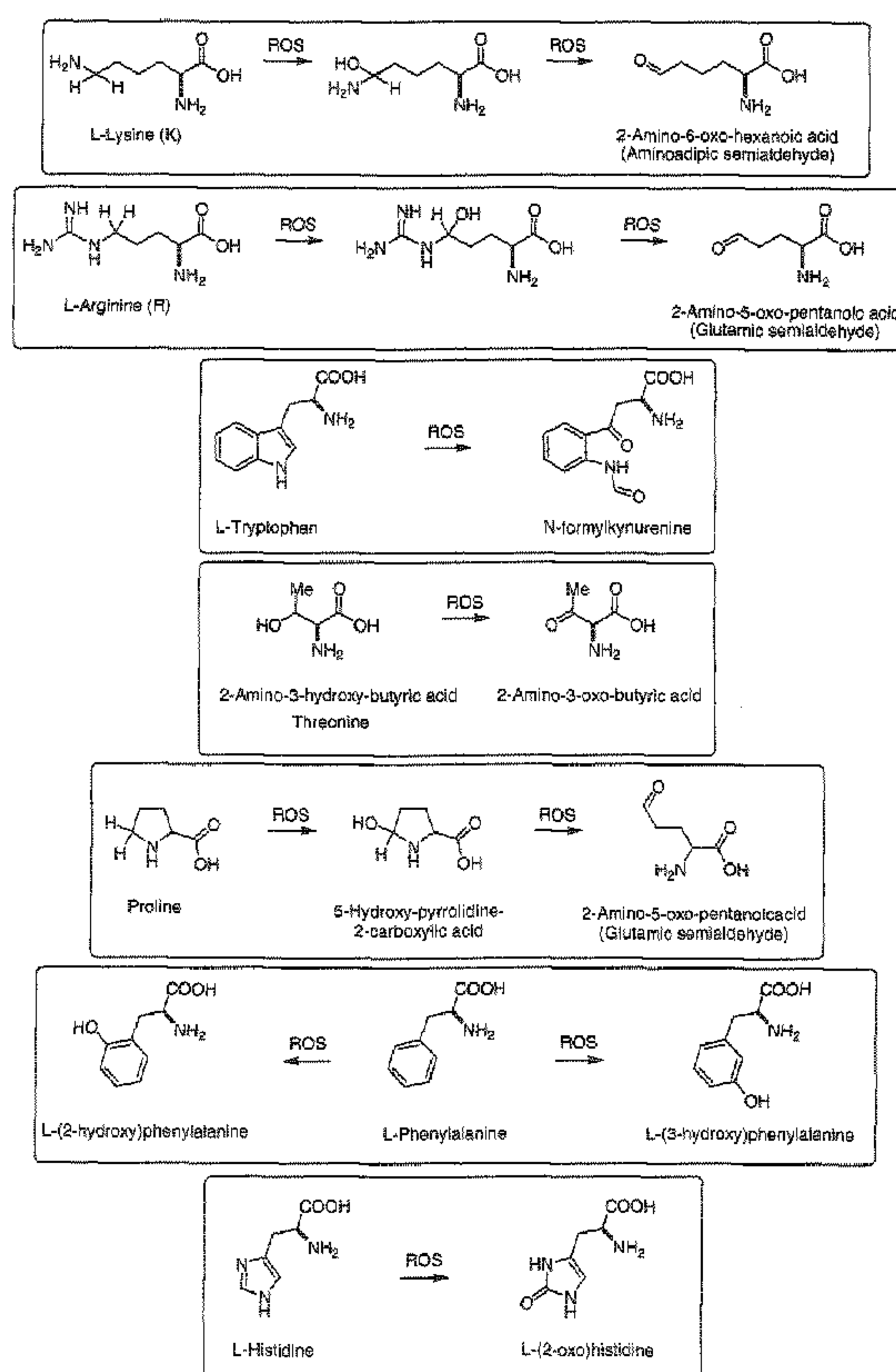




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(54) **Titre : COMPOSES ISOTOPIQUEMENT MODIFIES ET LEURS UTILISATIONS EN TANT QUE SUPPLEMENTS ALIMENTAIRES**
 (54) **Title: ISOTOPICALLY MODIFIED COMPOUNDS AND THEIR USE AS FOOD SUPPLEMENTS**



(57) **Abrégé/Abstract:**

A nutrient composition comprises an essential nutrient in which at least one exchangeable H atom is ²H and/or at least one C atom is ¹³C. The nutrient is thus protected from, inter alia, reactive oxygen species.

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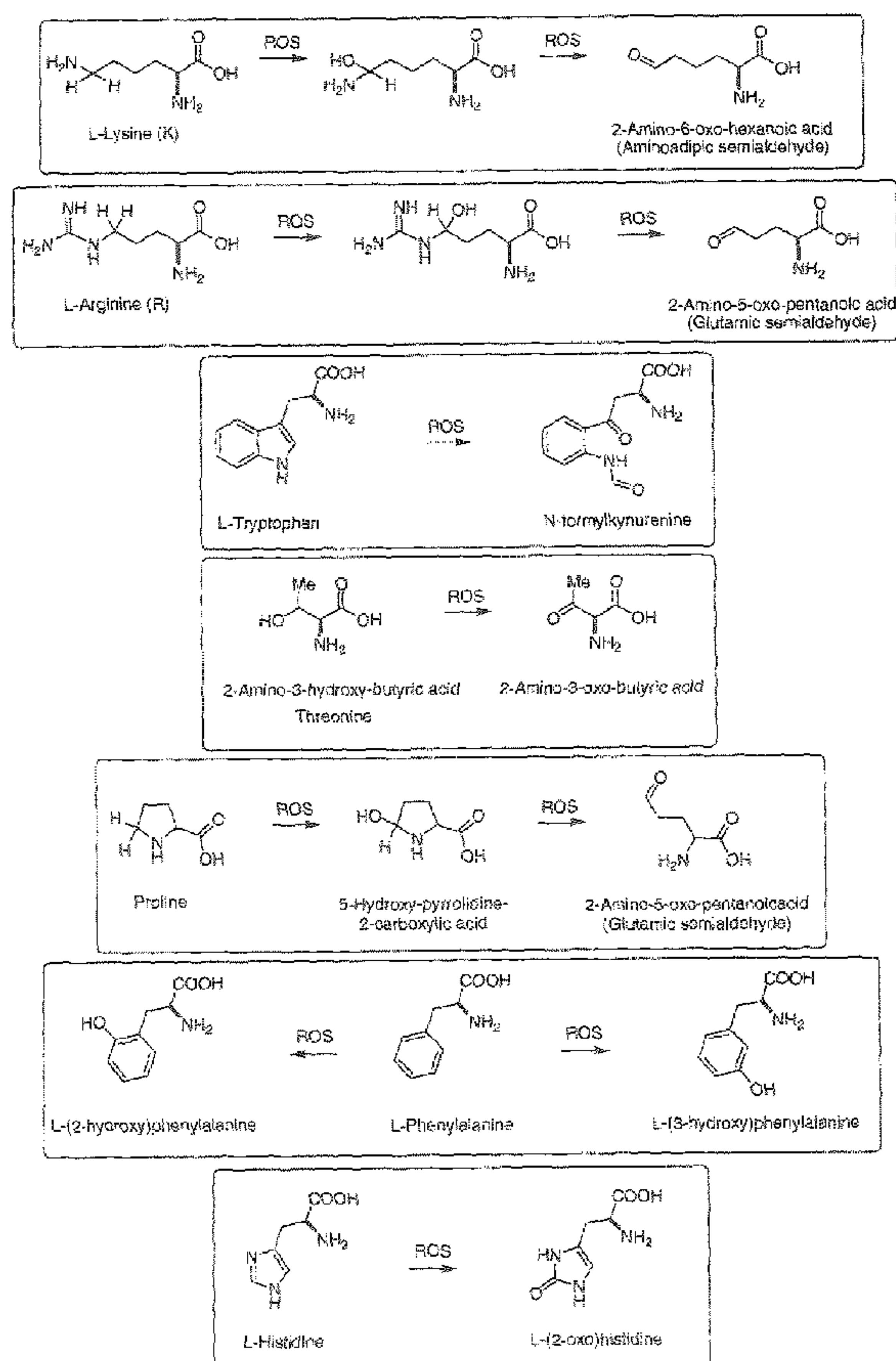
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(54) Title: ISOTOPICALLY MODIFIED COMPOUNDS AND THEIR USE AS FOOD SUPPLEMENTS

(57) Abstract: A nutrient composition
comprises an essential nutrient in which at least
one exchangeable H atom is ²H and/or at least
one C atom is ¹³C. The nutrient is thus protected
from, *inter alia*, reactive oxygen species.

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ISOTOPICALLY MODIFIED COMPOUNDS AND THEIR USE AS FOOD SUPPLEMENTS

Field of the Invention

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The present invention related to isotopically modified compounds and their use as food supplements.

Background of the Invention

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A currently accepted theory of ageing blames the irreversible changes in cell machinery and reduced efficiency of metabolic processes on the detrimental effects of free radicals and other reactive oxygen species (ROS) or reactive nitrogen species (RNS) which are normally present in the cell as part of the respiratory process. ROS and RNS oxidize/nitrate DNA, proteins, lipids and other cell components. Of these, protein oxidation, which converts arginine, lysine, threonine, thryptophan and proline into corresponding carbonyl compounds, cannot be repaired by proteases after a certain threshold number of amino acid residues have been oxidized.

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The damaged protein loses its catalytic or structural activity, but proteases are unable to disintegrate heavily carbonylised strands, so that the damaged species accumulate and aggregate, clogging up cellular passages. This rust-like process gradually wears down all cellular mechanisms, slowing everything down and ultimately causing cellular death.

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Apart from ageing, many diseases such as Alzheimer's, Parkinson's, dementia, cataract, arthritis, chronic renal failure, acute respiratory syndrome, cystic fibrosis, diabetes, psoriasis and sepsis, to give a few examples, are associated with increased protein carbonylation. Typically, physiological levels of protein carbonyls are at around 1 nmol/mg protein, whereas pathological levels go to 8 nmol/mg and above.

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For the two molecules involved in the process of oxidative damage of proteins, i.e. an oxidizer and its substrate, the oxidizer has been the subject of many studies aiming at neutralizing or removing it by means of increasing the number of antioxidants (vitamins, glutathione, peptides or enzymes). The substrate, e.g. amino acid (AA) residues which are converted into carbonyls, has received less attention.

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One common feature of all the AA residues (except proline) vulnerable to carbonylation is that they belong to the group of essential AAs, which cannot be synthesized by vertebrata and should be ingested, e.g. consumed with food. The group includes phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, arginine, lysine and leucine (arginine is essential for children of up to 5 years of age).

45

Oxidation of both Arg and Lys by ROS yields amino adipic semialdehyde and proceeds through sequential replacement of ω -hydrogens with hydroxyls. Oxidation of Lys, Arg, Trp, Thr, Phe and His is shown in Fig. 1. Side-chains undergo the same transformations if these AAs are part of polypeptides/proteins. Other essential AAs undergoing ROS-

driven oxidation include Leu (to 5-hydroxyleucine), Val (3-hydroxyvaline) and Ile (several products).

5 Other types of oxidative damages affecting essential AAs involve reactive nitrogen species (RNS). Examples are shown in Fig. 2.

10 Yet another process detrimental to proteins is a ROS-driven peptide bond cleavage, which is preceded by oxygen free radical-mediated protein oxidation. A hydrogen atom is abstracted from a C_α atom of the polypeptide chain, which then leads to formation of an alkoxyl radical. This can lead either to hydroxyl protein derivative, or to peptide bond cleavage by (1) diamide or (2) α-amidation pathway. This is illustrated in Fig. 3.

15 Nucleic acids are not normally considered as essential components of the diet, but are also damaged by ROS. An example particularly important for the mitochondrial functioning is the formation of 8-oxy-G, as illustrated in Fig. 4. This leads to mutations in the mitochondrial genome, which is not maintained and repaired as efficiently as the nuclear genome, with detrimental consequences to the efficiency of respiratory processes in the cell. Another cause of degradation is radiation.

20 The kinetic isotope effect is widely used when elucidating mechanisms and rate-determining stages of chemical and biochemical reactions. The rate of reaction involving C-¹H bond cleavage is typically 5 to 10 times faster than the corresponding C-²H (²H = D = deuterium) bond cleavage, due to the two-fold difference in the masses of H and D isotopes. The difference in reaction rates is even higher for tritium (³H or T) as it is 3
25 times heavier than hydrogen, but that isotope is unstable. The second component of the C-H bond, the carbon atom, can also be substituted for a heavier ¹³C isotope, but the bond cleavage rate decrease will be much smaller, since ¹³C is only a fraction heavier than ¹²C. See Park *et al*, JACS (2006) 128: 1868-72.

30 Oxidation reactions are a good example of the isotope effect, as the hydrogen subtraction by an oxidizer is usually a rate-limiting step of the process. Damgaard, Biochemistry (1981) 20: 5662-69, illustrates this: the kinetic isotope effect upon V/K for (1-R)[1-²H₂]- and (1-R)[1-³H₂]- ethanol oxidation by liver alcohol dehydrogenase (ADH) to acetaldehyde, measured at pH 6, was 3 (D(V/K)) and 6.5 (T(V/K)), decreasing to 1.5 and
35 2.5 respectively at pH 9. Lower than expected rates confirm the discrete role of the non-ADH systems as alternative pathways. *In vivo* experiments in perfused rat liver, as reported in Lundquist *et al*, Pharm. & Tox. (1989) 65: 55-62, gave the mean value of D(V/K) of 2.89. Therefore, in all cases the oxidation of deuterated ethanol was substantially slowed down.

40 Isotopically labelled material has been administered to animals, and also to humans, for diagnostic purposes. Gregg *et al*, Life Sciences (1973) 13: 755-82, discloses the administration to weanling mice of a diet in which the digestible carbon fraction contained 80 atom % ¹³C. The additive was ¹³C-labelled acetic acid. Tissue examination
45 revealed no abnormalities clearly attributable to the high isotopic enrichment.

