COMPOSITIONS OF PROKARYOTIC PHENYLALANINE AMMONIA-LYASE AND METHODS OF USING SAID COMPOSITIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of United States Application Number 11/807,227, filed May 25, 2007, which is herein incorporated by reference in its entirety.

TECHNICAL FIELD OF INVENTION

Provided herein are prokaryotic phenylalanine ammonia-lyase (PAL) and compositions thereof, and optimization of such compositions to enhance PAL catalytic activity and stability while reducing immunogenicity and/or proteolytic sensitivity of PAL. Also provided herein is the use of such optimal compositions of PAL for therapeutic and industrial purposes.

BACKGROUND OF THE INVENTION

PAL is a non-mammalian enzyme widely distributed in plants (Koukol, *et al.*, J. Biol. Chem. 236:2692-2698 (1961); Hanson, *et al.*, The Enzymes 7:75-166 (1972); Poppe, *et al.*, Curr. Org. Chem. 7:1297-1315 (2003)), some fungi (Rao, *et al.*, Can. J. Biochem. 4512:1863-1872 (1967); Abell, *et al.*, Methods Enzymol. 142:242-253 (1987)) and bacteria (Bezanson, *et al.*, Can. J. Microbiol. 16:147-151. (1970); Xiang, et al, J. Biol. Chem. 277:32505-32509 (2002); Hill, *et al.*, Chem. Commun. 1358-1359 (2003)) and can be recombinantly produced in Escherichia coli.

A representative list of PALs includes: Q9ATN7 Agastache rugosa; O93967 Amanita muscaria (Fly agaric); P35510, P45724, P45725, Q9SS45, Q8RWP4 Arabidopsis thaliana (Mouse-ear cress); Q6ST23 Bambusa oldhamii (Giant timber bamboo); Q42609 Bromheadia finlaysoniana (Orchid); P45726 Camellia sinensis (Tea); Q9MAX1 Catharanthus roseus (Rosy periwinkle) (Madagascar periwinkle); Q9SMK9 Cicer arietinum (Chickpea); Q9XFX5, Q9XFX6 Citrus clementina x Citrus reticulate; Q42667 Citrus limon (Lemon); Q8H6V9, Q8H6W0 Coffea canephora (Robusta coffee); Q852S1 Daucus carota (Carrot); O23924) Digitalis lanata (Foxglove); O23865) Daucus carota (Carrot); P27991 Glycine max (Soybean); O04058) Helianthus annuus (Common sunflower); P14166, (Q42858) Ipomoea

batatas (Sweet potato); Q8GZR8, Q8W2E4 Lactuca sativa (Garden lettuce); O49835, O49836 Lithospermum erythrorhizon; P35511, P26600 Lycopersicon esculentum (Tomato); P35512 Malus domestica (Apple) (Malus sylvestris); Q94C45, Q94F89 Manihot esculenta (Cassava) (Manioc); P27990 Medicago sativa (Alfalfa); P25872, P35513, P45733 Nicotiana tabacum (Common tobacco); Q6T1C9 Quercus suber (Cork oak); P14717, P53443, Q7M1Q5, Q84VE0, Q84VE0 Oryza sativa (Rice); P45727 Persea americana (Avocado); Q9AXI5 Pharbitis nil (Violet) (Japanese morning glory); P52777 Pinus taeda (Loblolly pine); Q01861, Q04593 Pisum sativum (Garden pea); P24481, P45728, P45729 Petroselinum crispum (Parsley) (Petroselinum hortense); Q84LI2 Phalaenopsis x Doritaenopsis hybrid cultivar; P07218, P19142, P19143 Phaseolus vulgaris (Kidney bean) (French bean); Q7XJC3, Q7XJC4 Pinus pinaster (Maritime pine); Q6UD65 Populus balsamifera subsp. trichocarpa x Populus deltoides; P45731, Q43052, O24266 Populus kitakamiensis (Aspen); Q8H6V5, Q8H6V6 Populus tremuloides (Quaking aspen); P45730 Populus trichocarpa (Western balsam poplar); O64963 Prunus avium (Cherry); Q94EN0 Rehmannia glutinosa; P11544 Rhodosporidium toruloides (Yeast) (Rhodotorula gracilis); P10248 Rhodotorula rubra (Yeast) (Rhodotorula mucilaginosa); Q9M568, Q9M567 Rubus idaeus (Raspberry); P31425, P31426 Solanum tuberosum (Potato); Q6SPE8 Stellaria longipes (Longstalk starwort); P45732 Stylosanthes humilis (Townsville stylo); P45734 Trifolium subterraneum (Subterranean clover); Q43210, Q43664 Triticum aestivum (Wheat); Q96V77 Ustilago maydis (Smut fungus); P45735 Vitis vinifera (Grape); and Q8VXG7 Zea mays (Maize).

Numerous studies have focused on the use of the enzyme phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) for enzyme substitution treatment of phenylketonuria (PKU) (Hoskins, *et al.*, Lancet 1(8165):392-394 (1980); Gilbert, *et al.*, Biochem. J. 199(3):715-723 (1981); Hoskins, J.A., *et al.*, Res. Commun. Chem. Pathol. Pharmacol. 35(2):275-282 (1982); Sarkissian, *et al.*, Proc. Natl. Acad. Sci. USA 96(5):2339-2344 (1999); Liu, *et al.*, Artif. Cells Blood Substit. Immobil. Biotechnol. 30(4):243-257 (2002); Wieder, K.J., J Biol. Chem. 254(24):12579-12587 (1979); Gamez, *et al.*, Mol. Ther. 11(6):986-989 (2005); Ambrus, *et al.*, J. Pharmacol. Exp. Ther. 224(3):598-602 (1983); Ambrus, *et al.*, Science 201(4358):837-839 (1978); Kalghatgi, Res. Commun. Chem. Pathol. Pharmacol. 27(3):551-561 (1980); Ambrus, Res. Commun. Chem. Pathol. Pharmacol. 37(1):105-111 (1982); Gilbert, *et al.*, Biochem. Biophys. Res. Commun. 131(2):557-563 (1985); Pedersen, Res. Commun. Chem. Pathol. Pharmacol. 20(3):559-569 (1978); Marconi, *et al.*, Biochimie 62(8-9):575-580 (1980); Larue, *et al.*, Dev. Pharmacol. Ther. 9(2):73-81 (1986); Ambrus,

C.M., *et al.*, Ann. Intern. Med. 106(4):531-537 (1987); Bourget, *et al.*, Appl. Biochem. Biotechnol. 10:57-59 (1984); Bourget, *et al.*, FEBS Lett. 180(1):5-8 (1985); Bourget, *et al.*, Biochim. Biophys. Acta 883(3):432-438 (1986); Chang, *et al.*, Artif. Cells Blood Substit. Immobil. Biotechnol. 23(1):1-21 (1995); Chang, *et al.*, Mol Biotechnol. 17(3):249-260 (2001); U.S. Patent No. 5,753,487).

PKU is an inborn error of amino acid metabolism that results from impaired activity of hepatic phenylalanine hydroxylase (PAH), the enzyme responsible for the metabolism of phenylalanine. Patients with PAH mutations that lead to PKU and hyperphenylalaninemia (HPA) display elevated levels of phenylalanine, impaired neurophysiologic functioning and reduced cognitive development. For patients with severe PKU, there is the potential for irreversible mental retardation unless phenylalanine levels are maintained at low levels using dietary restrictions. PAL converts phenylalanine to ammonia and trans-cinnamic acid, a harmless metabolite, which is further metabolized and excreted in the urine as hippurate (Hoskins *et al.*, (1980) *ibid.*; Hoskins, *et al.*, Biomed Mass Spectrom 11(6): 296-300 (1984)).

Current treatment for PKU involves the lifetime adherence to a diet that is low in the amino acid phenylalanine (Levy, Proc. Natl. Acad. Sci. USA 96(5):1811-1813 (1999)). This dietary therapy is difficult to maintain (Matalon, *et al.*, Genet. Med. 6(1): 27-32 (2004); Woolf, *et al.*, Arch. Dis. Child. 33(167):31-45 (1958); Kim, Mol Ther. 10(2):220-224 (2004)) and does not always eliminate the damaging neurological effects that can be caused by elevated phenylalanine levels (Sarkissian, *et al.*, Mol. Genet. Metab. 69:188-194 (2000)). Less than ideal dietary control during pregnancy can lead to birth defects (Levy, (1999) *ibid.*). In addition, it is very difficult for PKU/HPA patients to live a normal life while following the restrictive diet, and dietary therapy can be associated with deficiencies of several nutrients, some of which are detrimental for brain development (Levy, (1999) *ibid.*). Most low phenylalanine diet products have organoleptic properties sufficiently unsatisfactory that compliance with this treatment is compromised (Levy, (1999) *ibid.*). Therefore, development of a therapeutic treatment would replace or supplement the current dietary treatment and prevent the neurological damages inflicted on those individuals with PKU, particularly for those patients with the most severe forms of the disease.

