronic degenerative conditions of the central nervous system related to inflammation comprising the step of administering AOS protein found in rubber plant *Parthenium argentatum* or a functionally equivalent variant thereof to a subject.

In a preferred embodiment the conditions related to oxidative stress which may be treated with AOS are vascular-degenerative conditions. Oxidative stress is one of the major causes and mechanisms of vascular damage/degeneration. The role of vascular degeneration and dysfunction in brain aging has been suggested by clinical research that demonstrates the reduction of cerebral flow and volume from middle age. Therefore a further preferred embodiment of the invention is the use of AOS to treat/mitigate the effects of aging on the central nervous system.

AOS is an enzymatic anti-oxidant, which provides the additional benefit over traditional chemical/non-enzymatic anti-oxidants, such as vitamin E, of having the capability to take part in thousands of anti-oxidation reactions per molecule. Most chemical/non-enzymatic anti-oxidants only have the ability to take part in one or two reactions before they are no longer active. This capability of enzymatic anti-oxidant may allow lower dose rates to be administered to subjects. AOS is effective at miniscule concentrations making it less toxic to the patient than conventional anti-oxidant drugs

In addition AOS is particularly suited to treatment of chronic degenerative condition because it is an enzyme anti-oxidant that functions long after administration.

Further, virtually all antioxidant enzymes, such as catalase and superoxide dismutase, also generate a secondary pro-oxidant radical species that requires a second enzyme to remove it. AOS is believed to act against lipid hydroperoxides and is able to act alone, without a second enzyme.

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In some cases AOS it is beneficial to administer AOS prophylactically. For example where there is a family history of a chronic degenerative condition of the central nervous system, it may be beneficial to administer AOS prior to the appearance of noticeable symptoms, as initial vascular damage may not be noticeable without extensive testing. AOS is also useful in 10 delaying degeneration of the central nervous system associated with aging. In this case, it is beneficial to administer AOS prior to the appearance of significant symptoms associated with aging of the central nervous system. Oxidative stress is believed to be an early event of degenerative cascades, which is an ongoing process during degeneration of the central nervous system. The ability of AOS to mitigate oxidative stress makes it particularly suited to 15 prophylactic use, early use, or prior to the appearance of significant/noticeable symptoms.

The preferred compositions and methods of treatment will thus include controlled slow release options. Treatments of such conditions are likely to be relatively long term and generally not a single/immediate treatment. Therefore controlled slow release options allow for reduced 20 numbers of treatments which will be more convenient for the user. This will also most likely improve uptake of the treatment by the user.

A therapeutically effective amount of AOS is administered in accordance with the invention in form of a composition including AOS and pharmaceutically acceptable excipient, adjuvant, 25 carrier, buffer or stabiliser. The pharmaceutically acceptable excipient, adjuvant, carrier, buffer or stabiliser should be non-toxic and should not detrimentally interfere with the efficacy of the AOS. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, such as cutaneous, subcutaneous, or intravenous injection.

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A "therapeutically effective amount", is to be understood as an amount of an AOS that is sufficient to show retardation of the progress of the chronic condition. In some cases there may be cessation of the progress of the chronic condition. However, in the case of some conditions, for example aging, cessation would not be expected. The actual amount, rate and time-course 35 of administration, will depend on the nature and severity of the condition being treated.

Prescription of treatment is within the responsibility of general practitioners and other medical doctors.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. A capsule may comprise a solid carrier such as gelatin.

For intravenous, cutaneous or subcutaneous injection, the AOS will be in the form of a parenterally acceptable solution which has a suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride injection, Ringer's injection, Lactated Ringer's injection. Preservatives, stabilisers, buffers antioxidants and/or other additives may be included as required.

As discussed above, the invention makes use of AOS protein found in rubber plant *Parthenium argentatum* or a functionally equivalent variant thereof.

In one embodiment of the invention uses AOS protein as described on Genbank CAA55025.2 or a functionally equivalent variant of this protein.

The phrase "functionally equivalent variants" as used herein, includes those peptides or proteins having one or more (for example, 1 to 50, 1 to 30, 1 to 20, 1 to 10 or 1 to 5): deletions, additions and/or substitutions; while substantially retaining the desired function of the protein. In one particular embodiment, the amino acid substitutions are conservative amino acid substitutions.

The functionally equivalent variants will have anti-oxidant activity. In one particular embodiment they will have the ability to convert lipid peroxides/hydroperoxides to lipid epoxides. In one particular embodiment, they will have the ability to convert lipid peroxides/hydroperoxides to lipid epoxides at the lipid/cell membrane interface.