Summary of the Invention

The present invention is based on the realisation that isotopic substitution can be used to synthesize a class of compounds that, when ingested, result in the formation of bodily constituents (e.g. proteins, nucleic acids, fats, carbohydrates, etc) that are functionally equivalent to normal bodily constituents but which have a greater resistance to degradative/detrimental processes, e.g. those mediated by ROS and RNS or radiation. Therefore, according to this invention, a nutrient composition comprises a nutrient composition comprising an essential nutrient in which at least one exchangeable H atom is ^2H and/or at least one C atom is ^{13}C .

Compounds for use in the invention are identical to normal nutrients or constituents of food except that they contain stable isotopes which, when incorporated into bodily constituents make such bodily constituents more resistant to degradative processes than they would be otherwise. They provide a method for protecting the preferred functionality of natural biomolecules; the method comprises supply of a compound in such a way that it becomes incorporated into biomolecules and in so doing confers properties on the biomolecule that protect against damaging or unwanted chemical changes.

Compounds for use in the invention may be chemically synthesized and, when ingested by an organism, are metabolized in a way that results in the incorporation of the compound into a functional biomolecule; the incorporation of the compound resulting in the biomolecule having a higher degree of resistance to damaging molecular changes than would be the case for the equivalent biomolecule that did not comprise the compound. Such compounds may act as mimics of naturally occurring precursor elements of biomolecules. They may mimic an essential amino acid. The organism is typically a plant, microbe, animal or human.

A compound for use in the invention is typically not degraded by enzymes of the P450 pathway. It can therefore accumulate in a subject for which it is essential.

Description of the Drawings

Figs. 1 to 4 each show reactions that degrade essential nutrients.

Description of the Invention

The present invention relates to the fact that essential supplements may undergo irreversible chemical transformations such as oxidation, nitration, etc, leading to the onset of senescence or diseases. Essential food components cannot be synthesised de novo by an organism, e.g. mammal, primate or human, and therefore need to be supplied with the diet. For the purposes of this specification, a nucleic acid is essential, although it may be more properly be described as conditionally essential. Conditionally essential nutrients need to be supplied with the diet under certain circumstances.

For humans, 10 amino acids are essential, i.e. Phe, Val, Trp, Thr, Ile, Met, His, Leu, Lys and Arg (up to the age of five). Purine and pyrimidine nucleosides are conditionally essential. Essential fatty acids are ω -3 and ω -6, while monounsaturated oleic acid is generally non-essential.

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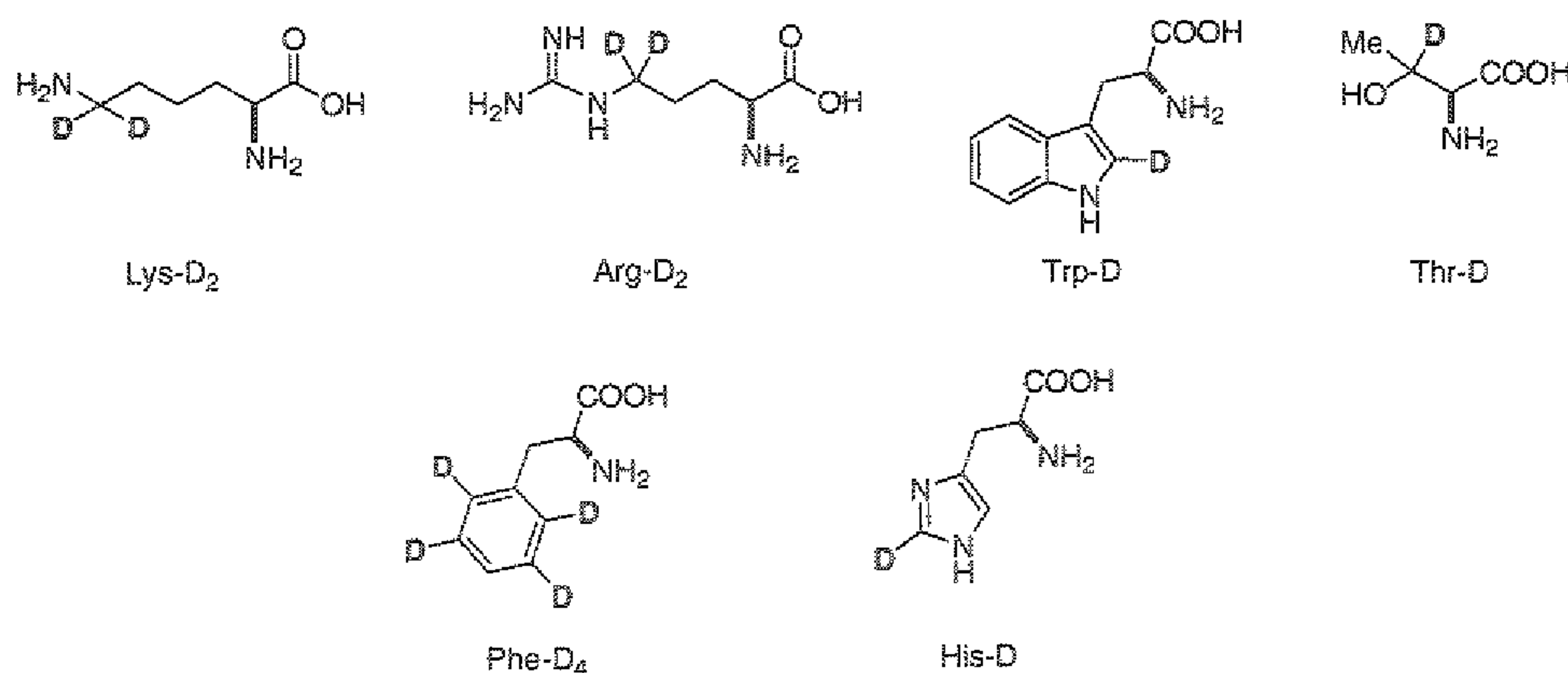
According to this invention, the proposed undesired effects such as ageing/diseases can be slowed down. The compounds consumed should be modified to slow down the undesired reactions, while still retaining their chemical identity. This can be achieved in one embodiment by substituting hydrogen atoms subjected to abstraction during oxidation/oxidative substitution at the most reactive carbon sites, or the sites known to undergo the ROS/RNS inflicted damage as illustrated on Figs. 1-4, with deuteriums, which due to the isotope effect slow down the rate of reactions. Substituting carbons instead of or in addition to H atom substitution may require a greater degree of substitution since one does not add so much to the reaction rate decrease (D is twice the weight of H, and ^{13}C is less than 10% heavier than ^{12}C).

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Depending in part of the method of preparation, a compound for use in the invention may comprise partial or total isotopic substitution. For example, deuterium substitution may be only at the one or two hydrogen atoms that are considered chemically exchangeable, e.g. at OH or CH_2 adjacent to a functional group. Total rather than partial ^{13}C substitution may often be achieved more effectively.

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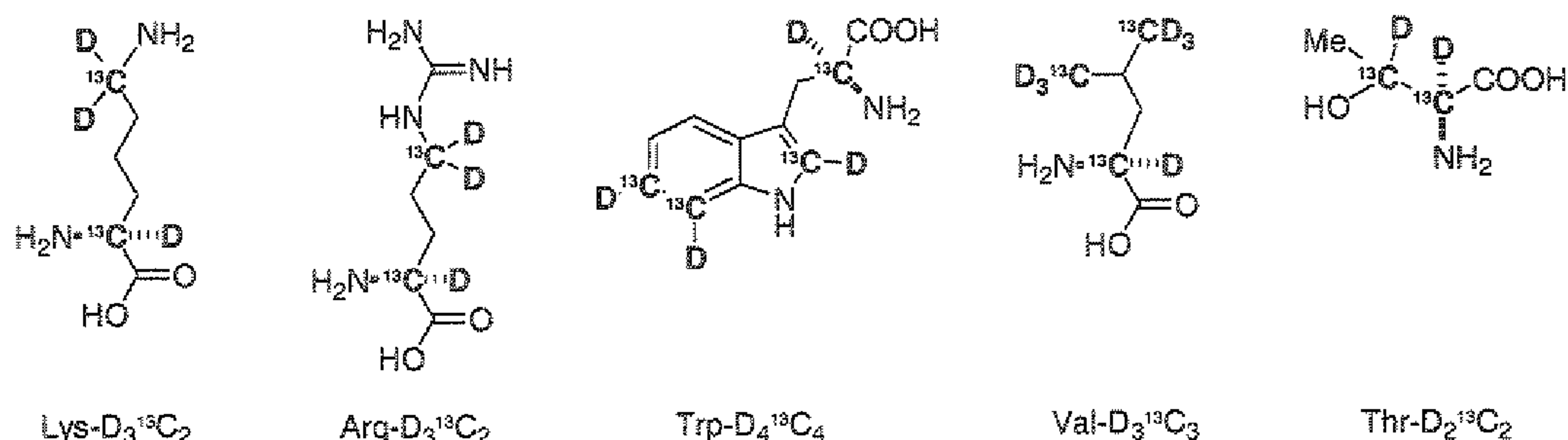
In a preferred embodiment of the invention, the (or only the) oxidation-sensitive hydrogens should be substituted with deuteriums, to minimize the risk of other metabolic processes slowing down when fragments of these AAs are used to build up other structures. In special cases, to further increase the resistance to oxidation, both ^1H and ^{12}C of a H-C bond can be substituted by ^2H and ^{13}C . To minimize any possible negative effect of isotopes, such as unwanted slowing down of biochemical reactions that utilise fragments of AAs protected with isotopes, preferably only the most sensitive parts of the AAs should be derivatised, for example, ω -atoms of Lys and Arg. Preferred compounds of this type are

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If the oxidative stress is so severe that benefits from protecting the vulnerable sites outweigh potential damaging effects from slowing down other metabolic pathways (as

is the case with some diseases), then AAs more heavily protected with isotopes can be employed, as shown in the following, illustrative formulae



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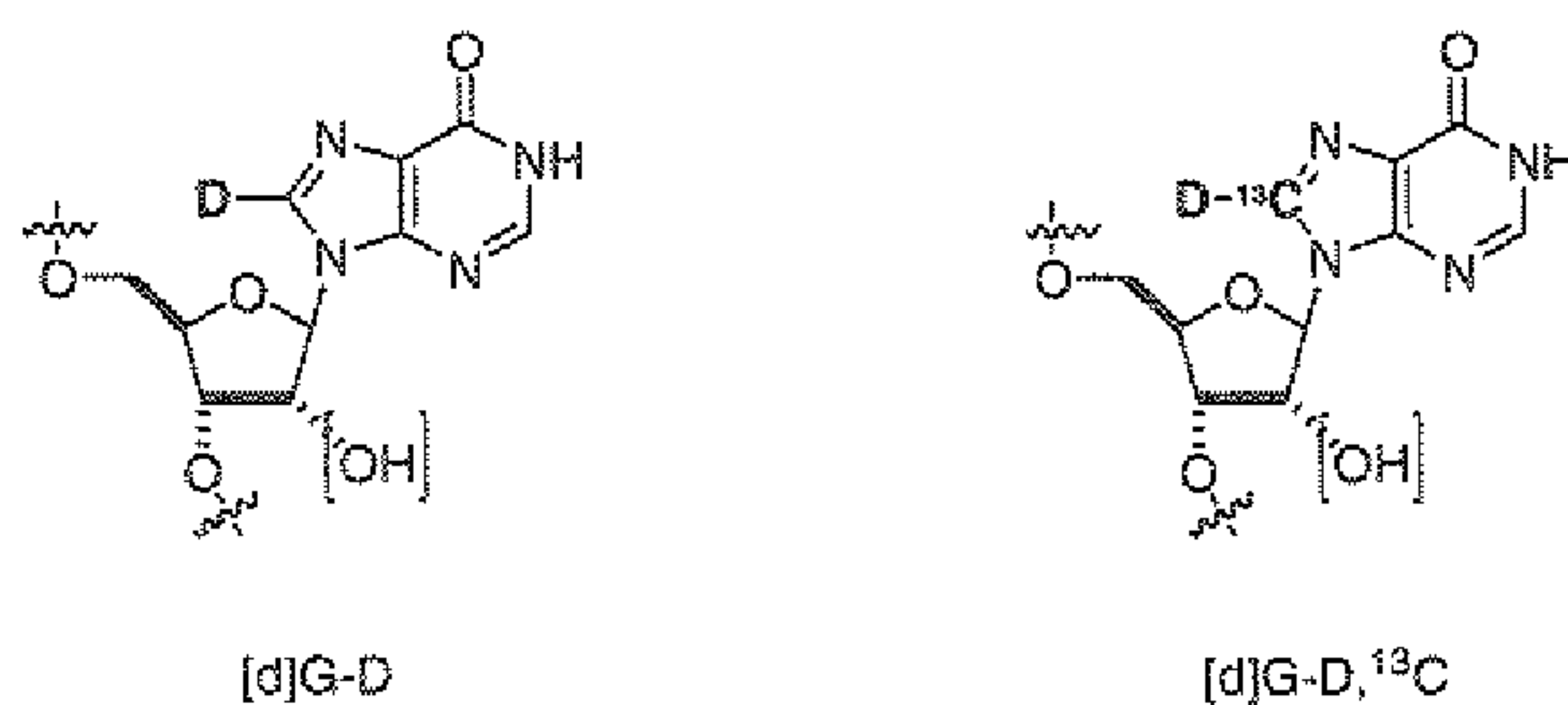
Such derivatives confer protection from the detrimental effects illustrated in Figs. 1-4.

As all vertebrata have lost the ability to synthesise the essential AAs and require the outside supply of essential AAs or fatty acids, non-painful ways of delivering the deuterated/deuterated and ¹³C-modified AAs into human food sources are possible. For AAs, one example of process is to create essential AAs-deficient yeast/algae/bacteria/etc, growing them on appropriate isotopically 'protected' media/substrates and then feeding the obtained biomass to fish or livestock. The fish or livestock can then be introduced into the food chain in the normal manner. Another example is by a direct pill/supplement-based delivery.

15

Non-essential components of food are the compounds that can be produced by an organism, such as nucleic acid bases. But when these are consumed as food, some of the non-essential components are digested/used as precursors for other compounds, but a certain fraction is utilized directly in metabolic processes, e.g. nucleic acid (NA) bases, incorporated into DNA. Therefore, as an example, some of the NA bases supplied with food may be isotopically protected, as shown in the following, illustrative formulae

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Such species are less vulnerable to oxidation upon incorporation into DNA. In other words, the oxidation rate of DNA, including mitochondrial DNA, can be reduced.