In 1999, Scriver and colleagues reported their initial studies on the use of the enzyme PAL from Rhodosporidium toruloides (Sarkissian, *et al.*, (1999) *ibid.*) for PKU enzyme substitution applications. Mouse PKU and HPA model studies demonstrated that

PAL administration (either by i.p. injection or orally using either PAL in combination with aprotinin protease inhibitor or PAL recombinantly expressed and present inside *E. coli* cells) was associated with lower blood plasma phenylalanine levels. In addition, preliminary studies describing the use of PAL with PKU patients have shown reduction in phenylalanine levels using PAL administered in enteric-coated gelatin capsules (Hoskins, *et al.*, (1980) *ibid.*) or using an extracorporeal enzyme factory (Ambrus, *et al.*, Ann. Intern. Med. 106(4):531-537 (1987)). These studies suggest that if PAL is protected against proteolytic degradation, significant reductions of plasma Phe can be achieved upon oral administration.

The use of PAL for cancer treatment has also been suggested based on its ability to limit the nutrient supply of phenylalanine to cancer cells and thereby inhibit neoplastic growth (Fritz, *et al.*, J Biol Chem. 251(15):726 (1976); Roberts, *et al.*, Cancer Treat Rep. 60(3):261-263 (1976); Shen, *et al.*, Cancer Res. 37(4):1051-1056 (1977); Shen, *et al.*, Cancer Treat Rep. 63(6):1063-1068 (1979); Wieder, *et al.*, J Biol Chem. 254(24):12579-12587 (1979)). However, intravenously injected pegylated PAL was cleared rapidly from circulating blood after the 13th injection. In addition, PAL-mediated reduction in phenylalanine prevented the proliferation of murine leukemia and metastatic melanoma (Abell, *et al.*, Cancer Res. 33:2529-2532 (1973); Roberts, *et al.*, (1976) *ibid.*; Shen, *et al.*, (1977) *ibid.*).

Bacterial PAL from the marine bacterium Streptomyces maritimus may serve as an important source of the bacteriostatic agent enterocin. S. maritimus PAL, EncP, catalyzes the initial step in enterocin synthesis, which is the conversion of phenylalanine to trans-cinnamic acid (Xiang, *et al.*, J. Biol. Chem. 277:32505-32509 (2002)).

PAL may be used for the industrial synthesis of L-phenylalanine methyl ester (for Aspartame production) (D'Cunha, *et al.*, Enzyme and Microbial Technology 19(6):421-427 (1996); Hamilton, *et al.*, Trends in Biotechnol. 3(3):64-68 (1985)) and other substituted L-phenylalanine derivatives that are used as pharmaceutical precursors (U.S. Patent App. 20020102712).

PAL may also have agricultural applications, wherein PAL participates in the initial enzymatic process leading to the phenylpropanoids that produce lignins, coumarins, and flavanoids in plants, fungi, and bacteria. Hence, modulation of PAL activity can influence a number of agricultural phenomena such as the browning of fruit. In addition,

structure-based drug design of active site PAL inhibitors could lead to effective herbicides (Poppe, *et al.*, (2003) *ibid.*).

Although PAL potentially has various industrial and therapeutic applications, the use of PAL may be limited by reduced specific activity and proteolytic instability. Similar to other therapeutic proteins, use of PAL as an enzyme therapy is accompanied by several disadvantages such as immunogenicity and proteolytic sensitivity. Further, a delicate balance is required between substrate affinity and enzyme activity to achieve and maintain control of plasma phenylalanine levels within a normal somewhat narrow range in disorders characterized by hyperphenylalanemia. As yet, a concerted effort toward improving these parameters has not been made due to a paucity of structural and biochemical knowledge regarding this protein.

Thus, there remains a need for PAL molecules with optimal kinetic characteristics including potent catalytic activity and greater biological half-life, greater biochemical stability and/or attenuated immunogenicity.

SUMMARY

Several bacterial PAL have been already identified as part of the HAL/PAL family, including but not limited to PAL from Streptomyces maritimus (also known as EncP, SEQ ID NO:5, FIGURE 4), PAL/HAL from Nostoc punctiforme (Accession ZP_00105927 from Nostoc punctiforme ATCC 29133, submitted October 1, 2004, NCBI Microbial Genomes Annotation Project) (SEQ ID NO:2, FIGURE 4), PAL/HAL from Anabaena variabilis (Gene ID 3679622, Ava_3988 phenylalanine/histidine ammonia-lyase[Anabaena variabilis ATCC 29413, March 31, 2006 (SEQ ID NO:4, FIGURE 4), the photosynthetic prokaryote Anacystis nidulans (Lofflehardt, Z. Naturforsch 31(11-12):693-9 (1976)), the gram-negative bacteria from the family Enterobacteriaceae, Photorabdus luminescens TT01 (Williams, *et al.*, Microbiology 151:2543-2550 (2005)), and Streptomyces verticillatus (Bezanson, *et al.*, Can. J. Microbiol. 16(3):147-51 (1970)). Further, PAL activity has been evaluated in Streptomyces maritimus (Xiang, L., *et al.*, J. Biol. Chem. 277:32505-32509 (2002)). Cyanobacteria, such as Anabaena and Nostoc have been studied with respect to their production of bioactive natural products that are generated *via* mixed polyketide-peptide biosynthetic pathways (Moore, B.S., Natural Products Reports 22(5):580-593 (2005); Becker, *et al.*, Gene 325:35-42 (2004); Hoffman, *et al.*, Gene 311:171-180 (2003)). The invention is

based on the finding that prokaryotic or bacterial PAL may serve as an effective treatment for PKU and other disorders. The invention contemplates compositions of bacterial PAL and biologically active fragments, mutants, variants and analogs thereof, with enhanced properties, such as more potent catalytic activity, greater biochemical stability and, for therapeutic applications, attenuated immunogenicity and greater biological half-life. The present invention also provides methods of production and purification of bacterial PAL and biologically active fragments, mutants, variants and analogs thereof, and methods of using such compositions for therapeutic and industrial purposes.

As used herein, "bacterial PAL" and "prokaryotic PAL" are used interchangeably to mean (1) wild-type PAL from prokaryotic organisms, including but not limited to PAL from Streptomyces maritimus (also known as EncP, SEQ ID NO:5, FIGURE 4), Nostoc punctiforme (SEQ ID NO:2, FIGURE 4), Anabaena variabilis (SEQ ID NO:4, FIGURE 4), Anacystis nidulans (Lofflehardt, (1976) *ibid.*), Photorabdus luminescens TT01 (Williams, *et al.*, (2005) *ibid.*), and Streptomyces verticillatus (Bezanson, *et al.*, (1970) *ibid.*); (2) fragments, mutants, variants and analogues of such wild-type enzymes that retain similar (*i.e.*, at least 50%) catalytic activity for phenylalanine, and that, in some embodiments, exhibit increased catalytic activity and/or increased half-life and/or decreased immunogenicity, and (3) chemically modified versions of such wild-type enzymes, fragments, mutants, variants and analogs thereof that have been are linked to other chemical moieties that provide other advantageous effects, such as enhanced half-life. For example, any references to methods of making or using prokaryotic PAL, fragments, mutants, variants, analogs or chemically modified versions thereof, and compositions of such enzyme(s), for either therapeutic or industrial purposes, are meant to refer to methods of making, using or formulating all such wild-type, fragments, mutants, variants, analogs or chemical modifications thereof.

In a first aspect, there is provided an *Anabaena variabilis* phenylalanine ammonia-lyase (AvPAL) variant, wherein one or more cysteine residues selected from the group consisting of cysteine residues at positions 503 and 565 has been substituted by a serine residue, and wherein said AvPAL variant has a greater phenylalanine-converting activity and/or a reduced immunogenicity as compared to a wild-type AvPAL (SEQ ID NO:4).

In one example, the present disclosure provides bacterial PAL and biologically active fragments, mutants, variants or analogs thereof. One example is a bacterial PAL from Nostoc punctiforme (SEQ ID NO:2) or biologically active fragment, mutant, variant or analog thereof. Another example is a bacterial PAL from Anabaena variabilis (SEQ ID NO:4) or biologically active fragment, mutant, variant or analog thereof. In some examples, the variants retain the wild-type active site residues at positions corresponding to Ser 210 Ala-Ser-Gly triad (211-213) Asp214, Leu 215, Asn270, Val269, Leu266, Leu 134, His 137, Lys468, Glu496, Gin 500 in PAL from Rhodosporidium

toruloides or conservative substitution(s) of these active site residue(s), of which the Ala-Ser-Gly triad at 211-213 is believed to be the binding site for phenylalanine.