It should be appreciated that a "functionally equivalent variant" may have a level of activity

higher or lower than the protein of which it is a variant. In various embodiments of the invention a functionally equivalent variant has at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% of the level of activity of the protein of which it is a variant.

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Skilled persons will readily be able to assess function and determine the level of activity of a protein or functionally equivalent variants of use in the invention, based on the information contained herein, and using techniques known in the art. However, by way of example, one can determine anti-oxidant activity using the methods described in Pinchuk et al, Chemistry
10 and Physics of Lipids 164 (2001) 42-48 or using a commercially available assay kit available thought Sigma-Aldrich the details of which are set out in Example 1. .

As used herein "conservative amino acid substitution(s)" should be taken broadly to mean substitution of amino acids that have similar biochemical properties. Persons skilled in the art
15 will appreciate appropriate conservative amino acid substitutions based on the relative similarity between different amino acids, including the similarity of the amino-acid side chain substituents (for example, their size, charge, hydrophilicity, hydrophobicity and the like). By way of example, a conservative substitution includes substitution of one aliphatic amino acid for another aliphatic amino acid, substitution of an amino acid with a hydroxyl- or sulphur-
20 containing side chain with another amino acid with a hydroxyl- or sulphur-containing side chain, substitution of an aromatic amino acid with another aromatic amino acid, substitution of a basic amino acid with another basic amino acid, or substitution of an acidic amino acid with another acid amino acid. By way of further example, "conservative amino acid substitution(s)" include:

- substitution of Glycine, Alanine, Valine, Leucine, or Isoleucine, one for another
- 25 substitution of Serine, Cysteine, Theronine, or Methionine, one for another
- substitution of Phenylalanine, Tyrosine, or Tryptophan, one or another
- substitution of Histidine, Lysine, or Arginine, one for another
- substitution of Aspartic acid, Glutamic acid, Asparagine or Glutamine, one for another

30 Functionally equivalent variants will preferably retain at least 70%, 80%, 90%, 95% or 99% amino acid sequence similarity to an AOS specifically referred to herein (for example AOS on Genbank CAA55025.2). In one embodiment, the functionally equivalent variant has at least 70%, 80% 90%, 95% or 99% sequence identity with an AOS protein specifically referred to herein.

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Functionally equivalent variants may be composed of L-amino acids, D-amino acids or a mixture thereof and may include non-naturally occurring amino acids.

A protein or peptide of use in the invention may be isolated from natural sources, or derived by chemical synthesis (for example, fmoc solid phase peptide synthesis as described in Fields GB, Lauer-Fields JL, Liu RQ and Barany G (2002) Principles and Practice of Solid-Phase peptide Synthesis; Grant G (2002) Evaluation of the Synthetic Product. Synthetic Peptides, A User's Guide, Grant GA, Second Edition, 93-219; 220-291, Oxford University Press, New York) or genetic expression techniques, methods for which are readily known. Standard recombinant DNA and molecular cloning techniques are described for example in: Sambrook, and Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Silhavy et al., Experiments with Gene Fusions, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1984); and, Ausubel et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987). The inventor's also contemplate production of a protein or peptide of use in the invention by an appropriate transgenic animal, microbe, or plant.

Those of general skill in the art to which the invention relates will readily be able to identify a variety of nucleic acids which encode the proteins and functionally equivalent variants of use in the invention on the basis of the amino acid sequences provided herein, the genetic code and the understood degeneracy therein.

In one embodiment, the protein or functionally equivalent variant thereof may be connected to one or more additional compounds. For example, they may be connected to one or more additional compounds that aids the function or activity of the protein, protects the protein from degradation, otherwise improves its half life, aids in isolation and/or purification of the protein during manufacture (for example ubiquitin, a his-tag, or biotin), or assists with cell membrane translocation or cell-specific targeting.

The one or more additional compounds may be of any desirable nature and include, for example, peptides, nucleic acids, lipids and carbohydrates.

The compounds may be connected to the protein, or synthesised as a part of a construct, using any appropriate means which allows the protein to retain at least a level of its desired function. The word "connected" or like terms should be taken broadly to encompass any form of

attachment, bonding, fusion or association between the peptide and the at least one compound (for example, but not limited to, covalent bonding, ionic bonding, hydrogen bonding, aromatic stacking interactions, amide bonds, disulfide bonding, chelation) and should not be taken to imply a particular strength of connection. The protein and the at least one compound may be connected in an irreversible or a reversible manner, such that upon administration the protein is released from the compound.