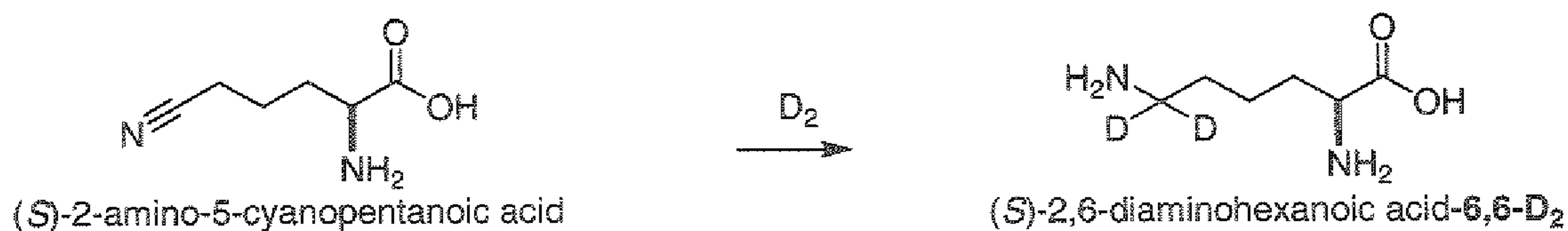
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Both essential and non-essential components may be administered through a digestive system to achieve a desired effect of slowing down detrimental changes associated with ageing process and various diseases. Nevertheless, ways other than through the digestive tract, for instance intravenous delivery, can be envisaged. The important aspect of any delivery system is to get the isotopically engineered compounds incorporated into bodily/biochemical constituents.

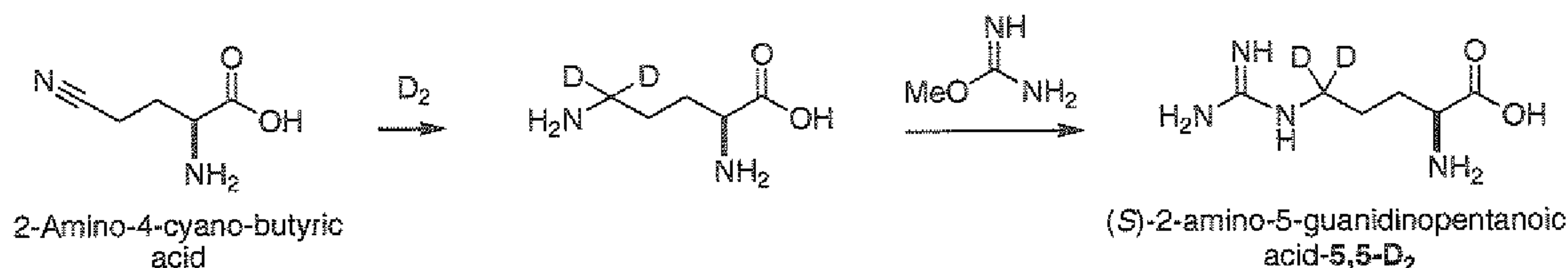
A composition of the invention can be provided like any food supplement. It typically comprises one or more nutrients in addition to the isotopically labelled essential component. It may comprise plant material, microbial material or animal material. The composition may be a normal foodstuff, a tablet or other solid medicament, or an injectable or other liquid.

The composition may comprise unmodified compounds in addition to those that have been labelled. The labelled compound is typically present in a larger amount, and certainly greater than that which may be present naturally.

Compounds for use in the invention may be prepared by procedures that are known or that can be modified as appropriate by one of ordinary skill in the art. For example, the deuterated analogue of Lys, 2,6-diaminohexanoic acid-6,6-D₂, may be synthesized from a precursor nitrile by hydrogenolysis in D₂ according to standard procedures.



The deuterated analogue of Arg, 2-amino-5-guanidinopentanoic acid-5,5-D₂, may be synthesized from a corresponding nitrile.



Ornithine-D₂, obtained by hydrogenolysis in a way similar to that described above for Lys, was dissolved in water and mixed with an equal volume of 0.5M O-methylisourea, pH 10.5, adjusted with NaOH. After 4-5 h, 1% TFA was added to stop the reaction. The compound was purified by a RP HPLC (Buffers were A: 0.1% TFA/H₂O; B: 0.1% TFA/(80%MeCN / 20% H₂O)), 0-65 % B over 40 min. See Kimmel, *Methods Enzymol.* (1967), **11**: 584-589, and Bonetto *et al.*, *Anal. Chem.* (1997), **69**: 1315-1319.

Cyano-aminoacids are precursors to amino acids. Synthesis of cyano-aminoacids can be carried out by several routes, starting from a variety of precursors. Alcohols (Davis & Untch, *J. Org. Chem.* (1981);46: 2985-2987), amines (Mihailovic *et al.*, *Tet. Lett.* (1965) 461-464), amides (Yamato & Sugasawa, *Tet. Lett.* (1970) 4383-4384) and glycine (Belokon *et al.*, *JACS* (1985)107: 4252-4259) can all serve as starting materials in such syntheses. Some methods can yield both ¹³C and ²H-substituted compounds, while others are only compatible with deuteration.

Deuteration can be carried out using deuterium gas (for example, as described in White *et*

al, JACS (1994) 116: 1831-1838) or different deuterides, for example NaBD₄ (Sato *et al*, Tet. Let. (1969) 4555-4558); the choice between these methods should be made based on the availability and price of the corresponding deuterium derivatives. Some of the strategies tested are described in detail below.

5

The sites to be protected within essential fatty acids for the purpose of the present invention are the methylene groups of the 1,4-diene systems ('bis-allyl' positions). They are the most reactive, and can easily be derivatised using a variety of methods. Bromination of this position followed by reduction with ²H₂ results in the substitution of one hydrogen at a time. To substitute both, the procedure should be repeated twice. A more attractive method may be a direct one-step substitution in heavy water. An example of such exchange is given below (Example 6) for 8-deuteration of deoxyguanosine.

An alternative approach to the synthesis of deuterated unsaturated fatty acids is based on strong base treatment of 1,4-dienes followed by quenching with heavy water. This is illustrated in Example 7.

There are literature examples for substitutions at any position for all major nucleotide bases, with all major types of isotopes (²H₂, ³H₂, ¹³C, ¹⁴C, ¹⁵N, ¹⁸O etc). Described below are just two procedures, based on the previously published work, for selective deuteration of purines (Esaki *et al*, Heterocycles (2005) 66: 361-369, and Chiriac *et al*, Labelled Compd. Radiopharm. (1999) 42: 377-385). Numerous other protocols are suitable as well. It is often possible to exchange hydrogens for deuteriums on an existing nucleic acid base/nucleoside, while to incorporate ¹³C, the bases should be assembled (for example, see Folesi, *et al*, Nucleosides Nucleotides Nucleic Acids (2000).

Syntheses of some isotopically 'reinforced' essential dietary components suitable for use in the present invention are known; see for instance, 6,6-²H₂,1,1-¹³C₂-L-Lys: Lichtenstein *et al*, J. Lipid Res. (1990) 31: 1693-1701 and 8-deutero-deoxy-guanosine: Toyama *et al*, J. Raman Spectrosc. (2002) 33: 699-708).

The invention is not limited by the synthetic organic chemistry methods described above, as there exists a large arsenal of different methods that can also be used to prepare the above mentioned and other isotopically protected components suitable for use in the present invention. For instance, in addition to the methods disclosed in the Examples, other methods suitable for conversion of a primary amino group function into a CN function (with the aim of subsequent deuteration of the alpha-(relative to N) carbon atom) can be employed, such as:

- 40 - a direct oxidation by oxygen catalysed by cuprous chloride-dioxygen-pyridine system (Nicolaou *et al*, Synthesis (1986) 453-461; Capdevielle *et al*, Tet. Lett. (1990) 31: 3305-3308)
- a direct conversion using bromosuccinimide (Gottardi, Monatsh. Chem. (1973) 104: 1690-1695)
- 45 - a direct iodosobenzene oxidation (Moriarty *et al*, Tet. Lett. (1988) 29: 6913-6916)
- a two-step conversion via a di-tosyl derivative and an iodo derivative (DeChristopher *et al*, JACS (1969) 91: 2384-2385).

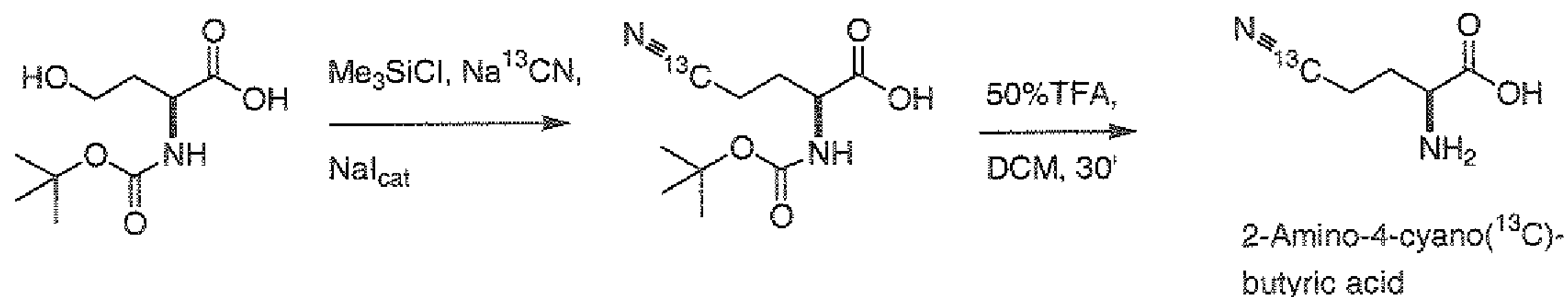
The following Examples 1 to 9 illustrate the preparation of materials suitable for use in the invention.

5 (MA)LDI-TOF mass spectra were obtained using a Voyager Elite Biospectrometry Research Station (PerSeptive Biosystems, Vestec Mass Spectrometry Products) in a positive ion mode; FAB spectra were acquired using a Varian instrument. Analytical thin-layer chromatography was performed on the Kieselgel 60 F₂₅₄ precoated aluminium plates (Merck) or aluminium oxide 60 F₂₅₄ precoated aluminium plates (Merck), spots were visualized under UV or as specified. Column chromatography was performed on silica gel (Merck Kieselgel 60 0.040–0.063 mm) or aluminium oxide (Aldrich aluminium oxide, activated, neutral, Brockmann I, 150 mesh, 58 Å).

15 Reagents for biological experiments, unless otherwise specified, were from Sigma-Aldrich. ¹³C-glucose was from Sigma and Reakhim (Russia).

Reagents obtained from commercial suppliers were used as received. All solvents were from Aldrich; trifluoroacetic acid was from Pierce; HPLC grade solvents were from Chimmed (Russia), and were used without further purification. (S)-2-Amino-5-cyanopentanoic acid was from Genolex (Russia). Deuterium gas was generated by electrolysis by a GC Hydrogen Supply Module (output 6 atm; Himelectronika, Moscow, Russia), using heavy water as a source. Heavy water (²H₂O, D₂O), NaBD₄ and Na¹³CN were from Reakhim (Russia) and Gas-Oil JSC (Russia). DMF was freshly distilled under reduced pressure and stored over 4Å molecular sieves under nitrogen. DCM was always used freshly distilled over CaH₂. THF was distilled over LiAlH₄.

Example 1 - (S)-2-Amino-4-cyano(¹³C)-butyric acid (a precursor for ¹³C-Arg and ¹³C, ²H₂-Arg)

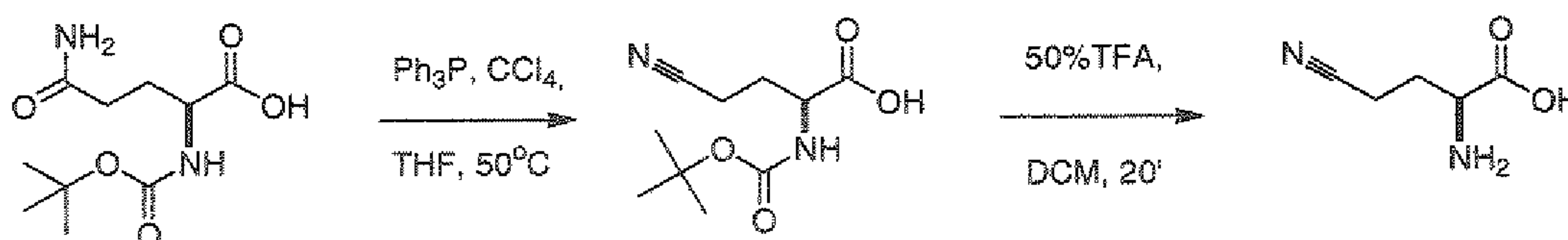


2.19 g (10 mmol) of N-Boc-homo-Serine (Bachem; desiccated overnight over P₂O₅) was dissolved in 10 ml of a mixture of acetonitrile/dimethylformamide (1:1). Dry Na¹³CN (Gas-Oil JSC, Russia; 1 g, 2 eqv) and NaI (10 mg, cat) were added, and the mixture was degassed. Me₃SiCl (2.55 ml, 2 eqv) was then added with a syringe at RT under argon. The reaction mixture was stirred under argon at 60°C for 6 h, with monitoring by TLC (chloroform/methanol 2:1, visualization in iodine vapor). Upon completion, the reaction mixture was cooled to RT, diluted with water (100 ml) and extracted with diethyl ether (2 x 50 ml). The organic phase was washed with water (4 x 50 ml) and brine (50 ml), dried (Na₂SO₄), decanted and concentrated *in vacuo* to yield (2.07 g, 91%) of colorless oil. The structure of the Boc-nitrile was confirmed by MALDI-TOF (Voyager Elite, PerSeptive Biosystems), with HPA as a matrix. Found: 229.115 (M⁺), 230.114 (M⁺ +

H⁺), 252.104 (MI + Na⁺). No peaks related to the starting material were detected.