Desirable variants may include proteins in which one or more cysteine residues have been replaced with another amino acid (*e.g.,* serine) residue to reduce protein aggregation that can be associated with decreased enzyme activity, increased immunogenicity, and/or other disadvantageous effects *in vivo.*

In a second aspect, there is provided an AvPAL variant, wherein the cysteine residue at position 503 of AvPAL has been substituted with a serine residue (SEQ ID NO:9).

In a third aspect, there is provided an AvPAL variant, wherein the cysteine residue at position 565 of AvPAL has been substituted with a serine residue (SEQ ID NO:10).

In a fourth aspect, there is provided an AvPAL variant, wherein the cysteine residues at positions 503 and 565 have been substituted with serine residues (SEQ ID NO:11).

Desirable variants may include fusion proteins in which the enzyme has been fused to another heterologous polypeptide, such as a native or modified constant region of an immunoglobulin or a fragment thereof that retains the salvage epitope, known in the art to increase half-life.

The disclosure further contemplates chemically modified versions of such polypeptides, which have been linked to a chemical moiety that provides other advantageous effects. For example, nonspecific or site-specific (*e.g.,* N-terminal) linkage of water-soluble polymers to polypeptides is known in the art to improve half-life, and linkage of chemical moieties may also reduce immunogenicity and improve protease resistance.

Such bacterial PAL is isolated and purified in accordance with the methods described herein and is thereby present in amounts which enable using the enzyme therapeutically. In some examples, a cDNA encoding for a complete or wild-type bacterial PAL is used. However, in other examples, a cDNA encoding for a biologically active fragment or mutant thereof may be used. Further, the present disclosure provides compositions of optimized bacterial PAL obtained by structure-based molecular engineering approaches and/or chemically-modified (*e.g.,* pegylated) forms of PAL. Specific

examples contemplate optimal compositions of PAL with improved specific activity, enhanced stability, reduced immunogenicity and/or proteolytic sensitivity appropriate for therapeutic use. One example is a pegylated form of Nostoc punctiforme PAL with improved specific activity. Another example is a pegylated form of Anabaena variabilis PAL with improved specific activity.

In various examples, the pegylation of PAL variant is described in reference to the ratio of PAL:PEG or PEG:PAL. As used herein, and unless otherwise indicated, the ratio of "PAL:PEG" refers to the ratio of lysine residues on the PAL variant to PEG molecules in the pegylation reaction. Similarly, as used herein, and unless otherwise

indicated, the ratio of "PEG:PAL" refers to the ratio of PEG molecules to lysine residues on the PAL variant in the pegylation reaction.

In one example, the disclosure contemplates pegylated PAL variants with reduced immunogenicity. Another example is a pegylated form of Nostoc punctiforme PAL (NpPAL) variant with reduced immunogenicity. Another example is a pegylated form of Anabaena variabilis PAL (AvPAL) variant with reduced immunogenicity. Specific examples contemplate NpPAL or AvPAL variants in which pegylation is achieved by
reacting the NpPAL or AvPAL variant with a water-soluble polymer, e.g., polyethylene glycol (PEG). In some examples, pegylation is achieved by reacting the NpPAL or AvPAL variant once with PEG at a ratio of at least 1:1, at least 1:1.5, at least 1:2, at least 1:3, or at least 1:4 PAL:PEG. In one example, the PAL variant is an AvPAL variant, and the pegylation is achieved using a PAL:PEG ratio of 1:3.

The disclosure also contemplates re-pegylating the pegylated PAL variants for reduced immunogenicity. In some examples, the pegylation is achieved by reacting the NpPAL or AvPAL variant twice or more, with PEG, each pegylation at a ratio of at least 1:1, at least 1:1.5, at least 1:2, at least 1:3., or at least 1:4 PAL:PEG. In some examples, the pegylation is achieved by reacting the NpPAL or AvPAL variant twice, three times, four times, five times, or more, with PEG, each pegylation at a ratio of at least 1:1, at least 1:1.5, at least 1:2, at least 1:3., or at least 1:4 PAL:PEG. In one example, the PAL variant is an AvPAL variant, and the pegylation is achieved using a PAL:PEG ratio of 1:3 for both the first and second pegylation reactions.

In some examples, the biologically active sites of wild-type PAL according to the disclosure may be modified as desired to optimize PAL kinetic characteristics. In one example, a modified PAL has sufficient activity to reduce but also maintain plasma phenylalanine levels within the optimal range of about 120 μM to about 240 μM. In other examples, the biologically active modified PAL has a kcat of at least about 0.1 s-1 or greater than about 0.5 s-1. In some examples, the biologically active modified PAL has a kcat of at least about 0.2 s-1 or greater than about 1.0 s-1. In other examples, the biologically active modified PAL has a Km of between about 10 μM to about 1000 μM. In other examples, the biologically active modified PAL has a Km of between about 100 μM to about 1000 μM. In other examples, the biologically active modified PAL exhibits enzymatic activity that is from about two-fold to about 1000-fold times greater than that of the wild-type. In other examples, the biologically active modified PAL exhibits

enzymatic activity that is from about 10% to 100% higher than that of the wild-type. Such biological active modified PAL proteins may be formed using methods well known in the art, such as by site-directed mutagenesis. In further examples, the disclosure contemplates use of bacterial PAL that metabolizes phenylalanine (*i.e.,* converts phenylalanine to another substance) in preparation of a medicament for the treatment of a deficiency in PAH activity, in mammals, in particular, humans, as well as a pharmaceutical composition containing bacterial PAL for use in treating a deficiency in PAH activity. In some examples, the pharmaceutical composition comprises highly purified PAL derived from bacteria, or a biologically active fragment, mutant or analog thereof alone or in combination with a pharmaceutically suitable carrier. In some examples, preparations contain bacterial PAL with a purity greater than 95%, 96%, 97%, 98%, 99%, 99.2%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9%. In other examples, the relative specific activity of the bacterial PAL according to the present disclosure is greater than about 110% of the specific activity of wild-type PAL.

In one example, the present disclosure features novel methods of using PAL compositions for therapeutic and industrial purposes. In one example, the disclosure contemplates methods of treating disorders caused all or in part by a deficiency in PAH activity by administering a therapeutically effective amount of a pharmaceutical composition comprising bacterial PAL to a subject in need of such treatment. The deficiency in PAH activity can be observed, *e.g.,* as activity levels of 50% or less, 25% or less, or 10% or less or 1% or less, compared to normal levels of PAH activity and can manifest as elevated phenylalanine levels, for example, as in hyperphenylalanemia, mild phenylketonuria or classic severe phenylketonuria. In some examples, the disease is phenylketonuria (PKU).

In specific examples, the subject is one who has been diagnosed as having a mutant phenylalanine hydroxylase (PAH). The mutant PAH may comprise a mutation in the catalytic domain of PAH. Exemplary such mutations include but are not limited to mutations F39L, L48S, I65T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241C, R261Q, A300S, L308F, A313T, K320N, A373T, V388M E390G, A395P, P407S, and Y414C.

In a fifth aspect, there is provided a pharmaceutical composition, comprising the AvPAL variant of the present invention and a pharmaceutically acceptable carrier.

In a sixth aspect, there is provided the use of the AvPAL variant of the present invention in preparation of a medicament for treating a disease in a subject caused all or in part by a deficiency in phenylalanine hydroxylase (PAH).

Also contemplated is a method of treating a subject having above normal concentration of plasma phenylalanine (*e.g.*, greater than 180 μM or 360 μM) comprising administering to the subject a bacterial PAL composition in an amount effective to produce a decrease in the plasma phenylalanine concentration of the subject. The subject will likely have a plasma phenylalanine concentration greater than 180 μM prior to administration of the

bacterial PAL. More particularly, the subject has a plasma phenylalanine concentration of between 120 μM and 200 μM. In other examples, the subject has a plasma phenylalanine concentration of between 200 μM and 600 μM. In still other examples, the subject has a plasma phenylalanine concentration of between 600 μM and 1200 μM. Yet another class of subjects to be treated is those that have an unrestricted plasma phenylalanine concentration greater than 1200 μM.