The at least one compound may be connected to the protein at its N-terminus, its C-terminus or at any other location.

It should be appreciated that while the protein and at least one compound may be connected directly to one another, linker molecules could also be used.

Example 1

The activity of AOS and functional equivalents thereof which are of use in the invention can be determined using the following method which is given by way of example only. A kit of use in performing the assay is available from Sigma Aldrich.

Materials and equipment

A UV-spectrophotometer with advanced kinetics capabilities. Dual beam and scanning functions are also desirable. Two sets of 1 cm-path length, quartz cuvettes: a set of 3 ml quartz cuvettes to prepare the substrate and a second set of 1 ml or 0.5 ml cuvettes measure the AOS reactions. A set of micropipettors (100-1000 µl, 10-100 µl and 0-10 µl range) along with a sheet parafilm to mix reagents in the cuvettes.

This kit contains the ingredients needed to make the AOS substrate, which is linoleic hydroperoxide (LOOH) and a highly enriched sample of AOS. Once the LOOH substrate is prepared it should be used for the AOS assays within 4-8 hours.

The kit comprises:

1. Reagent A (20X KPhos Buffer): 1M Potassium Phosphate buffer, pH 7.0 (Store 4°C)
2. Reagent B (150X Linoleic Acid): Linoleic Acid in 0.4M Borate buffer (Store -20°C, shelf-life 6 mo)
3. Lipoxygenase (LOX): 10 mg as a lyophilized powder (Store powder at -20°C) (Store suspension at 4°C)

4. Allene Oxide Synthase (AOS): highly enriched and purified AOS in PBS buffer (Store -20°C)

Methods

5 To begin, prepare a 50 mM KPhos, pH 7.0, 1X working buffer from the 20X, Reagent A stock (see Step 1 below). This is used to dilute the poly-unsaturated fatty acid (linoleic acid) substrate that is used to generate the LOOH by the reaction of a lipoxygenase (LOX) enzyme supplied as a dry powder in the kit. The 50 mM KPhos is also used to resuspend the LOX powder. The LOX reaction can be followed by the generation of LOOH that has an absorbance at 234nm.
10 The LOOH that is produced is then used as the substrate for AOS, which causes a drop in 234nm. The rate of disappearance of LOOH A_{234} is how AOS activity is determined. The 1 ml sample of AOS provided in the kit contains approx. 14.5 mg of AOS equal to 25000 units of per ml AOS.

15 Step 1. Preparing the 1X KPhos Working Buffer from 20X KPhos Reagent A: Thaw the frozen 15 ml Falcon tube containing the 1M (20X) KPhos Buffer. Remove 2.5 ml to a 50 ml sterile Falcon tube and dilute to 50 ml with sterile, distilled water. This yields the 1X, 50 mM KPhos working buffer that is use for all other reactions. Stored at 4°C.

20 Step 2. Preparing the LOX suspension: The lipoxygenase (LOX) enzyme is shipped as a 10 mg lyophilized powder that can be stored at -20°C. Resuspend approx. 1 mg of powder in 100 µl of 1X KPhos Working Buffer. Mix well. The powder will never completely dissolve in the buffer, but it is active when stored at 4°C. Mix before removing pipetting. Use about 1-10 µl per 3.0 ml reaction.

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Step 3. Preparing the 1X Linoleic Acid Working Solution from 150X Reagent B (Linoleic Acid): Thaw the frozen 2 ml tube of 150X Reagent B (Linoleic Acid) solution. This contains 25 µl linoleic acid per 10 ml of 0.4M Borate buffer, pH 9.0. Mix 40 µl of 1X Reagent B with 6.0 ml of 1X KPhos Reagent A working buffer. Transfer 3 ml of this into a 3 ml quartz cuvette and run a
30 UV scan on this from 200-260nm against 1X KPHos buffer (zeroed) in the reference cell. The scan of linoleic acid should look something Figure 4. Linoleic acid that has oxidized will have a hump between 220-240 nm and should not be used.

Step 4. Set Up UV Spectrophotometer to Run LOX Reactions in Scanning Mode: If scanning
35 capabilities are available on UV spectrophotometer, set the following parameters:

Start Wavelength: 200 nm; Stop Wavelength: 260 nm; Upper Absorbance: 4.0; Lower Absorbance: -0.2; Scan speed: Rapid; Reference cuvette: 3 ml of 1X LINOLEIC ACID; and Sample cuvette: 3 ml of 1X LINOLEIC ACID.