The removal of the Boc protecting group and the work-up were carried out using a standard peptide synthesis protocol (50% TFA in DCM, 30 min, RT). The structure of the nitrile was confirmed by MALDI-TOF (Voyager Elite, PerSeptive Biosystems), with HPA as a matrix. Found: 129.062 (MI), 130.070 (MI + H⁺). No signal related to the starting material was detected.

Example 2 - (S)-2-Amino-4-cyano-butyric acid (a precursor for ²H₂-Arg)



4.93 g (20 mmol) of N-Boc-L-Glutamine (Sigma) was dissolved in 30 ml of anhydrous THF and added with stirring to a mixture of triphenylphosphine (10.49 g, 40 mmol, Aldrich) and 40 ml of anhydrous tetrachloromethane. The reaction mixture was stirred with gentle heating for 3 h (control by TLC, chloroform/methanol 2:1, visualization in iodine vapour), cooled and the precipitate of triphenylphosphine oxide filtered off. The oil obtained upon evaporation and re-evaporation with an additional 15 ml of THF was diluted with 30 ml of water. The aqueous fraction was saturated with brine, washed with diethyl ether (2 x 20 ml), and acidified to pH 3.5 with sulphuric acid. The product was extracted with ethyl acetate (2 x 20 ml). Combined organic fractions were dried (brine, Na₂SO₄) decanted and evaporated to give 3.46 g (76%) of colorless oil. The structure of the Boc-nitrile was confirmed by MALDI-TOF (Voyager Elite, PerSeptive Biosystems), with HPA as a matrix. Found: 228.114 (MI), 229.114 (MI + H⁺), 251.103 (MI + Na⁺). No peaks related to the starting material were detected.

The removal of the Boc protecting group and the work-up were carried out using a standard peptide synthesis protocol (50% TFA in DCM, 30 min, RT). The structure of the nitrile was confirmed by MALDI-TOF (Voyager Elite, PerSeptive Biosystems), with HPA as a matrix. Found: 128.069 (MI), 129.075 (MI + H⁺). No signal related to the starting material was detected.

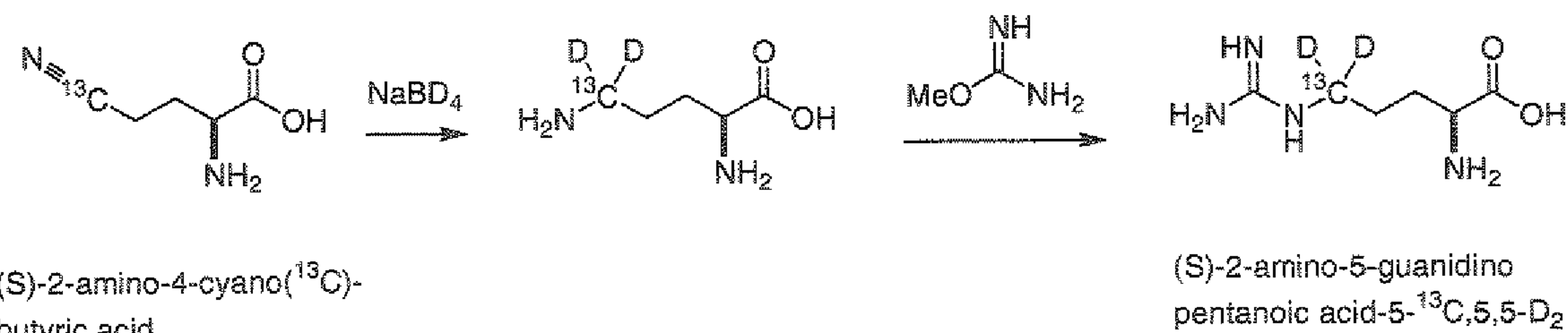
Example 3 - Lys-²H₂



(S)-2-amino-5-cyanopentanoic acid (Genolex, Russia; 14.21 g, 100 mmol) was dissolved in 100 ml of methanol. To this, Raney nickel, prepared from 4 g of alloy (30% Ni) according to (Adkins H. *et al*, *Org. Syntheses*. Coll. Vol. III, 1955, p. 180) was added, and the reaction mixture was shaken under deuterium (100 atm) at 90°C for 24 h. (TLC:

n-butanol-pyridine-acetic acid-water: 15-10-3-12; visualization by iodine vapor and fluorescamine). The reaction mixture was filtered and evaporated in vacuo. The product was redissolved in water-ethanol (3:1; 20 ml) followed by evaporation in vacuo (x 4) and then crystallized from ethylacetate to give 11.55 g (78%) of the deuterated product. The structure of deuterated lysine was confirmed by MALDI-TOF (Voyager Elite, PerSeptive Biosystems), with HPA as a matrix. Found: 148.088 (MI), 149.089 (MI + H⁺).

Example 4 - (5-¹³C, 5,5-²H₂)-Arginine



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The (S)-2-Amino-4-cyano(¹³C)-butyric acid (182 mg, 1.41 mmol) and CoCl₂ x 6H₂O (Aldrich, 670 mg, 2.82 mmol) were dissolved in water (6 ml) and NaBD₄ (Reakhim, Russia; 540 mg, 14.1 mmol) was added in two portions over 20 min. The nitrile was reduced in 30 min (control by TLC: n-butanol-pyridine-acetic acid-water: 15-10-3-12; fluorescamine/UV detection for Boc-protected amino acids, iodine vapor visualisation for unprotected amino acids).

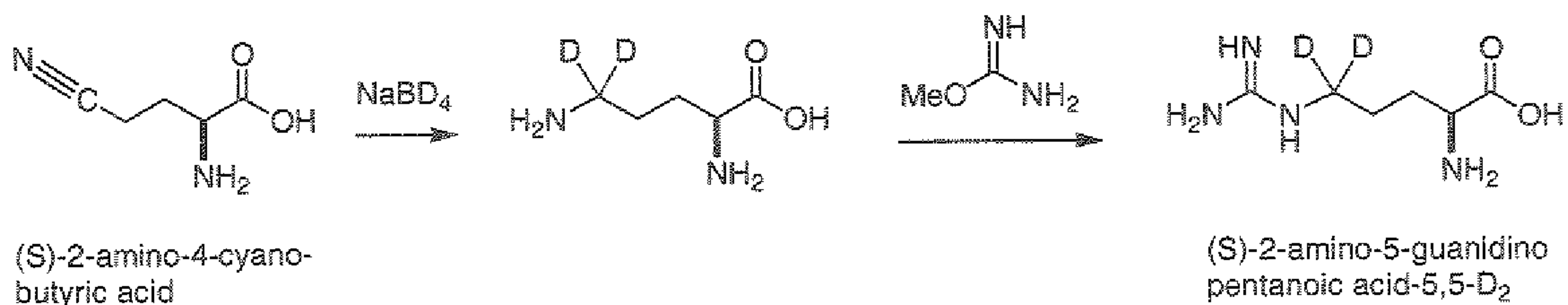
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The reaction mixture was quenched by acidification (1M HCl) followed by acetone, and purified by ion exchange (Amberlite IR120P (H⁺), Aldrich). The column was washed with water till neutral pH. The product was then recovered by washing the column with NH₄OH (0.3 M) followed by evaporation. The resulting ornitine-¹³C, ²H₂ (yield: 158 mg, 83%; MALDI-TOF (Voyager Elite, PerSeptive Biosystems), with HPA matrix. Found: 135.071 (MI), 136.068 (MI + H⁺) was dissolved in water and mixed with an equal volume of 0.5M O-methylisourea (Kimmel, *supra*), pH 10.5, adjusted with NaOH. After 4-5 h 1% TFA was added to stop the reaction (Bonetto *et al*, *supra*). The compound was purified by a RP HPLC (Buffers were A: 0.1% TFA/H₂O; B: 0.1% TFA/ (80%MeCN / 20% H₂O)), 0-65 % B over 40 min to give 140 mg (68%); MALDI-TOF (Voyager Elite, PerSeptive Biosystems), with HPA matrix; found: 177.402 (MI), 178.655 (MI + H⁺).

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Example 5 - (5,5-²H₂)-Arginine

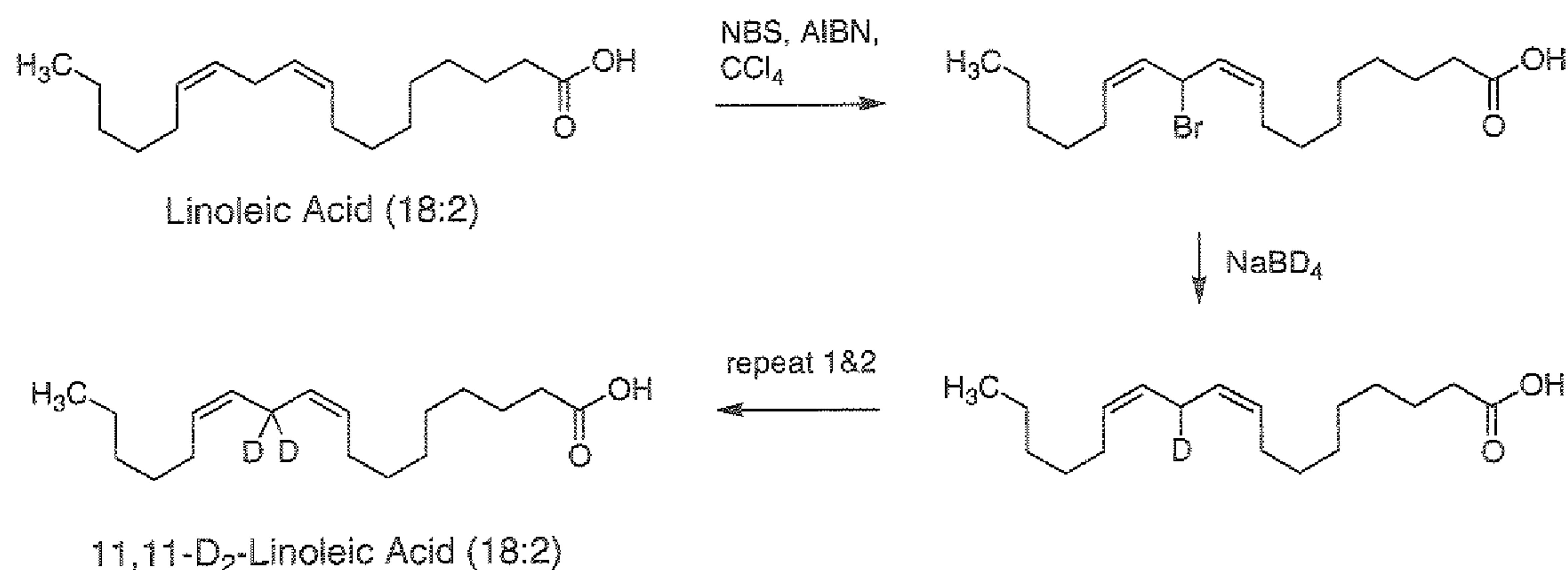


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The title compound was synthesized using the above protocol, starting from (S)-2-amino-4-cyano-butylric acid (Technohim, Russia). MALDI-TOF (Voyager Elite, PerSeptive

Biosystems), with HPA matrix; found: 176.377 (MI), 177.453 (MI + H⁺).

Example 6 - 11,11-di-deutero-linoleic acid (18:2)



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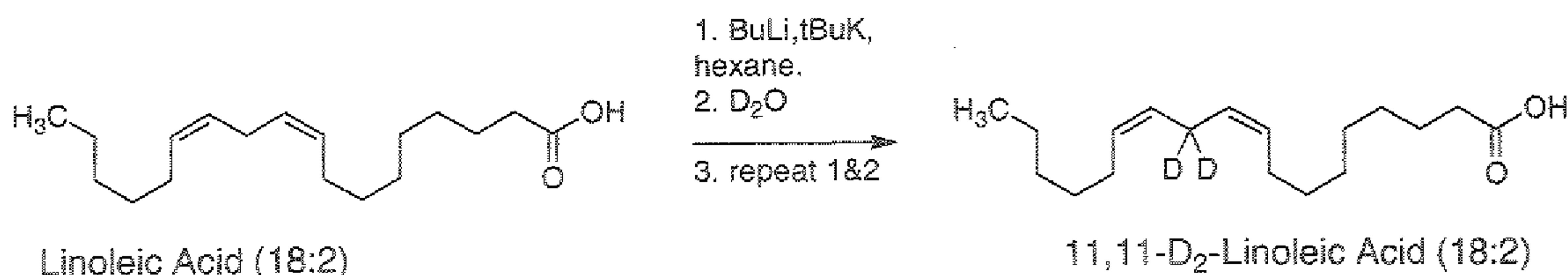
Linoleic acid (7 g, 25 mmol, Aldrich) was dissolved in 25 ml of carbon tetrachloride dried over P₂O₅. N-bromosuccinimide (4.425 g, 25 mmol, desiccated overnight over P₂O₅) and 0.05 g AIBN were added, and the reaction mixture in a flask with a reversed condenser was stirred with gentle heating till the reaction was initiated as manifested by an intense boiling (if the reflux is too intense the heating should be decreased). When succinimide stopped accumulating on the surface, the heating was continued for another 15 min (about 1 h in total). The reaction mixture was cooled to RT and the precipitate filtered off and washed with CCl₄ (2 x 5 ml). The combined organic fractions were evaporated and the 11-Bromolinoleic acid obtained was gradually added to a solution of NaBD₄ (390 mg, 10 mmol) in 30 ml of isopropanol. After an overnight stirring, a diluted solution of HCl was slowly added till there was no more deuterium gas produced. Upon a standard workup, the mono-deuterated acid was brominated and reduced again to yield a target di-deutero derivative (bp 230-231°C/15mm, 4.4 g, 63%). MALDI-TOF MS: mono-bromo derivative, found: 358.202, 360.191 (doublet, approx 1:1, MI); di-deutero derivative, found: 282.251 (MI).