In specific examples, the subject is an infant, more particularly, an infant having a plasma phenylalanine concentration greater than 1200 μM. The disclosure contemplates methods of treating an infant having phenylketonuria, comprising administering a bacterial PAL composition to the subject in an amount effective to produce a decrease in the plasma phenylalanine concentration of the infant wherein the infant is between 0 and 3 years of age and the infant has a plasma phenylalanine concentration of between about 360 μM to about 4800 μM. Prior to the administering of bacterial PAL, the infant has a phenylalanine concentration of about 1200 μM and the administering of bacterial PAL decreases the plasma phenylalanine concentration to about 1000 μM. In other examples, prior to the administering of bacterial PAL the infant has a phenylalanine concentration of about 800 μM and the administering of PAL decreases the plasma phenylalanine concentration to about 600 μM. In still further examples, prior to the administering of PAL the infant has a phenylalanine concentration of about 400 μM and the administering of PAL decreases the plasma phenylalanine concentration to about 300 μM. In some examples, the therapeutic methods contemplated herein should reduce the plasma phenylalanine concentration of the infant to a range of between about 120 μM to about 360 μM or a range of between about 120 μM to about 240 μM.

In a seventh aspect, there is provided the use of the AvPAL variant of the present invention in preparation of a medicament for treating phenylketonuria (PKU) in a subject.

In an eighth aspect, there is provided the use of a composition comprising a AvPAL variant of the present invention in the preparation of a medicament for treating a pregnant female having hyperphenylalaninemia (HPA) in combination with a protein-restricted diet, wherein the combined administration of the composition and protein-restricted diet is effective to lower the phenylalanine concentration in the plasma of the subject as compared to said concentration in the absence of said combined administration.

Also contemplated herein is a method for the treating a pregnant female having hyperphenylalaninemia (HPA) comprising administering to the subject bacterial PAL alone or in combination with a protein-restricted diet, wherein administration of bacterial PAL alone or in combination with the protein-restricted diet is effective to lower the phenylalanine concentration in the plasma of the subject as compared to the concentration in the absence of the combined administration. In certain examples, the subject has an unrestricted plasma phenylalanine concentration of greater than 180 μM but less than 600 μM. In other examples, the subject has an unrestricted plasma phenylalanine concentration of greater than 500 μM but less than 1200 μM. In still other examples, the subject has an unrestricted plasma phenylalanine concentration of greater than 1200 μM.

Pregnant subjects with a plasma phenylalanine concentration greater than 1200 μM are particularly attractive candidates for this type of therapy, as are subject who are females of child-bearing age that are contemplating pregnancy. In those examples, in which the subject has a plasma phenylalanine concentration greater than 1200 μM, and the method further comprises administering a protein-restricted diet to the subject.

In a ninth aspect, there is provided the use of a composition comprising a AvPAL variant of the present invention in the preparation of a medicament for treating an infant having phenylketonuria (PKU), in an amount effective to produce a decrease in the plasma phenylalanine concentration of said infant, wherein said infant is between 0 and 3 years of age and said infant has a plasma phenylalanine concentration of between about 360 μM to about 4800 μM.

The disclosure describes methods of treating classic severe phenylketonuria (PKU) in a subject comprising administering to the subject a bacterial PAL or a biologically active fragment, mutant, variant or analog thereof wherein the administration of bacterial PAL is effective to lower the phenylalanine concentration in the plasma of the subject as compared to the concentration in the absence of bacterial PAL administration. A subject selected for treatment according to the methods of the disclosure will have an elevated plasma Phe concentration, such a concentration may be greater than 1800 μM in the absence of the therapeutic. Other examples contemplate a subject that has a plasma phenylalanine concentration of greater than 1000 μM in the absence of a therapeutic regimen. In some examples, the combined administration methods of the disclosure decrease the plasma phenylalanine concentration of the subject to less than 600 μM. In one example, it is decreased to less than 500 μM. In another example, the combined administration decreases the plasma phenylalanine concentration of the subject in the range from about 120 μM to about 360 μM. In another example, the plasma phenylalanine concentration of the subject is reduced in the range from about 120 μM to about 240 μM.

Certain examples include optimizing the dosage to the needs of the organism to be treated, for example, mammals or humans, to effectively ameliorate the disease symptoms. PAL may be administered in a single daily dose, multiple doses on a daily basis, in a single weekly dose or multiple doses on a weekly basis. In some examples, the PAL therapy is not continuous, but rather PAL is administered on a daily basis until the plasma phenylalanine concentration of the subject is decreased to less than 360 μM. In some examples, wherein the plasma phenylalanine concentration of the subject is monitored on a daily basis and the PAL is administered when a 10% increase in plasma phenylalanine concentration is observed. In other examples, doses are delivered once weekly. The disclosure contemplates doses of at least 0.001 mg/kg, 0.005 mg/kg, 0.01 mg/kg, 0.05 mg/kg, and may range up to 0.1 mg/kg, 0.5mg/kg, 1.0 mg/kg or higher per week. In some examples, the dose is 1 mg/kg/week, 0.1 mg/kg/week, or 0.01 mg/kg/week.

A variety of parenteral or nonparenteral routes of administration, including oral, transdermal, transmucosal, intrapulmonary (including aerosolized), intramuscular, subcutaneous, or intravenous that deliver equivalent dosages are contemplated. Administration by bolus injection or infusion directly into the joints or CSF is also specifically contemplated, such as intrathecal, intracerebral, intraventricular, *via* lumbar puncture, or *via* the cisterna magna. In some examples, the doses are delivered subcutaneously or orally.

Other means of increasing PAL activity in the human subjects are also contemplated, including gene therapy. Transfer of a PAL gene is possible through a variety of means known in the art, including viral vectors, homologous recombination, or direct DNA injection. Within the scope of this aspect are examples featuring nucleic acid sequences encoding all or a part of bacterial PAL or a biologically active mutant or analogs thereof, which may be administered *in vivo* into cells affected with PAH deficiency.

In another example, bacterial PAL may also be administered in combination with a protein-restricted diet. The protein-restricted diet administered in the methods herein is one that is a phenylalanine-restricted diet wherein the total phenylalanine intake of the subject is restricted to less than 600 mg per day. In other examples, the protein-restricted diet is a phenylalanine-restricted diet wherein the total phenylalanine is restricted to less than 300 mg per day. In still other examples, the protein-restricted diet is one supplemented with amino acids, such as tyrosine, valine, isoleucine and leucine. Also contemplated is a composition comprising bacterial PAL and a pharmaceutically acceptable carrier, diluent or excipient. The composition may further comprise a medical protein supplement. In other examples, the PAL composition is part of an infant formula. In still other examples, the protein supplement is phenylalanine free. The protein supplement may be fortified with L-tyrosine, L-glutamine, L-carnitine at a concentration of 20 mg/100 g supplement, L-taurine at a concentration of 40 mg/100 g supplement and selenium. It may further comprise the recommended daily doses of minerals, *e.g.,* calcium, phosphorus and magnesium. The supplement further may comprise the recommended daily dose of one or more amino acids selected from the group consisting of L-leucine, L-proline, L-lysine acetate, L-valine, L-isoleucine, L-arginine, L-alanine, glycine, L-asparagine monohydrate, L-tryptophan, L-serine, L-threonine, L-histidine, L-methionine, L-glutamic acid, and L-aspartic acid. In addition, the supplement may be fortified with the recommended daily dosage of vitamins A, D and E. The supplement may comprise a fat content that provides at least 40%

of the energy of the supplement. Such a supplement may be provided in the form of a powder supplement or in the form of a protein bar.

The disclosure contemplates methods of treating various forms of neoplastic growth and cancer, including but not limited to lymphoblastic leukemia, mammary tumors, and melanomas.

The disclosure contemplates methods of using bacterial PAL for the commercial production of phenylalanine from ammonia and trans-cinnamate. Phenylalanine is used in aspartame, a sweetener and other food products, including beverages, cereals, cakes, desserts, egg and cheese dishes, fats, oils, fish and other seafoods, meat and meat products, milk and milk products, nuts, sauces and condiments, soups, sugars, jams and spreads, and vegetables.

It is further contemplated that bacterial PAL may be used for the production of herbicides and antimicrobial agents including enterocin and erythromycin.

In one example, the present disclosure features a method to produce prokaryotic PAL or a biologically active fragment, mutant, variant or analog thereof in amounts which enable using the enzyme therapeutically. The present disclosure contemplates PAL produced by bacteria including, but not limited to, Streptomyces, Sorangium, Pseudomonas, and cyanobacteria such as Nostoc and Anabaena. In some examples, PAL
is produced by the bacterial species Streptomyces maritimus, S. verticiliatus, Soragium cellulosum, Nostoc punctiforme, Nostoc tobacum, Anabaena variabilis, and Pseudomonas putida. In another example, prokaryotic PAL enzyme activity is generated using cDNA or DNA sequences that are derived from sequences sometimes described as coding for HAL activity or featuring a PAL-HAL motif, but possessing key PAL residues that differ from HAL.