5 Run a baseline scan to zero the instrument. Once a good baseline is established, remove the sample cuvette and add 3-10 µl of the LOX suspension to the 3 ml of 1X LINOLEIC ACID solution. Mix by inverting twice with parafilm and replace the cuvette and start a new scan. Repeated scans should show the development of peak at A_{234} that indicates the formation of LOOH. These progressive scans follow the production of LOOH, which has a peak absorbance
10 at 233-238 nm. The reaction is completed after about 10 – 20 minutes. Note that with this particular instrument, the peak absorbance is at 234 nm. This will be the wavelength used in future kinetics assays.

The material made in the 3.0 ml cuvette will then be used as a substrate for the AOS reaction,
15 which is essentially a reverse of this LOX reaction. Use 500 µl of this LOOH substrate per AOS reaction. Run each reaction in a 1.0 or 0.5 ml quartz cuvette.

The scanning mode allows one to choose the best wavelength for monitoring the reaction. However, it is preferred to use the kinetics mode to get accurate activity measurements.

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Step 5. Set Up UV Spectrophotometer to Run LOX Reactions in Kinetics Mode: With the spectrophotometer in kinetics mode, set up the following parameters: Measuring Wavelength: 236 nm; Internal Reference Wavelength: 260 nm; Wavelength reference (for dual beam): ON Upper Absorbance: 4.0; Lower Absorbance: -0.2; Interval time: 2 sec (interval between
25 readings); Run time: 500 sec; Initial delay (sec): 0; Reference cuvette: 3 ml of 1X LINOLEIC ACID in 3.0 ml quartz cuvette; Sample cuvette: 3 ml of 1X LINOLEIC ACID in 3.0 ml quartz cuvette.

With these settings in place, remove the 3 ml sample cuvette and add 3–10 µl of the LOX
30 suspension. Mix by inverting against parafilm, place it in the cuvette and begin the kinetics measurement.

The LOOH substrate in the 3 ml sample cuvette can now be used for the AOS reactions. For example, use 500 µl in a 0.5 ml quartz cuvette.

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Step 6. Set Up UV Spectrophotometer to Run AOS Reaction: With the spectrophotometer in kinetics mode, set up the following parameters: Measuring Wavelength: 234 nm; Internal Reference Wavelength: 260 nm; Wavelength reference (for dual beam): ON
Upper Absorbance: 4.0; Lower Absorbance: -0.2; Interval time: 1 sec (interval between readings); Run time: 100 sec; Initial delay (sec): 0; Reference cuvette: 500 μl of 1X LINOLEIC ACID in 1.0 or 0.5 ml cuvette; Sample cuvette: 500 μl of LOOH in 1.0 or 0.5 ml quartz cuvette.

Before adding any AOS to the sample cuvette, start measuring in the kinetics mode. As soon the trace of the scan is seen, at an absorbance at A234 of 1.2, quickly remove the sample and add 1 μl or less of the AOS sample. Quickly mix by inverting with parafilm and return to the spectrophotometer. The trace of the absorbance should begin to drop.

Note: The absorbance at the beginning of the run is at 1.342. When the spectrophotometer lid is lifted, a rapid drop in absorbance occurs. Ideally, keep the spectrophotometer running while the sample cuvette is removed, add a small sample of AOS to the LOOH in the cuvette, quickly mix by inverting with parafilm, reinserting the cuvette to the sample holder and closing the lid. The absorbance drops due to the disappearance of the LOOH by the action of AOS activity. In the reaction above, approximately -2.517 Units of AOS was added in an equivalent of 0.1 μl or - 25 U

Step 7. Dilute AOS and Run Reaction Dilute 1 μl of the AOS sample provided with 50 μl of 1X KPhos buffer. Use 1, 2 and 5 μl of this in a reaction as described in Step. 6. Dilute further if necessary to get an accurate kinetic measurement. Adding too much AOS will cause the complete disappearance of the substrate before you can close the lid of the spectrophotometer. Store the remaining AOS at -20 °C.

Step 8. Calculating AOS Activity: Dilute 1 μl of the AOS sample provided with 50 μl of 1X KPhos buffer. Use 1, 2 and 5 μl of this in a reaction as described in Step. 7. The activity is determined by taking calculating the steepest slope at the earliest point of the drop. 1 Unit of activity is equal to the drop of 1 absorbance unit at A234.

Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like, are to be construed in an inclusive sense as

opposed to an exclusive or exhaustive sense, that is to say, in the sense of "including, but not limited to".