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Example 7



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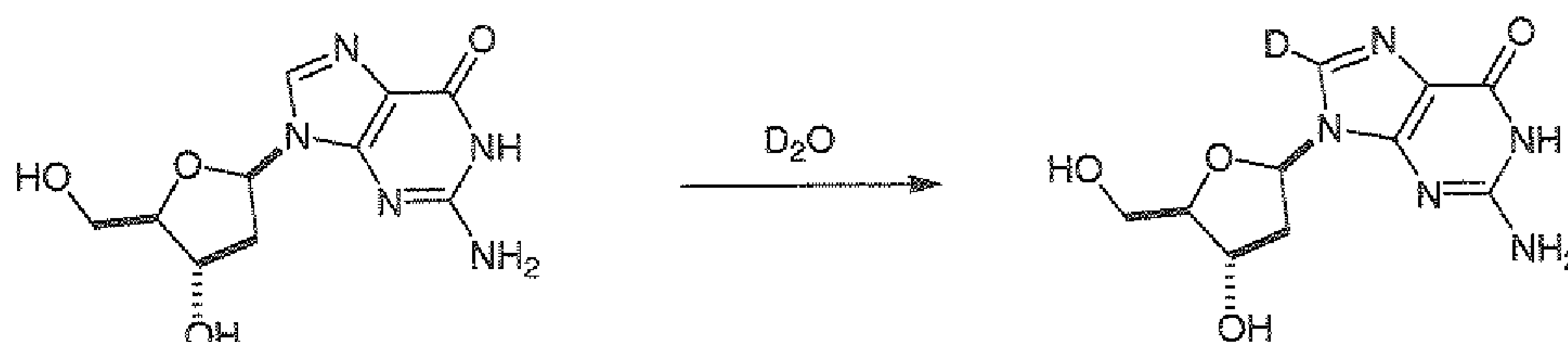
11,11-D₂-Linoleic acid (18:2) was synthesized by treating linoleic acid with an eqv of a BuLi-tBuK (Sigma-Aldrich) mix in hexane followed by quenching with D₂O. To improve yields this procedure needs to be repeated 3-4 times. It was found that this procedure also generates a detectable amount of alpha-deuterated product (FAB MS, Xe ions, thioglycerine: found: 283.34 (72; MI + 1)⁺, 284.33 (11; alpha-

30

monodeuteroderivative, $MI + 1$)⁺, 285.34 (10; alpha-dideuteroderivative, $MI + 1$)⁺; the nature of '284' and '285' peaks was established using MS/MS. The substitution at alpha-position can be prevented by utilizing transient ortho-ester protection (Corey & Raju Tetrahedron Lett. (1983) 24: 5571), but this step makes the preparation more expensive.

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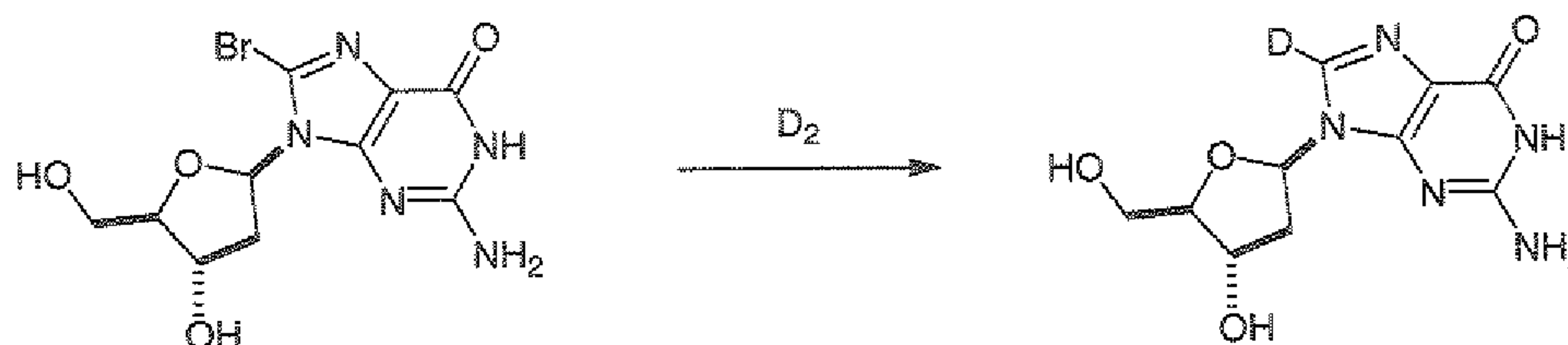
Example 8 - 8-D-Deoxyguanosine from deoxyguanosine



10 Deoxyguanosine (268 mg, 1 mmol, Aldrich) was dissolved in 4 ml of D₂O. 10% Pd/C (27 mg, 10 wt% of the substrate, Aldrich) was added, and the mixture was stirred at 160°C in a sealed tube under H₂ atmosphere for 24 h. After cooling to RT, the reaction mixture was filtered using a membrane filter (Millipore Millex[®]-LG). The filtered catalyst was washed with boiling water (150 ml), and the combined aqueous fractions were evaporated in vacuo to give deoxyguanoside-*d* as a white solid (246 mg, 92 %).
15 The structure of the nucleoside was confirmed by MALDI-TOF (Voyager Elite, PerSeptive Biosystems), with HPA as a matrix. Found: 268.112 (MI).

Example 9 - 8-D-deoxyguanosine from 8-bromodeoxyguanosine

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7% Pd/C catalyst, prepared from PdCl₂ as described in Chiriac *et al* (1999) 42: 377-385, was added to a solution of 8-bromodeoxyguanosine (Sigma) and NaOH in water. The mixture was stirred in D₂ (2 atm) at 30°C. The catalyst was filtered off and the reaction mixture was neutralized with 2N HCl. The procedure provides approx. 85-90% yield of the product. Other reducing agents can be employed, such as NaBD₄ (see the synthesis of D,D-linoleic acid).
25

30 The following Examples 10 to 12 illustrate the utility of the invention. In order to establish a range of a potential heavy isotope substitutions for the invention (from 100% light isotope to 100% heavy isotope, as well as the localized site protection such as that shown in Figs. 1-4, using compounds as shown above), and to test for a possible toxicity of large amounts of heavy isotopes on an organism, the influence of heavy carbon (¹³C) and specifically 'protected' building blocks of biopolymers (nucleic acid components (nucleosides), lipids and amino acids) on the life span was tested on a nematode *Caenorhabditis elegans*.
35

Previous studies of the model organism *C. elegans* have almost exclusively employed cultivation on a bacterial diet. Such cultivation introduces bacterial metabolism as a secondary concern in drug and environmental toxicology studies (specific metabolite-deficient bacterial strains can be employed to evaluate the influence of particular essential nutrients on the nematode longevity). Axenic cultivation of *C. elegans* can avoid these problems, yet some earlier work suggests that axenic growth is unhealthy for *C. elegans*. (Szewczyk *et al*, *Journal of Experimental Biology* 209, 4129-4139 (2006)). For the present invention, both NGM and axenic diets were employed in combination with isotopically enriched nutraceutical components.

Example 10

¹³C₆-glucose (99% enrichment; Sigma) was used as a carbon food source for culturing of *Escherichia coli*; the control was identical except for the ¹²C₆-glucose. *C. elegans* (N2, wild type) were grown on a standard (peptone, salts and cholesterol) media seeded with *Escherichia coli* prepared as described above. The only carbon-containing component apart from *E. coli* was ¹²C-cholesterol (Sigma; a hormone precursor that is essential for *C. elegans*), since the corresponding ¹³C-derivative was unavailable. Nematodes were thus grown on a 'heavy' and 'light' (control) diet in the temperature range of 15-25°C, in pools of 50-100 worms each. The animals on both diets developed normally with all major characteristics being very similar.

The longevity data was analyzed using Prism software package (GraphPad software, USA), according to published procedures (Larsen *et al*, *Genetics* 139: 1567 (1995)). It was found that animals on the 'heavy' diet have an increase of a lifespan of around 10% (in a typical experiment, 14 days for ¹²C animals versus about 15.5 days for the ¹³C-fed worms, for 25°C).

Example 11

Basic composition of the axenic media used was adapted from (Lu & Goetsch *Nematologica* (1993) 39: 303-311). Water-soluble and TEA-soluble components (vitamins and growth factors), salts, non-essential amino acids, nucleic acid substituents, other growth factors and the energy source were prepared as described (0.5L of 2x). To this, a mix of essential amino acids was added, containing (for 0.5L as 2x): 0.98g L-(D₂)-Arg (see above); 0.283g L-Hys; 1.05g L-(D₂)-Lys (see below); 0.184g L-Trp; 0.389g L-Met; 0.717g L-Thr; 1.439g L-Leu; 0.861g L-Ile; 1.02g L-Val, and 0.623g L-Phe. Prior to adding to the remaining components, this mixture was stirred at 55°C for 4 hours until a clear solution was formed, and then cooled to room temperature.

C. elegans (N2, wild type) were cultivated on this medium. For the control experiment, nematodes were grown on a medium prepared as above but containing standard L-Arg and L-Lys instead of the deuterated analogues, in the temperature range of 15-25°C, in pools of 50-100 worms each. The longevity data was analyzed using Prism software, as described in Example 10.

Example 12

5 A ^{12}C -NGM diet was enriched with 5,5-di-deutero-arginine and 6,6-di-deutero-lysine, 11,11-di-deutero-linoleic acid (18:2), and 8-D-deoxyguanosine. *C. elegans* were grown on a standard (peptone, salts and cholesterol) medium seeded with *Escherichia coli* prepared as described above, to which deuterium-‘reinforced’ derivatives (see above) were added, to a total concentration of 1g/L of each deuterated compound. Nematodes were thus grown on a ‘heavy’ and ‘light’ (control- whereby non-deuterated L-Arginine, L-Lysine, linoleic acid (18:2), and deoxyguanosine were used instead of deuterated analogues in 1g/L concentrations) diet in the temperature range of 15-25°C, in pools of 10 50-100 worms each. The longevity data was analyzed using Prism software package, as described in Example 10.

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CLAIMS:

1. An isotopically modified component for use in the preparation of a nutrient composition for reducing biomolecule oxidation in a human subject, wherein the isotopically modified component comprises a fatty acid or fat, nucleic acid or nucleotide, or amino acid that is isotopically modified at an oxidation-sensitive position at a level above the naturally-occurring abundance level of said isotopically modified component in said composition; wherein the isotopically modified component is for use in regular administration to the subject and the isotopically modified component or metabolite thereof is for incorporation into the subject's body to reduce the extent of biomolecule oxidation in the subject; and wherein the isotope of the isotopic modification is deuterium, carbon-13, or combinations thereof.
2. The isotopically modified component of claim 1, wherein the isotopically modified component is at least about 0.1% of the subject's diet.
3. The isotopically modified component of any one of claims 1-2 wherein the biomolecule oxidation is mediated by free radicals, reactive oxygen species, reactive nitrogen species, or radiation in the subject.
4. The isotopically modified component of any one of claims 1-3 wherein the biomolecule oxidation is carbonylation of a biomolecule *in vivo*.
5. The isotopically modified component of any one of the claims 1-4 wherein the isotopically modified component is a fatty acid or fat, nucleic acid or nucleotide, or amino acid that is not degraded by enzymes of the P450 pathway.
6. The isotopically modified component of any one of claims 1-5, for use in the treatment of an age related disease.
7. The isotopically modified component of any one of claims 1-6, wherein the isotopically modified component is an essential food component or conditionally essential food component.

8. The isotopically modified component of any one of claims 1-6, wherein the isotopically modified component is a non-essential food component.
9. The isotopically modified component of any one of claims 1-8, wherein the isotopically modified component is suitable for administration with non-isotopically modified essential food components.
10. The isotopically modified component of claim 9, wherein the ratio of isotopically modified component to non-isotopically modified component is greater than 1:1.
11. The isotopically modified component of any one of claims 1-10, wherein the isotopically modified component results in the accumulation of said isotopically modified component in the subject.
12. The isotopically modified component of any one of claims 1-11, wherein the isotope replaces an oxidation-sensitive hydrogen atom, a carbon atom possessing an oxidation-sensitive hydrogen atom, or both an oxidation-sensitive hydrogen atom and a carbon atom possessing an oxidation-sensitive hydrogen atom.
13. The isotopically modified component of any one of claims 1-12, wherein the isotopically modified component comprises two or more of an isotopically modified fatty acid or fat, nucleic acid or nucleotide, or amino acid.
14. The isotopically modified component of any one of claims 1-13, wherein the isotopically modified component is a fatty acid or fat and the biomolecule is a lipid.
15. The isotopically modified component of claim 14, wherein the fatty acid or fat is polyunsaturated.
16. The isotopically modified component of claim 15, wherein the fatty acid or fat is an omega-3 or omega-6 fatty acid.
17. The isotopically modified component of any one of claims 14-15, wherein the fatty acid or fat is isotopically modified at one or more bis-allylic positions.

18. The isotopically modified component of claim 17, wherein the fatty acid or fat is isotopically modified at one bis-allylic position.
19. The isotopically modified component of any one of claims 14-18, wherein the fatty acid is 11-D-linoleic acid or 11,11-D₂-linoleic acid.
20. The isotopically modified component of claim 19, wherein the fatty acid is 11,11-D₂-linoleic acid.
21. The isotopically modified component of any one of claims 14-20, wherein the fatty acid is a carboxylic acid.
22. The isotopically modified component of any one of claims 14-21, wherein the lipid oxidation is mediated by free radicals.
23. The isotopically modified component of any one of claims 1-13, wherein the isotopically modified component is a nucleic acid or nucleotide and the biomolecule is DNA.
24. The isotopically modified compound of claim 23, wherein the isotopically modified component is a purine nucleoside.
25. The isotopically modified compound of claim 23, wherein the isotopically modified component is a pyrimidine nucleoside.
26. The isotopically modified compound of any one of claims 23-25, wherein the isotopic substitution is present on the base of the nucleic acid or nucleotide.
27. The isotopically modified compound of any one of claims 23-26, wherein the isotopically modified component reduces the formation of 8-oxy-guanine.
28. The isotopically modified component of claim 23, wherein the isotopically modified component is 8-D-deoxyguanosine or 8-D-8-¹³C-deoxyguanosine.