In a broad example, the method comprises the step of transforming a cDNA or DNA encoding for all or a part of a prokaryotic PAL or a biologically active fragment, mutant, variant or analog thereof into a cell suitable for the expression thereof. In some examples, an expression vector is used to transfer the DNA into a suitable cell or cell line for expression thereof. In one example, the cDNA is transformed into *E. coli* and recombinant bacterial PAL is overexpressed as fusion protein. In a further example, the method of producing prokaryotic PAL comprises the steps of: (a) growing cells transformed with a cDNA encoding all or a biologically active variant, fragment or mutant of

prokaryotic PAL in a suitable growth medium to an appropriate density to produce a seed culture, (b) introducing the transformed cells into a bioreactor, (c) supplying a suitable growth medium to the bioreactor, and (d) separating the transfected cells from the media containing the enzyme.

In one example, recombinant PAL is over-expressed as an N-terminal octahistidyl-tagged fusion protein in a vector such as E coli BL21(DE3)/pLyseS (Invitrogen) with an inducible promoter such as with IPTG (isopropyl-beta-D-thiogalactopyranoside). In another example, recombinant PAL is over-expressed in *E. coli* BL21(DE3)/pLysS cells without an N-terminal tag. In another example, the method of producing prokaryotic PAL comprises the steps of: (1) growing a seed culture for a bioreactor/fermenter from a glycerol stock in shake flasks; (2) introducing such seed culture into a controlled bioreactor in fed-batch mode; (3) growing said culture in glucose-supplemented media, pH (7.8), > 20% dissolved oxygen, agitation up to 1200 rpm, 30°C until reaching a cell density of OD600 of 70-100 (~22-25 hrs); (4) inducing said culture with 0.4 mM IPTG; (5) growing said culture at a reduced temperature of 22 to 26°C until activity change is < 0.1 IU/ml (approximately 40-48 hrs and an OD600 typically of 200); and (5) harvesting bacteria by continuous centrifugation. In one example, the cell culture media is typically defined and composed of yeast extract protein, peptone-tryptone, glucose, glycerol, casamino acids, trace salts and phosphate buffering salts.

In one example, the present disclosure features a method to purify bacterial PAL or a biologically active fragment, mutant or analog thereof. According to one example, a transformed cell mass is grown and ruptured leaving crude recombinant enzyme. Exogenous materials are normally separated from the crude bulk to prevent fouling of the columns. Chromatographic purification is conducted using one or several chromatographic resins. Subsequently, the purified protein is formulated into a buffer designed to provide stable activity over an extended period of time. In another example, the method to purify the bacterial PAL comprises the steps of: (a) lysis of the bacteria containing recombinant PAL; (b) treatment of lysate with heat to inactivate viruses; (c) clarification of this lysate using a second continuous centrifugation step and/or depth filtration; (d) passage of clarified lysate through a charcoal filtration step; (e) passage of filtrate in (d) through a final filtration step (as with a Sartorious Sartopore 0.2 um filter); (f) passage of final filtrate over a hydrophobic interaction chromatography resin, such as a butyl hydrophobic interaction chromatography; (g) passage of eluate in (f) over an anionic

chromatography resin, such as a Q ion exchange column; (h) recovery of final product by buffer exchange with tangential flow filtration; and (i) sterilization of the final product. Those skilled in the art readily appreciate that one or more of the chromatography steps may be omitted or substituted, or that the order of the chromatography steps may be changed within the scope of the present disclosure. Finally, appropriate sterilizing steps may be performed as desired.

In one example, the present disclosure contemplates screening assays for identifying bacterial PAL that can prevent, ameliorate, or treat enhanced levels of phenylalanine by contacting a cell containing elevated levels of phenylalanine with the bacterial PAL and determining whether the bacterial PAL reduces such elevated levels of phenylalanine. Such screening assays may also include the steps of creating variants that include conservative or non-conservative substitutions in the active sites, *e.g.* Gly 142, Thr-Ser-Gly triad (143-145), Asp 146, Leu 147, Asn 196, Ile 195, Leu 192, Leu 76, Asn 79, Met 400, Thr 428, Gln 432 in EncP from Streptomyces maritimus which are equivalent to residues Ser 210 Ala-Ser-Gly triad (211-213) Asp214, Leu 215, Asn270, Val269, Leu266, Leu 134, His 137, Lys468, Glu496, Gin 500 in PAL from Rhodosporidium toruloides, in regions adjacent to the active sites, or throughout the polypeptide sequence, followed by testing the variants for *in vitro* phenylalanine converting activity. In certain examples, the method is a high throughput assay. In one example, complete genomes of the bacterial species are sequenced and screened for the presence of PAL homologs using a bioinformatics approach. In yet another example, PAL catalytic activity of the protein product of such homologs is confirmed, such as by testing ability to convert phenylalanine to trans-cinnamate *in vitro*.

In one example, the disclosure provides methods of using bacterial PAL compositions for the diagnosis of diseases, including but not limited to disorders caused all or in part by a deficiency in PAH activity. In one example, bacterial PAL is used to measure levels of phenylalanine in blood samples. In a further example, the disclosure contemplates a diagnostic kit comprising bacterial PAL for use in monitoring blood samples of subjects with elevated levels of phenylalanine.

Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating particular embodiments of the invention, are given by way of illustration only, because various changes and modifications

within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. FIGURE 1A: Gene sequence of Nostoc punctiforme PAL (SEQ ID NO:1); FIGURE 1B: Protein sequence of Nostoc punctiforme PAL (SEQ ID NO:2).

FIGURE 2. FIGURE 2A: Gene sequence of Anabaena variabilis PAL (SEQ ID NO:3); FIGURE 2B: Protein sequence of Anabaena variabilis PAL (SEQ ID NO:4).

FIGURE 3. Relatedness tree of aromatic amino acid ammonia-lyases from prokaryotes and eukaryotes. Sequences were retrieved from GenBank (accession numbers are given in parentheses) and aligned with ClustalX (1.83) using the Neighbor Joining Method.

FIGURE 4. Alignment of cyanobacterial protein sequences of N. punctiforme PAL (SEQ ID NO:2) and A. variabilis PAL (SEQ ID NO:4) with EncP PAL (SEQ ID. No. 5) and P. putida HAL (SEQ ID NO:6). Active site residues, which correspond to PAL or HAL activity, are highlighted.

FIGURE 5. Effect of (A) non-pegylated NpPAL (1.0 IU/mL) and (B) pegylated NpPAL 1:3 PAL:PEG (Nippon Oil and Fat, NOF Corporation) (1.0 IU/mL) on phenylalanine levels (μM) over a 12-day period.

FIGURE 6. Effect of (A) non-pegylated AvPAL (1.0 IU/mL) and (B) pegylated AvPAL 1:3 PAL:PEG (Nippon Oil and Fat, NOF Corporation) (1.0 IU/mL) on phenylalanine levels (μM) over a 12-day period.

FIGURE 7. Effect of pegylated NpPAL 1:3 PAL:PEG (Nippon Oil and Fat, NOF Corporation) (1.0 IU/mL) on phenylalanine levels (μM) in a 90-day chronic tolerance study.

FIGURE 8. FIGURE 8A: Protein sequence of Anabaena variabilis phenylalanine ammonia-lyase (PAL) with a cysteine to serine substitution at position 64 (AvPAL_C64S, SEQ ID NO:7); FIGURE 8B: Protein sequence of Anabaena variabilis PAL with a cysteine to serine substitution at position 318 (AvPAL_C318S, SEQ ID NO:8); FIGURE 8C: Protein sequence of Anabaena variabilis PAL with a cysteine to serine substitution at position 503 (AvPAL_C503S, SEQ ID NO:9); FIGURE 8D: Protein sequence

of Anabaena variabilis PALwith a cysteine to serine substitution at position 565 (AvPAL_C565S, SEQ ID NO: 10); FIGURE 8E: Protein sequence of Anabaena variabilis PAL with cysteine to serine substitutions at positions 503 and 565 (AvPAL_C565SC503S, SEQ ID NO:11). Cysteine to serine substitutions are underlined in bold.

FIGURE 9. FIGURE 9A: Effect of cysteine to serine substitutions at position 565 or both positions 565 and 503 of unpegylated AvPAL on *in vitro* PAL specific enzyme activity after incubation for various lengths of time at 37°C. FIGURE 9B: Effect of cysteine to serine substitutions at position 565 or both positions 565 and 503 of pegylated AvPAL on *in vitro* PAL specific enzyme activity after incubation for various lengths of time at 37°C.

FIGURE 10. FIGURE 10A: Effect of cysteine to serine substitutions in AvPAL on formation of protein aggregates in solution as analyzed by gel electrophoresis under denaturing conditions (left panel) or native conditions (right panel). FIGURE 10B: Effect of cysteine to serine substitutions in AvPAL on formation of protein aggregates in solution as analyzed by SEC-HPLC.