The entire disclosures of all applications, patents and publications cited above and below, if
5 any, are herein incorporated by reference.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgement or any form of suggestion that that prior art forms part of the common general knowledge in the field of endeavour in any country in the world.

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The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, in any or all combinations of two or more of said parts, elements or features.

15 Wherein the foregoing description reference has been made to integers or components having known equivalents thereof, those integers are herein incorporated as if individually set forth.

It should be noted that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and
20 modifications may be made without departing from the spirit and scope of the invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be included within the scope of the invention.

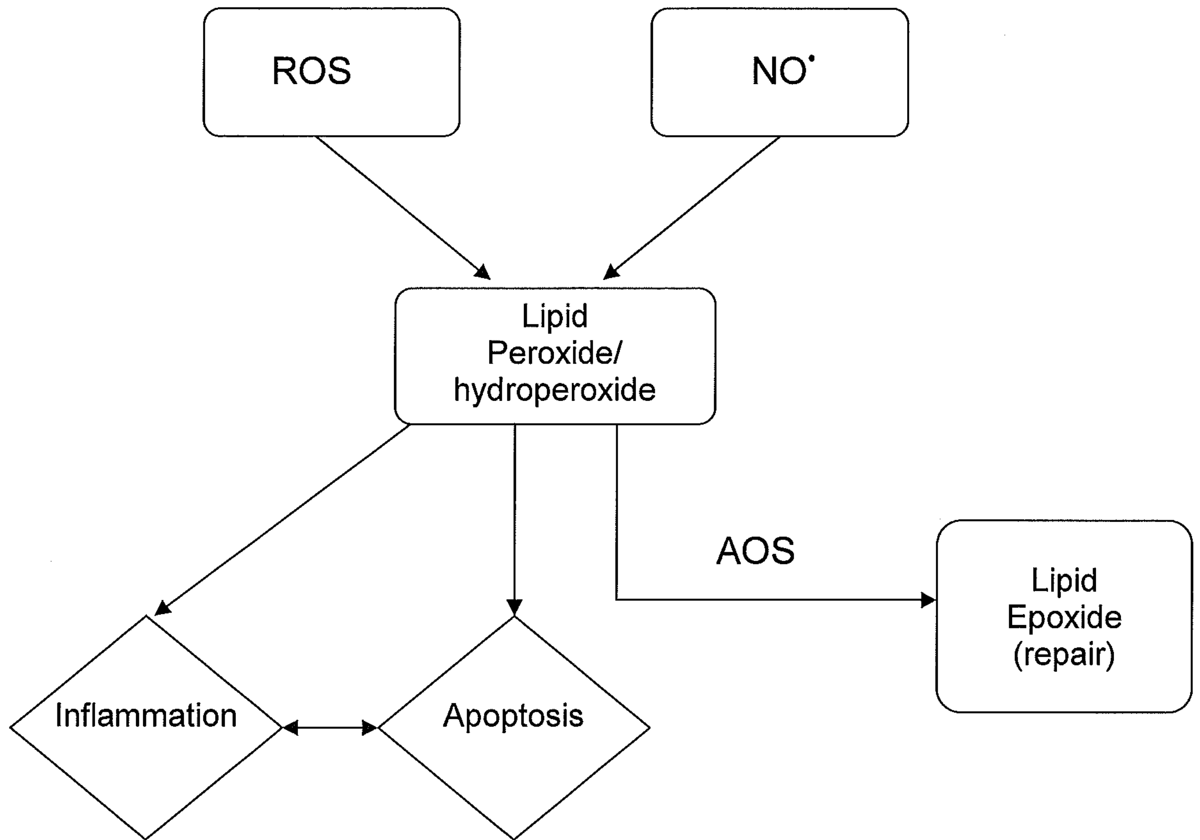

Figure 1

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  1 mdpsskplre ipgsygipff qpikdrleyf ygtggrdeyf rsrmqkyqst vfranmppgp
 61 fvssnpkviv lldaksfpil fdvskvekkd lftgtympst kltggyrvls yldpseprha
121 qlknllffml knssnrvipq fettytelfe gleaelakng kaafndvgeq aafrflgray
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301 tqlaeeirga iksygdgnvt leaieqmplt ksvvyeslri eppvppqygk aksnftiesh
361 datfevkkge mlfgyqpfat kdpkvfdrpe eyvpdrfvgd geallkyvww sngpetespt
421 venkqcagkd fvvlitrlfv ielfrrydsf eielgesplg aavtltflkr asi
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Figure 2