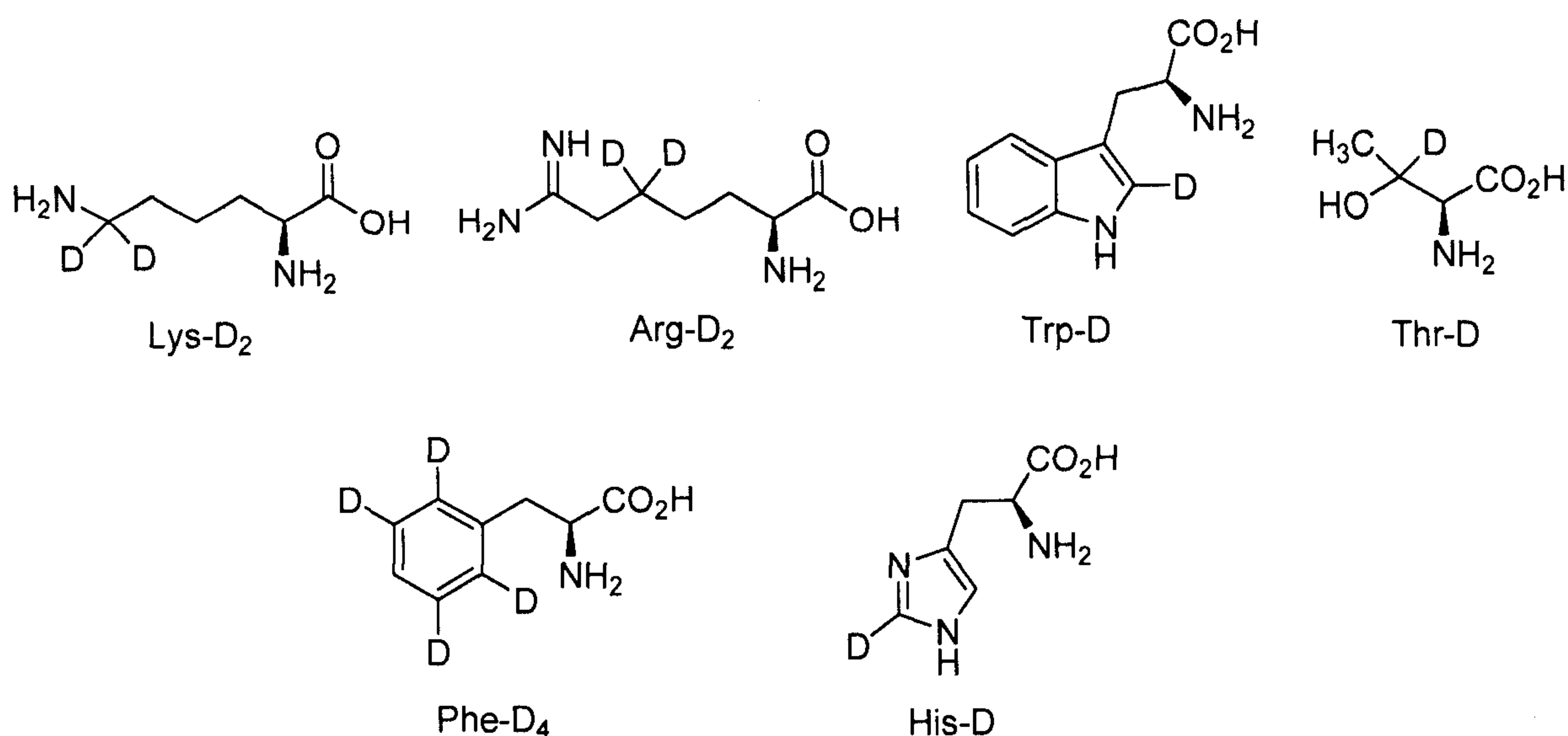
29. The isotopically modified compound of any one of claims 23-24 or claims 26-28, wherein the isotopically modified component reduces the formation of 8-oxy-guanine in the subject's mitochondria.

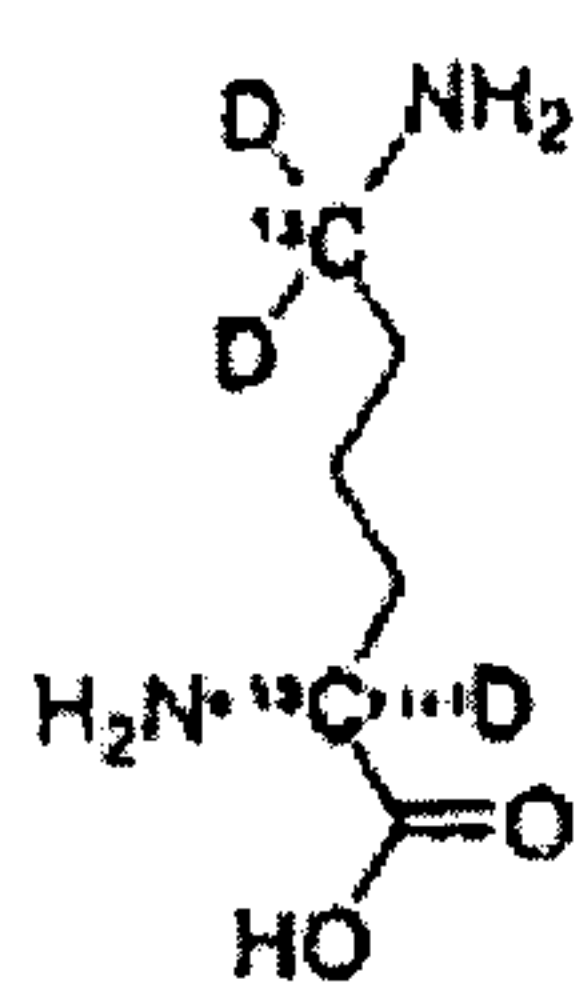
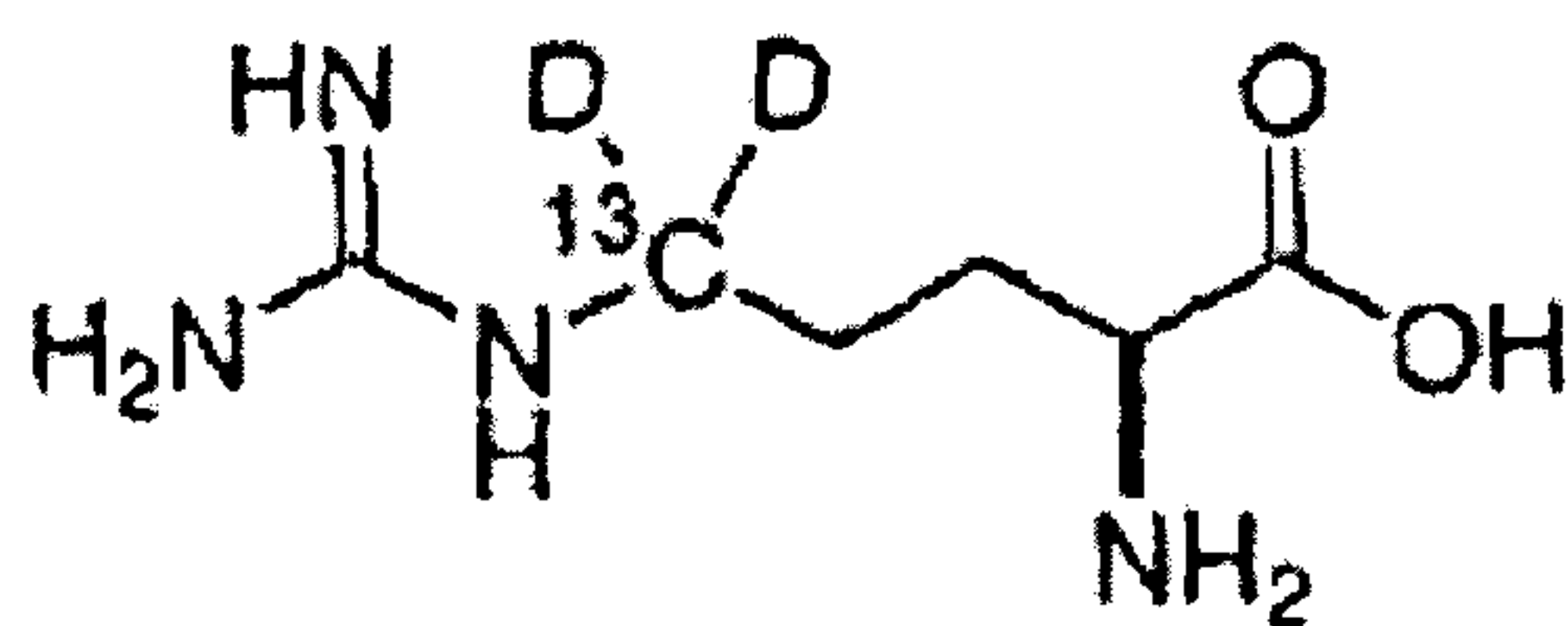
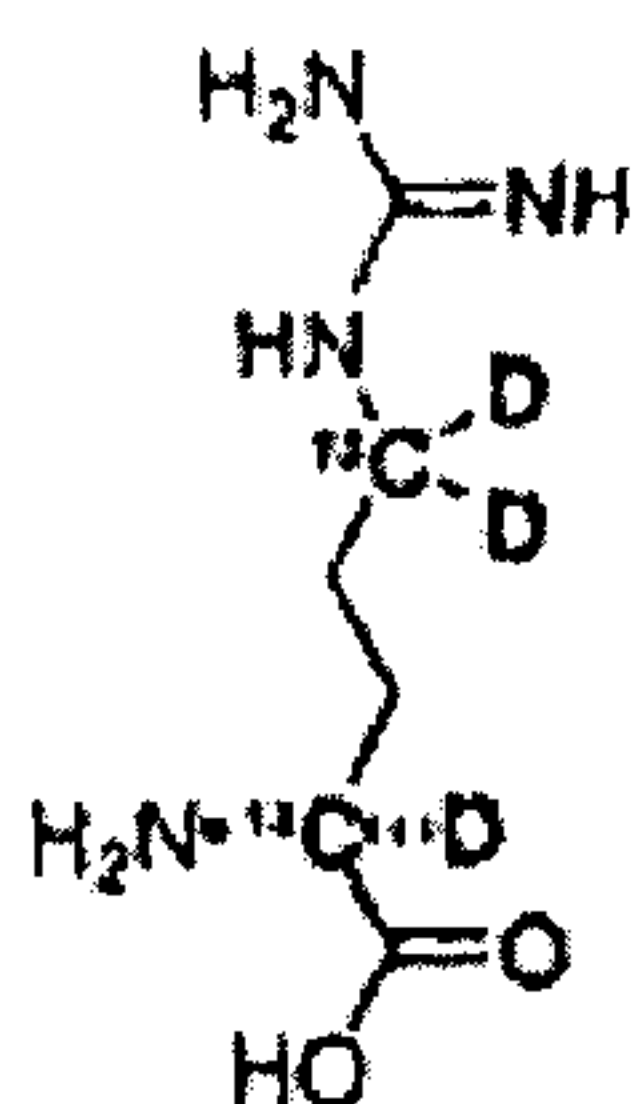
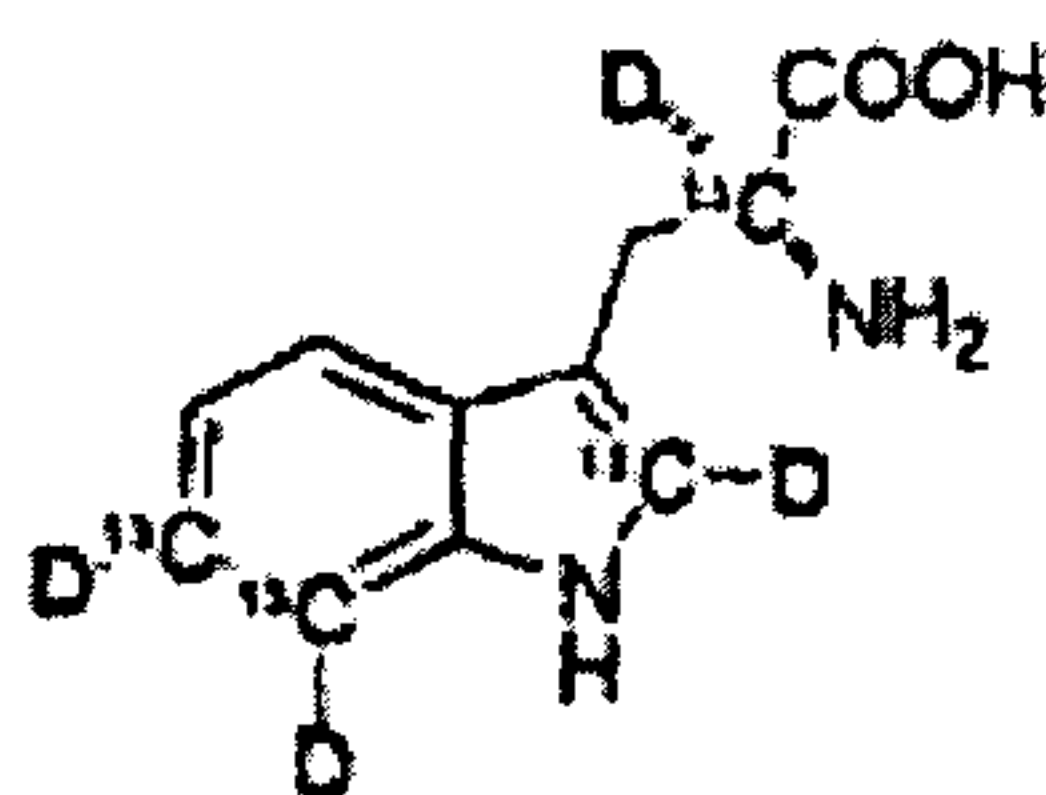
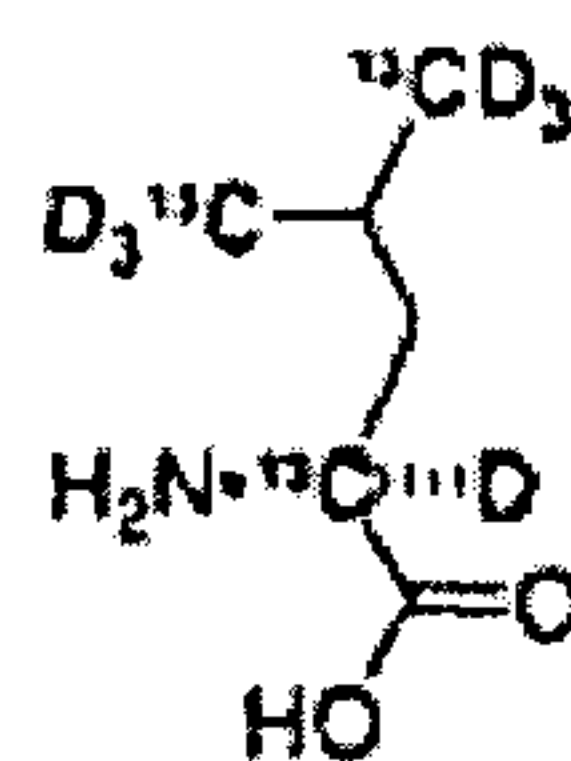
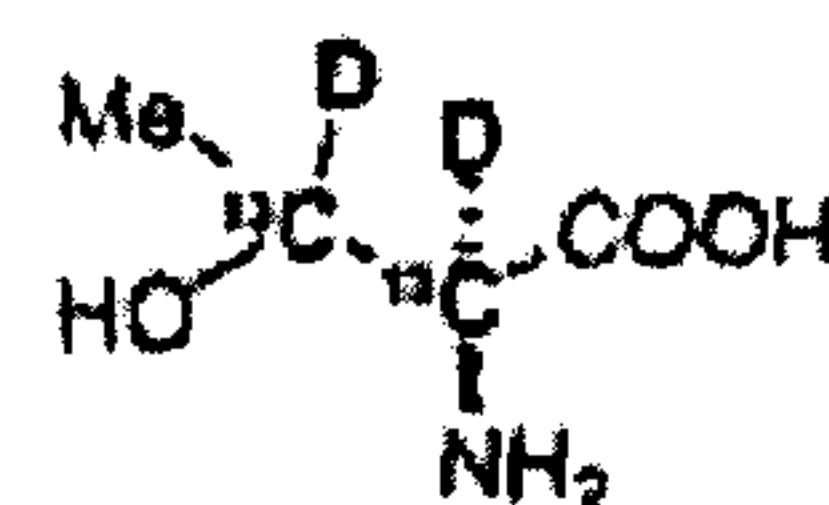
30. The isotopically modified component of any one of claims 1-13, wherein the isotopically modified component is an amino acid and the biomolecule is a protein.