FIGURE 11. Effect of cysteine to serine substitutions at positions 565 and 503 (dbl Mutant) in AvPAL on site-specific pegylation at various PEG concentrations.

FIGURE 12. Effect of treatment of AvPAL with 0.05% Tween80 or 10 mM EDTA on formation of protein aggregates in solution as analyzed by SEC-HPLC.

FIGURE 13. FIGURE 13A: Effect of treatment of AvPAL by dithiotreitol (DTT) on formation of protein aggregates in solution as analyzed by SEC-HPLC. FIGURE 13B: Effect of treatment of AvPAL by DTT and N-ethylmaleimide (NEM) on formation of protein aggregates in solution as analyzed by SEC-HPLC.

FIGURE 14. Effect of cysteine to serine substitutions at positions 565 and 503 (AvPAL_C565SC503S) in pegylated AvPAL on *in vivo* phenylalanine (Phe) levels in ENU2 mice dosed with 0.25 IU (top panel), 1.0 IU (middle panel) or 4.0 IU (bottom panel) enzyme as compared to ENU2 mice dosed with vehicle or 4.0 IU wild-type pegylated AvPAL.

FIGURE 15. Effect of cysteine to serine substitutions at positions 565 and 503 (AvPAL_C565SC503S) in pegylated AvPAL on body weights of ENU2 mice dosed with 0.25 IU, 1.0 IU or 4.0 IU enzyme as compared to ENU2 mice dosed with vehicle or 4.0 IU wild-type pegylated AvPAL

FIGURE 16. Effect of cysteine to serine substitutions at positions 565 and 503 (AvPAL C503S/565S) in pegylated AvPAL on *in vivo* phenylalanine (Phe) levels in ENU2 mice dosed with 4 IU enzyme at various AvPAL:PEG ratios: 1:1.6 (top panel), 1:2.4 (middle panel) or 1:3 (bottom panel), as compared to ENU2 mice dosed with vehicle or 4.0 IU wild-type pegylated AvPAL at an AvPAL:PEG ratio of 1:3.

FIGURE 17. Effect of cysteine to serine substitutions at positions 565 and 503 (AvPAL_C565SC503S) in pegylated AvPAL on body weights of ENU2 mice dosed with 4 IU enzyme at various AvPAL:PEG ratios: 1:1.6, 1:2.4 or 1:3, as compared to ENU2 mice dosed with vehicle or 4.0 IU wild-type pegylated AvPAL at an AvPAL:PEG ratio of 1:3.

FIGURE 18. Effect of re-pegylation of pegylated AvPAL_C565SC503S on AvPAL specific activity *in vitro*. (Top) Recovery of specific activity of rAvPAL-PEG, first pegylated at a PAL:PEG ratio of 1:3 (2 mM lysine residues:6 mM PEG, filled circles), after re-pegylation under a variety of PAL:PEG ratios and concentrations of lysine residues and PEG molecules as indicated. (Bottom) Recovery of specific activity of rAvPAL-PEG, first pegylated at a PAL:PEG ratio of 1:1 (2 mM lysine residues:2 mM PEG, filled squares), or 1:3 2 mM lysine residues:6 mM PEG, filled circles), after re-pegylation under a variety of PAL:PEG ratios and concentrations of lysine residues and PEG molecules as indicated.

FIGURE 19. FIGURE 19A: Effect of increasing the PAL:PEG ratio in the re-pegylation reaction on overall pegylation of rAVPAL-PEG, first pegylated at a PAL:PEG ratio of 1:3 (2 mM lysine residues:6 mM PEG), as determined by Capillary Gel Electrophoresis (CGE) analysis. FIGURE 19B: Effect of increasing the PAL:PEG ratio in the first pegylation reaction on overall pegylation of rAVPAL-PEG where the re-pegylation reaction is performed at a fixed PAL:PEG ratio of 1:3 (2 mM lysine residues:6 mM PEG), as determined by CGE analysis.

FIGURE 20. Effect of re-pegylation of AvPAL_C565SC503S, first pegylated at a PAL:PEG ratio of 1:3 (2 mM lysine residues:6 mM PEG), on the percent pegylation of specific lysine residues in AvPAL after re-pegylation under a variety of PAL:PEG ratios and concentrations of lysine residues and PEG molecules as indicated. The lysine residue at position 2 (K2) is 100% pegylated under all the conditions tested (not shown).

FIGURE 21. Effect of re-pegylation of AvPAL_C565SC503S, first pegylated at a PAL:PEG ratio of 1:3 (2 mM lysine residues:6 mM PEG), and re-pegylated at the samePAL:PEG ratio and concentration of lysine residues and PEG molecules, on *in vivo*

plasma phenylalanine (Phe) levels in ENU2 mice dosed subcutaneously as indicated in Table 14.

DETAILED DESCRIPTION OF THE INVENTION

Although PAL is a ubiquitous higher plant enzyme that catalyzes the nonoxidative deamination of phenylalanine to cinnamic acid in the committed step to phenylpropanoid metabolites (Hahlbrock, *et al.*, Annu. Rev. Plant Phys. Plant Mol. Biol. 40:347-369 (1989)), PAL has only been encountered in a few bacteria where it is involved in benzoyl-CoA biosynthesis in "S. maritimus" (Xiang, *et al.*, J. Biol. Chem. 277:32505-32509 (2002)) and Sorangium cellulosum (Hill, *et al.*, Chem. Commun. 1358-1359 (2003)) and in the biosynthesis of cinnamamide in Streptomyces verticillatus (Bezanson, *et al.*, Can. J. Microbiol. 16:147-151 (1970)). The bacteriostatic agent enterocin is a natural product of the marine bacterium "Streptomyces maritimus" whose biosynthesis involves a number of unusual features (Hertweck, *et al.*, Chem. Biol. 11:461-468 (2004); Piel, *et al.*, Chem. Biol. 7:943-955 (2000); Piel, *et al.*, J. Am. Chem. Soc. 122:5415-5416 (2000); Xiang, *et al.*, Proc. Natl. Acad. Sci. USA 101:15609-15614 (2004)). Among these is the formation of the rare polyketide synthase (PKS) starter unit benzoyl-coenzyme A (CoA) (Moore, *et al.*, Nat. Prod. Rep. 19:70-99 (2002)). The initial biochemical reaction involves the conversion of the amino acid L-phenylalanine to trans-cinnamic acid by the novel bacterial phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) EncP (Xiang, *et al.*, J. Biol. Chem. 277:32505-32509 (2002)). Activation of cinnamic acid to its CoA thioester followed by a single round of beta-oxidation yields benzoyl-CoA (Hertweck, *et al.*, Chem Bio Chem 2:784-786 (2001); Hertweck, *et al.*, Tetrahedron 56:9115-9120 (2000); Xiang, *et al.*, J. Bacteriol. 185:399-404 (2003)), which primes the enterocin type II PKS for chain extension with seven molecules of malonyl-CoA.

The first prokaryotic PAL-encoding gene (encP) (SEQ ID NO:5) was characterized and its inactivation resulted in the abolishment of de novo cinnamic acid and enterocin synthesis in "S. maritimus" (Kalaitzis, *et al.*, J. Am. Chem. Soc. 125:9290-9291 (2003); Xiang, *et al.*, J. Biol. Chem. 277:32505-32509 (2002)). Enterocin biosynthesis could be restored in encP-inactivated mutants through supplementation with cinnamic or benzoic acids as well as complementation with plasmid-borne encP. Furthermore, the heterologous expression of the encP gene under the control of the ermE* promoter in Streptomyces coelicolor led to the production of cinnamic acid in the fermented cultures (Xiang, *et al.*, J.

Biol. Chem. 277:32505-32509 (2002)). The encP gene encodes a 522 amino acid protein that is considerably smaller than eukaryotic PALs by nearly 200 amino acid residues. Although sequence homologous to plant PALs such as from Petroselinum crispum (Röther, *et al.*, Eur. J. Biochem. 269:3065-3075 (2002)) (CAA57056, 30% identical and 48% similar), it rather shares greater homology to bacterial histidine ammonia-lyases (HALs, EC 4.3.1.3) such as from Pseudomonas putida (Schwede, *et al.*, Biochemistry 27:5355-5361 (1999)) (A35251, 36% identical and 54% similar, SEQ ID NO:6, FIGURE 4) and to tyrosine ammonia-lyase (TAL) from Rhodobacter capsulatus (Kyndt, *et al.*, FEBS Lett. 512:240-244 (2002)) (FIGURE 3). The homology includes the conserved active site serine residue at position 143 of the phenylalanine/histidine/tyrosine family of ammonia-lyases that is the probable precursor of the modified dehydroalanine residue in the 4-methylideneimidazole-5-one (MIO) prosthetic group (Langer, *et al.*, Adv. Prot. Chem. 58:175-188 (2001); Poppe, Curr. Opin. Chem. Biol. 5:512-524 (2001); Schwede, *et al.*, Biochemistry 27:5355-5361 (1999)). EncP shares greatest sequence homology to AdmH (AAO39102, 63% identical and 76% similar), a putative phenylalanine aminomutase involved in andrimid biosynthesis in Pantoea agglomerans that is related to the tyrosine aminomutase Sgc4 from Streptomyces globisporus (Christenson, *et al.*, J. Am. Chem. Soc. 125:6062-6063 (2003); Christenson, *et al.*, Biochemistry 42:12708-12718 (2003)). Notably, because encP has closer homology to a human protein (HAL) encP may be potentially less immunogenic. Also because they are very similar it could be possible to mutate encP to look like human HAL, a humanized version of encP.

HAL and PAL were shown to share in common a mechanism for the chemically difficult elimination of ammonia from histidine and phenylalanine, respectively. With both enzymes, a superelectrophilic prosthetic group 5 methylene-3,5-dihydroimidazol-4-one (MIO) activates the non-acidic beta hydrogen atoms of their respective substrates by a Friedel-Crafts-type attack at the aromatic ring. The sigma complex that is generated prevents the extraction of protons from the ring by excluding any bases from access to the binding pocket of the enzyme. The formation of an exocyclic double bond is key in the elimination of ammonia, rearomatization, and fragmentation. The prosthetic MIO group is regenerated and the product urocanate or cinnamate is formed (Poppe, *et al.*, Angew. Chem. Int. Ed. 44:3668-3688 (2005)).

Because of the high homology between HAL and PAL, the conserved regions of HAL and PAL are referred to as the HAL/PAL conserved region. This high homology can

create some ambiguities in databases like NCBI on the potential enzyme activity of a "PAL-HAL" protein conducting to mislabeling, such as with protein sequences listed in the NCBI database for Nostoc punctiforme and Anabaena variabilis. Therefore some PAL enzymes can be mislabeled HAL enzymes. Although the active sites of PALs and HALs are very similar, they are predicted to differ in some key residues (Calabrese *et al.*, Biochemistry 43(36):11403-11416 (2004); Xiang *et al.*, (2002) *ibid.*; Williams *et al.*, (2005) *ibid.*). Particularly in HAL, the methionine 382 and glutamine 414 from Pseudomonas putida (SEQ ID NO:6) are highly conserved in all HALs but are always replaced in all the PALs described so far (eukaryotic or prokaryotic) by lysine and glutamine, respectively (FIGURE 4). So it can be said that all proteins with a "PAL-HAL" region and having the homologues of lysine382 and glutamine414 have the sequence signature of a protein with PAL activity. This relatively newly described PAL signature (Williams *et al.*, (2005) *ibid.*) allows to label properly some enzymes from HAL to PAL and could be used to identify some new PAL enzymes from already published genes and proteins database.

The present invention relates to compositions of such prokaryotic PAL and biologically active fragments, mutants and variants thereof and their uses for therapeutic and industrial purposes.

A. DEFINITIONS

Unless otherwise stated, the following terms used in this application, including the specification and claims, have the definitions given below. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Definition of standard chemistry terms may be found in reference works, including Carey and Sundberg, Advanced Organic Chemistry, 3rd Edition, Vols. A and B (Plenum Press, New York 1992). The practice of the present invention will employ, unless otherwise indicated, conventional methods of synthetic organic chemistry, mass spectroscopy, preparative and analytical methods of chromatography, protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. See, *e.g.*, T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., 4th Edition, 2004); Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan

eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)
Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

"Polynucleotide" refers to a polymer composed of nucleotide units. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") as well as nucleic acid analogs. Nucleic acid analogs include those which include non-naturally occurring bases, nucleotides that engage in linkages with other nucleotides other than the naturally occurring phosphodiester bond or which include bases attached through linkages other than phosphodiester bonds. Thus, nucleotide analogs include, for example and without limitation, phosphorothioates, phosphorodithioates, phosphorotriesters, phosphoramidates, boranophosphates, methylphosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), and the like. Such polynucleotides can be synthesized, for example, using an automated DNA synthesizer. The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (*i.e.*, A, T, G, C), this also includes an RNA sequence (*i.e.*, A, U, G, C) in which "U" replaces "T."

"cDNA" refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as "upstream sequences"; sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences."

"Complementary" refers to the topological compatibility or matching together of interacting surfaces of two polynucleotides. Thus, the two molecules can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other. A first polynucleotide is complementary to a second polynucleotide if the nucleotide sequence of the first polynucleotide is identical to the nucleotide sequence of the polynucleotide-binding partner of the second polynucleotide. Thus, the polynucleotide whose sequence 5'-TATAC-3' is complementary to a polynucleotide whose sequence is 5'-GTATA-3'.

A nucleotide sequence is "substantially complementary" to a reference nucleotide sequence if the sequence complementary to the subject nucleotide sequence is substantially identical to the reference nucleotide sequence.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are

degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, *e.g.*, a "recombinant polypeptide." A recombinant polynucleotide may serve a non-coding function (*e.g.*, promoter, origin of replication, ribosome-binding site, *etc.*) as well.

"Expression control sequence" refers to a nucleotide sequence in a polynucleotide that regulates the expression (transcription and/or translation) of a nucleotide sequence operatively linked thereto. "Operatively linked" refers to a functional relationship between two parts in which the activity of one part (*e.g.*, the ability to regulate transcription) results in an action on the other part (*e.g.*, transcription of the sequence). Expression control sequences can include, for example and without limitation, sequences of promoters (*e.g.*, inducible or constitutive), enhancers, transcription terminators, a start codon (*i.e.*, ATG), splicing signals for introns, and stop codons.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or *in vitro* expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide.

"Amplification" refers to any means by which a polynucleotide sequence is copied and thus expanded into a larger number of polynucleotide molecules, *e.g.*, by reverse transcription, polymerase chain reaction, and ligase chain reaction.

"Primer" refers to a polynucleotide that is capable of specifically hybridizing to a designated polynucleotide template and providing a point of initiation for synthesis of a complementary polynucleotide. Such synthesis occurs when the polynucleotide primer is placed under conditions in which synthesis is induced, *i.e.*, in the presence of nucleotides, a complementary polynucleotide template, and an agent for polymerization such as DNA

polymerase. A primer is typically single-stranded, but may be double-stranded. Primers are typically deoxyribonucleic acids, but a wide variety of synthetic and naturally occurring primers are useful for many applications. A primer is complementary to the template to which it is designed to hybridize to serve as a site for the initiation of synthesis, but need not reflect the exact sequence of the template. In such a case, specific hybridization of the primer to the template depends on the stringency of the hybridization conditions. Primers can be labeled with, *e.g.*, chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

"Probe," when used in reference to a polynucleotide, refers to a polynucleotide that is capable of specifically hybridizing to a designated sequence of another polynucleotide. A probe specifically hybridizes to a target complementary polynucleotide, but need not reflect the exact complementary sequence of the template. In such a case, specific hybridization of the probe to the target depends on the stringency of the hybridization conditions. Probes can be labeled with, *e.g.*, chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

A first sequence is an "antisense sequence" with respect to a second sequence if a polynucleotide whose sequence is the first sequence specifically hybridizes with a polynucleotide whose sequence is the second sequence.

"Hybridizing specifically to" or "specific hybridization" or "selectively hybridize to" refers to the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.*, total cellular) DNA or RNA.

The term "stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal

melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the Tm for a particular probe.

An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65°C for 15 minutes (see, Sambrook, *et al.* (1989) *ibid.* for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

"Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked *via* peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides.

Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

"Conservative substitution" refers to the substitution in a polypeptide of an amino acid with a functionally similar amino acid. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Amino acids may also be grouped as follows:

(1) hydrophobic: Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr;

(3) acidic: Asp, Glu;

(4) basic: Asn, Gln, His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro; and

(6) aromatic: Trp,Tyr, Phe.

"Allelic variant" refers to any of two or more polymorphic forms of a gene occupying the same genetic locus. Allelic variations arise naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. "Allelic variants" also refer to cDNAs derived from mRNA transcripts of genetic allelic variants, as well as the proteins encoded by them.

The terms "identical" or percent "identity," in the context of two or more polynucleotide or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

The phrase "substantially homologous" or "substantially identical" in the context of two nucleic acids or polypeptides, generally refers to two or more sequences or subsequences that have at least 40%, 60%, 80%, 90%, 95%, 98% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. In some embodiments, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, over a region of at least about 100 residues, or over at least about 150 residues. In another embodiment, the sequences are substantially identical over the entire length of either or both comparison biopolymers.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if

necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection.