31. The isotopically modified component of claim 30, wherein the amino acid is selected from the group consisting of phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine, lysine, and arginine, and wherein the amino acid is isotopically modified at an oxidation-sensitive position of the amino acid side chain.

32. The isotopically modified component of claim 31, wherein the amino acid is selected from the group consisting of phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine, lysine, and arginine, and wherein the amino acid is isotopically modified at the alpha carbon position.

33. The isotopically modified component of any one of claims 30-32, wherein the amino acid is selected from the group consisting of



Lys-D₃¹³C₂Arg-D₃¹³C₂Trp-D₄¹³C₄Val-D₃¹³C₃Thr-D₂¹³C₂

and (S) -5,5-D₂-2,5-diaminopentanoic acid.

34. The isotopically modified component of any one of claims 30-33, wherein the amino acid is lysine or arginine and the ω -atoms of the amino acid are isotopically modified.

35. The isotopically modified component of claim 34, wherein the isotopic modification is deuterium.

36. The isotopically modified component of claim 34, wherein the isotopic modification is carbon-13.

37. The isotopically modified component of claim 34, wherein the isotopic modification is both deuterium and carbon-13.

38. The isotopically modified component of any one of claims 30-32, wherein the amino acid is selected from the group consisting of (S)-6-D-2,6-diaminohexanoic acid; (S)-6,6-D₂-2,6-diaminohexanoic acid; (S)-6-¹³C-2,6-diaminohexanoic acid; (S)-6-D-6-¹³C-2,6-diaminohexanoic acid; (S)-6,6-D₂-6-¹³C-2,6-diaminohexanoic acid; (S)-2-amino-5-D-5-guanidino-pentanoic acid; (S)-2-amino-5,5-D₂-5-guanidino-pentanoic acid; (S)-2-amino-5-¹³C-5-guanidino-pentanoic acid;

(S)-2-amino-5-D-5-¹³C-5-guanidino-pentanoic acid; (S)-2-amino-5,5-D₂-5-¹³C-5-guanidino-pentanoic acid; (S)-2-amino-3-(1*H*-indol-2-D-3-yl)propanoic acid; (S)-2-amino-3-(1*H*-indol-2-¹³C-3-yl)propanoic acid; (S)-2-amino-3-(1*H*-indol-2-D-2-¹³C-3-yl)propanoic acid; (S)-2-amino-3-(1*H*-indol-6-D-3-yl)propanoic acid; (S)-2-amino-3-(1*H*-indol-6-¹³C-3-yl)propanoic acid; (S)-2-amino-3-(1*H*-indol-6-D-6-¹³C-3-yl)propanoic acid; (S)-2-amino-3-D-3-hydroxybutanoic acid; (S)-2-amino-3-¹³C-3-hydroxybutanoic acid; (S)-2-amino-3-D-3-¹³C-3-hydroxybutanoic acid; (S)-pyrrolidine-5-D-2-carboxylic acid; (S)-pyrrolidine-5,5-D₂-2-carboxylic acid; (S)-pyrrolidine-5-¹³C-2-carboxylic acid; (S)-pyrrolidine-5-D-5-¹³C-2-carboxylic acid; (S)-pyrrolidine-5,5-D₂-5-¹³C-2-carboxylic acid; (S)-2-amino-3-(2-D-phenyl)propanoic acid; (S)-2-amino-3-(2-¹³C-phenyl)propanoic acid; (S)-2-amino-3-(2-D-2-¹³C-phenyl)propanoic acid; (S)-2-amino-3-(3-D-phenyl)propanoic acid; (S)-2-amino-3-(3-¹³C-phenyl)propanoic acid; (S)-2-amino-3-(3-D-3-¹³C-phenyl)propanoic acid; (S)-2-amino-3-(2-D-1*H*-imidazol-4-yl)propanoic acid; (S)-2-amino-3-(2-¹³C-1*H*-imidazol-4-yl)propanoic acid; and (S)-2-amino-3-(2-D-2-¹³C-1*H*-imidazol-4-yl)propanoic acid.

39. A use of an isotopically modified component for significantly increasing the resistance of a subject to a detrimental degradative process, wherein the isotopically modified component comprises a fatty acid, fat, nucleic acid or nucleotide, or amino acid that is isotopically modified at an oxidation-sensitive position at a level above the naturally-occurring abundance level of said isotopically modified component; wherein the isotopically modified component is for use in regular administration to the subject and the isotopically modified component or metabolite thereof is for incorporation into the subject's body to reduce the extent of biomolecule oxidation in the subject; and wherein the isotope of the isotopic modification is deuterium, carbon-13, or combinations thereof.

40. The use of claim 39, wherein the subject is a patient having a condition associated with oxidative stress or age-related diseases.

41. The use of any one of claims 39-40 wherein the resistance of a degradative process is mediated by reactive oxygen species, reactive nitrogen species, or radiation in the subject.

42. The use of any of the claims 39-41 wherein the degradative process is detrimental oxidation of a biomolecule *in vivo*.

43. The use of claim 42 wherein the detrimental oxidation of a biomolecule is carbonylation of an amino acid *in vivo*.

44. The use of any one of claims 39-43 wherein the isotopically modified component is in a compound that is not degraded by enzymes of the P450 pathway.

45. A composition comprising:

an isotopically modified component,

wherein the isotopically modified component comprises a fatty acid, fat, nucleic acid or nucleotide, or amino acid that is isotopically modified at an oxidation-sensitive position at a level above the naturally-occurring abundance level of said isotopically modified component in said composition; wherein the composition is for use in regular administration to the subject and the isotopically modified component or metabolite thereof is for incorporation into the subject's body to reduce the extent of biomolecule oxidation in the subject; and wherein the isotope of the isotopic modification is deuterium, carbon-13, or combinations thereof.

46. A use of an isotopically modified component for reducing lipid oxidation,

wherein the isotopically modified component is contained in a foodstuff and the isotopically modified component is at a level above the naturally-occurring abundance level of said isotopically modified component in said foodstuff;

wherein the isotopically modified component is a fatty acid or fat that is isotopically modified at one or more bis-allylic positions;

wherein the isotopically modified component is at least 0.1% of a patient's diet;

wherein the isotopically modified component is for use in regular administration to a patient; and

wherein the isotopically modified component or metabolite thereof is for incorporation into the patient's body following administration.

47. The use of claim 46, wherein the lipid oxidation comprises an oxidation mediated by free radicals, reactive oxygen species, reactive nitrogen species, or radiation in the subject.

48. The use of claim 46 or 47, wherein the isotopically modified component is in a fatty acid or fat that is not degraded by enzymes of the P450 pathway.

49. The use of any one of claims 46 to 48, wherein the isotopically modified component or metabolite thereof is a fatty acid or fat that is an essential food component.

50. The use of any one of claims 46 to 49, wherein the fatty acid or fat is isotopically modified at one bis-allylic position.

51. The use of any one of claims 46 to 50, wherein the fatty acid, or fat is 11-D-linoleic acid or 11,11-D2-linoleic acid.

52. The use of claim 51, wherein the fatty acid or fat is 11,11-D2-linoleic acid.

53. The use of claim 51, wherein the fatty acid or fat is suitable for administration with non-isotopically modified essential food components.

54. The use of claim 53, wherein the ratio of isotopically modified fatty acid or fat to non-isotopically modified fatty acid is greater than 1:1.

55. The use of any one of claims 46 to 54, wherein the isotopic modification comprises at least one ¹³C atom or at least one deuterium atom at the one or more bis-allylic positions, wherein the at least one ¹³C atom or the at least one deuterium atom are present at a level greater than the naturally-occurring abundance level of said isotope in said fatty acid fat.

56. The use of claim 55, wherein the fatty acid, or fat is 11-D-linoleic acid or 11,11-D2-linoleic acid.

57. The use of claim 56, wherein the fatty acid, or fat is 11,11-D2-linoleic acid.

58. The use of claim 57, wherein the fatty acid, or fat is a polyunsaturated substance.
59. The use of any one of claims 46 to 58, wherein the fatty acid or fat is 11-D-linoleic acid or 11,11-D2-linoleic acid.
60. The use of claim 59, wherein the fatty acid, or fat, is 11,11-D2-linoleic acid.
61. The use of claim 47, wherein the lipid oxidation is mediated by free radicals.
62. The use of any one of claims 46 to 61, wherein the fatty acid is a carboxylic acid.
63. A foodstuff for use in reducing lipid oxidation in a patient, comprising an isotopically modified component;
- wherein the isotopically modified component is a fatty acid, or fat that is isotopically modified at one or more bis-allylic positions; and
- wherein the amount of the isotopically modified component is at least 0.1% of the foodstuff.
64. The foodstuff of claim 63, wherein the amount of the isotopically modified component is sufficient to significantly reduce lipid oxidation mediated by free radicals, reactive oxygen species, reactive nitrogen species, or radiation in the subject.
65. The foodstuff of claim 63 or 64, wherein the amount of the isotopically modified component is sufficient to significantly reduce lipid oxidation mediated by free radicals.
66. The foodstuff of any one of claims 63 to 65, wherein the isotopically modified component resists degradation by enzymes of the P450 pathway.
67. The foodstuff of any one of claims 63 to 66, wherein the isotopically modified component or metabolite thereof is a fatty acid or fat that is an essential food component.
68. The foodstuff of any one of claims 63 to 67, wherein the fatty acid or fat is isotopically modified at one bis-allylic position.

69. The foodstuff of claim 68, wherein the fatty acid is 11-D-linoleic acid or 11,11-D2-linoleic acid.
70. The foodstuff of claim 69, wherein the fatty acid is 11,11-D2-linoleic acid.
71. The foodstuff of any one of claims 63 to 70, further comprising non-isotopically-modified essential nutrients.
72. The foodstuff of claim 71, wherein the ratio of the fatty acid or fat that is isotopically modified to the same fatty acid or fat that is non-isotopically modified fatty acid is greater than 1:1.
73. The foodstuff of any one of claims 63 to 72, wherein the isotopic modification comprises at least one ^{13}C atom or at least one deuterium atom at the one or more bis-allylic positions, wherein the at least one ^{13}C atom or the at least one deuterium atom are present at a level greater than the naturally-occurring abundance level of said isotope in said fatty acid.
74. The foodstuff of claim 73, wherein the isotopically modified fatty acid is 11-D-linoleic acid or 11,11-D2-linoleic acid.
75. The foodstuff of claim 74, wherein the isotopically modified fatty acid is 11-D-linoleic acid.
76. The foodstuff of any one of claims 63 to 75, wherein the fatty acid is a polyunsaturated substance.
77. The foodstuff of any one of claims 63 to 76, wherein the isotopically modified fatty acid is 11-D-linoleic acid or 11,11-D2-linoleic acid.
78. The foodstuff of claim 77, wherein the isotopically modified fatty acid is 11-D-linoleic acid.
79. The foodstuff of any one of claims 63 to 78, wherein the fatty acid is a carboxylic acid.

80. The foodstuff of any one of claims 63 to 79, wherein the foodstuff is suitable for human consumption.

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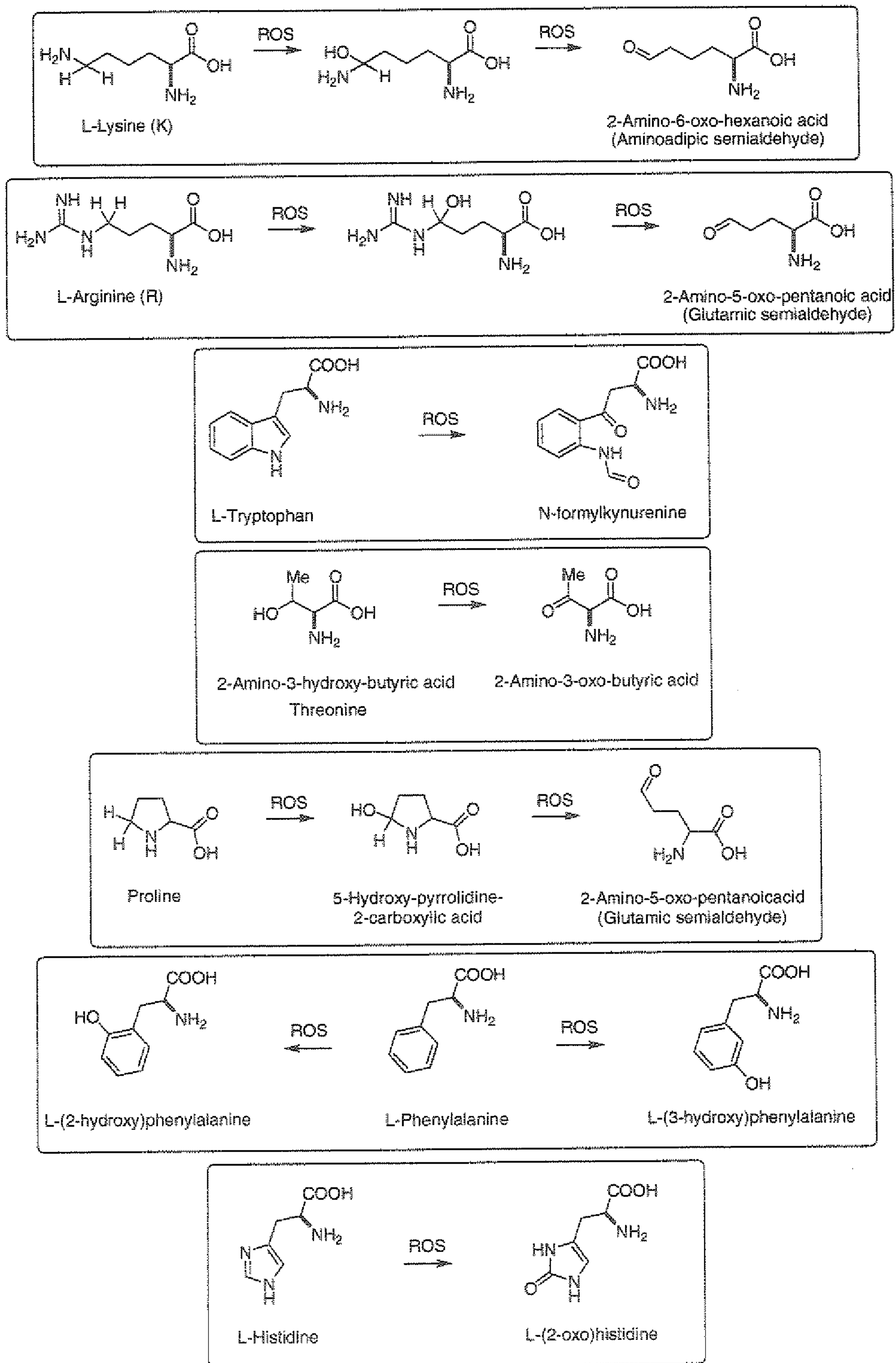


Figure 1

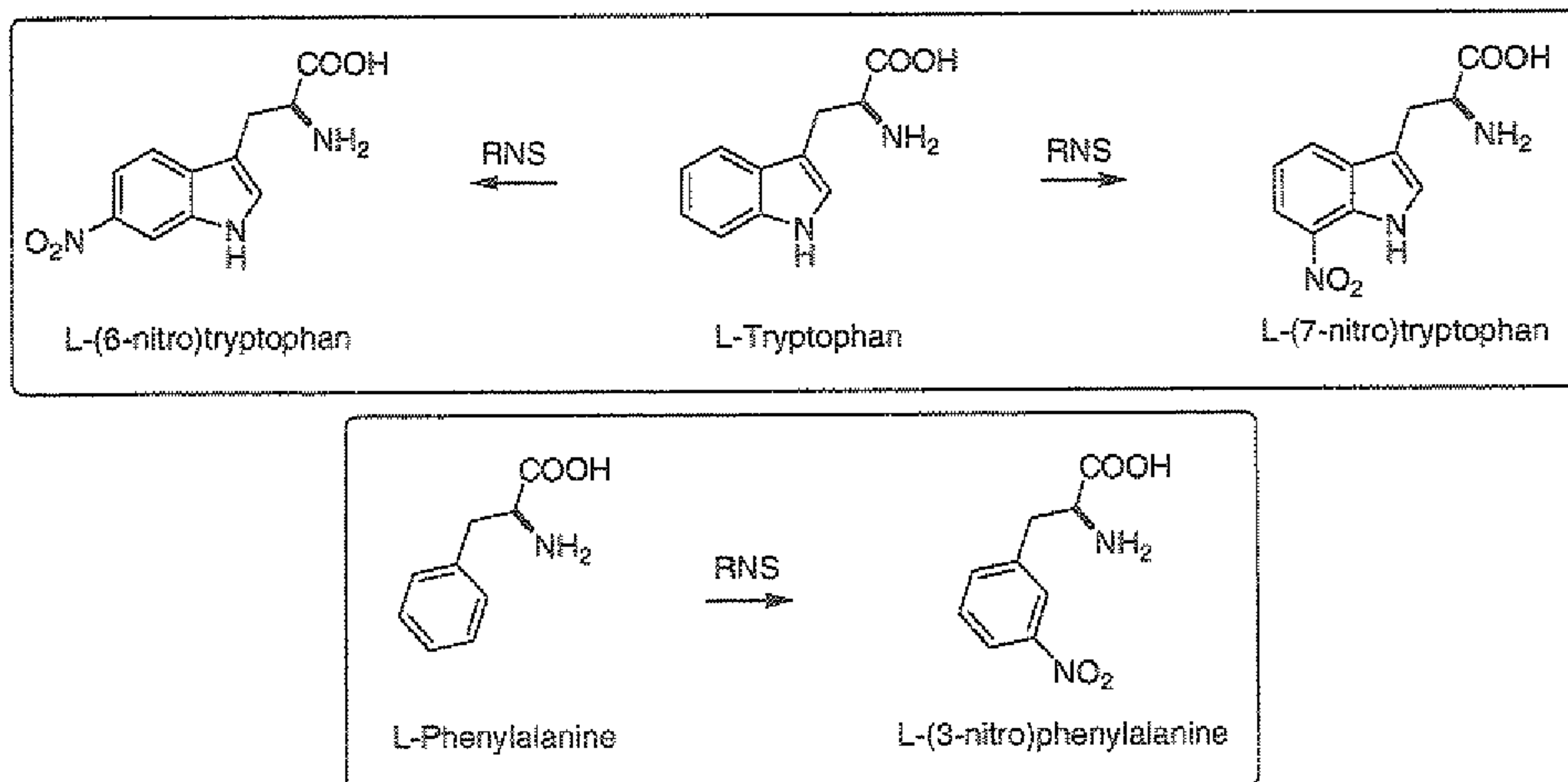


Figure 2

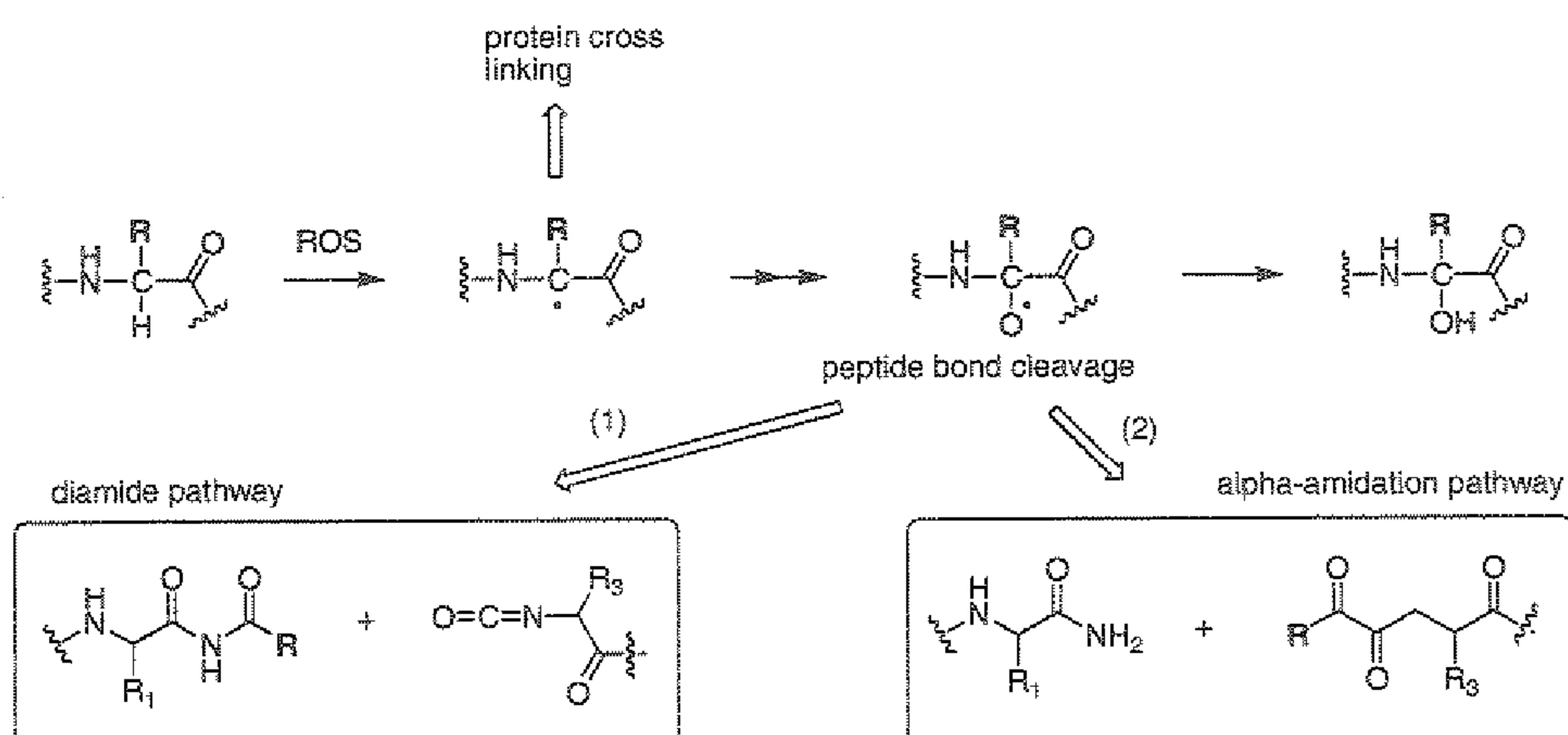


Figure 3

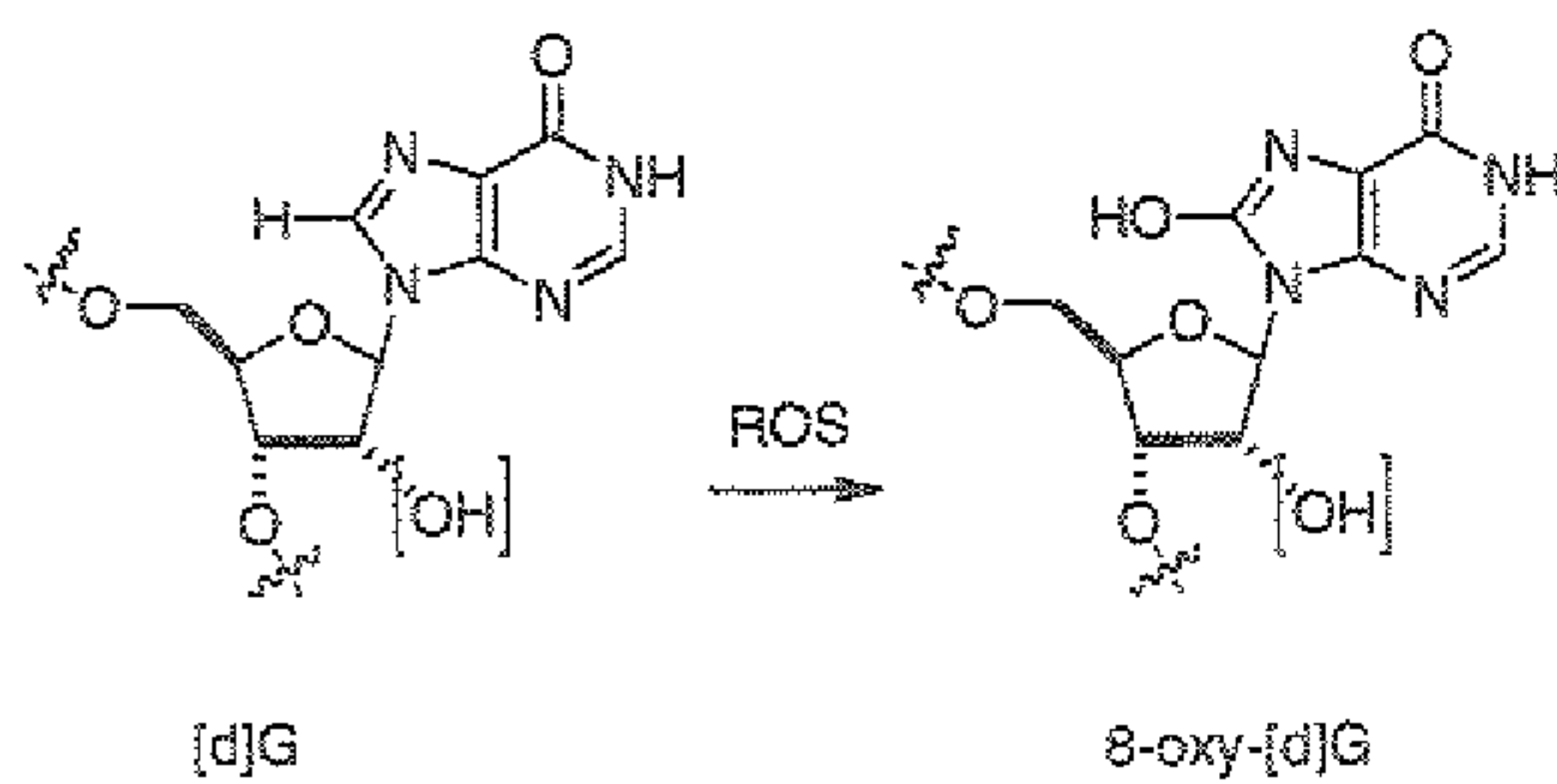


Figure 4