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol. 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, CABIOS 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. Another algorithm that is useful for generating multiple alignments of sequences is Clustal W (Thompson, *et al.*, Nucleic Acids Research 22: 4673-4680 (1994)).

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul, *et al.*, J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This

algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.* (1990), *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, less than about 0.01, or less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described herein.

"Substantially pure" or "isolated" means an object species is the predominant species present (*i.e.*, on a molar basis, more abundant than any other individual macromolecular species in the composition), and a substantially purified fraction is a composition wherein the object species comprises at least about 50% (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition means that about 80% to 90% or more of the macromolecular species present in the composition is the purified species of interest. The object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) if the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), stabilizers (*e.g.*, BSA), and elemental ion species are not considered macromolecular species for purposes of this definition. In some embodiments, the compounds or conjugates of the invention are substantially pure or isolated. In some embodiments, the compounds or conjugates of the invention are substantially pure or isolated with respect to the macromolecular starting materials used in their synthesis. In some embodiments, the pharmaceutical compositiona of the invention comprise a substantially purified or isolated conjugate of a PAL polypeptide and the active agent admixed with one or more pharmaceutically acceptable excipient.

"Naturally occurring" as applied to an object refers to the fact that the object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring.

"Wild-type" (wt) is a term referring to the natural genetic form of an organism. A wild-type is distinguished from a mutant form (an organism with a genetic mutation).

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention,

a "polypeptide" refers to a protein, which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations arising with hosts that produce the proteins or errors due to PCR amplification.

As used herein, an "analogue" or "derivative" is a compound, *e.g.*, a peptide, having more than about 70% sequence but less than 100% sequence similarity with a given compound, *e.g.*, a peptide. Such analogues or derivatives may be comprised of non-naturally occurring amino acid residues, including by way of example and not limitation, homoarginine, ornithine, penicillamine, and norvaline, as well as naturally occurring amino acid residues. Such analogues or derivatives may also be composed of one or a plurality of D-amino acid residues, and may contain non-peptide interlinkages between two or more amino acid residues.

The term "effective amount" means a dosage sufficient to produce a desired result on a health condition, pathology, and disease of a subject or for a diagnostic purpose. The desired result may comprise a subjective or objective improvement in the recipient of the dosage. "Therapeutically effective amount" refers to that amount of an agent effective to produce the intended beneficial effect on health. An appropriate "effective" amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

"Treatment" refers to prophylactic treatment or therapeutic treatment or diagnostic treatment.

A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing pathology. The compounds or conjugates of the invention may be given as a prophylactic treatment to reduce the likelihood of developing a pathology or to minimize the severity of the pathology, if developed.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs or symptoms of pathology for the purpose of diminishing or eliminating those signs or symptoms. The signs or symptoms may be biochemical, cellular, histological, functional, subjective or objective. The compounds or conjugates of the invention may be given as a therapeutic treatment or for diagnosis.

"Diagnostic" means identifying the presence or nature of a pathologic condition. Diagnostic methods differ in their specificity and selectivity. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

"Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in subject animal, including humans and mammals. A pharmaceutical composition comprises a pharmacologically effective amount of a PAL polypeptide and also comprises a pharmaceutically acceptable carrier. A pharmaceutical composition encompasses a composition comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product which results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present invention encompass any composition made by admixing a compound or conjugate of the present invention and a pharmaceutically acceptable carrier.

"Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and excipients, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents and/or adjuvants. Suitable pharmaceutical carriers and formulations are described in Remington's Pharmaceutical Sciences, 19th Ed. (Mack Publishing Co., Easton, 1995). Pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (*e.g.*, oral) or parenteral (*e.g.*, subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical, transdermal, or transmucosal administration). A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound or conjugate for pharmaceutical use including, *e.g.*, metal salts (sodium, potassium, magnesium, calcium, *etc.*) and salts of ammonia or organic amines.

By "pharmaceutically acceptable" or "pharmacologically acceptable" is meant a material which is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of compound or conjugate of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular compound or conjugate employed and the effect to be achieved, and the pharmacodynamics associated with each compound or conjugate in the host.

By "physiological pH" or a "pH in the physiological range" is meant a pH in the range of approximately 7.2 to 8.0 inclusive, more typically in the range of approximately 7.2 to 7.6 inclusive.

As used herein, the term "subject" encompasses mammals and non-mammals. Examples of mammals include, but are not limited to, any member of the mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. The term does not denote a particular age or gender.

In various embodiments, the pegylation of PAL variant is described in reference to the ratio of PAL:PEG or PEG:PAL. As used herein, and unless otherwise indicated, the ratio of "PAL:PEG" refers to the ratio of lysine residues on the PAL variant to PEG molecules in the pegylation reaction. Similarly, as used herein, and unless otherwise indicated, the ratio of "PEG:PAL" refers to the ratio of PEG molecules to lysine residues on the PAL variant in the pegylation reaction.

B. Structure-based Protein Engineering

Numerous methods are known for protein engineering using rational optimization based primarily on protein structural information (Brannigan, *et al.*, Nat. Rev. Mol. Cell. Biol. 3(12):964-970 (2002); Marshall, *et al.*, Drug Discov. Today 8(5):212-221 (2003)). Systematic replacement of structural features can lead to improved protein properties and/or a redesign of substrate specificity. Recruitment of function from one member of a gene family into another homologous member can be accomplished by the

introduction of a limited number of amino acid substitutions in the immediate substrate binding vicinity. Such improvements in protein function by generating improved protein variants can lead to useful proteins for industrial, agricultural, and therapeutic applications (Bocanegra, *et al.*, Biochemistry 32(11):2737-2740 (1993); Failla, *et al.*, Fold Des. 1(1):35-42 (1996); Hayes, *et al.*, Proc Natl Acad Sci USA 99(25):15926-15931 (2002); Voigt, *et al.*, Nat. Struct. Biol. 9(7):553-558 (2002); Malashkevich, *et al.*, Nat Struct Biol. 2(7):548-553 (1995); Wells, *et al.*, Proc Natl Acad Sci USA 84(15):5167-5171 (1987); Wilks, *et al.*, Science 242(4885):1541-1544 (1988)).

C. THE STRUCTURE OF PAL

The three dimensional structure of wild-type prokaryotic PAL can be determined using x-ray crystallography. The PAL protein is a homotetramer, with each monomer consisting of mainly alpha-helices and subdivisible into four domains – a central catalytic domain, an N-terminal domain, and a small C-terminal domain with similarity to the Pseudomonas putida histidine ammonia-lyase structure (HAL, Schwede, *et al.*, Biochemistry 38(17):5355-5361 (1999)) plus an additional domain inserted in the C-terminal region that protrudes from the ends of the intact tetramer molecule. The N-terminal first 25 residues are not visible in the structure for all four monomers in the tetramer, and this region is probably disordered. The loop regions between residues 109-123 and 350-353 are disordered for monomer B, with regions 103-123 and 350-353 disordered for monomers A, C, and D in the PAL tetramer. Two other X-ray structures of Rhodosporidium toruloides PAL have been determined using trans-cinnamate and NH4 ion addition during the crystallization process, with resolutions of 2.1 Å (P3221 space group) and 2.7 Å (P21 space group; Calabrese, *et al.*, Biochemistry 43(36):11403-11416 (2004)). The lower pH used for crystallization and the lower resolution of these structures led to more inherent disorder in the structures (especially in the N-terminal regions) and the inability to unambiguously assign additional electron density present on the MIO cofactor to an NH2 adduct.

There are a number of related structures (using DALI, Holm, *et al.*, J. Mol. Biol. 233:123-138 (1993)) in this family of tetrameric enzymes that catalyze the elimination of various groups from carboxylic acids, including the ammonia-lyases (PAL, HAL, and aspartate ammonia-lyase (AAL)), fumarase, and arginosuccinate lyase (Schwede, *et al.*, Biochemistry 38(17):5355-5361 (1999)). In addition, the δ-crystallin avian eye lens protein

has a similar fold but is a non-enzymatic form of this structural family (Schwede, *et al.*, (1999) *ibid.*).

The three-dimensional X-ray structures of HAL from P. putida (Röther, *et al.*, Eur. J. Biochem. 268:6011-6019 (2001); Schwede, *et al.*, Biochemistry 27:5355-5361 (1999), and more recently of PAL from Rhodosporidium toruloides (Calabrese, *et al.*, Biochemistry 43:11403-11416 (2004)), revealed the active site residues of these tetrameric enzymes that are important for substrate binding, catalysis and MIO formation. All active site residues in HAL are present in EncP except for H83 and E414, which are replaced with valine and glutamine residues, respectively (Xiang, L., *et al.*, J. Biol. Chem. 277:32505-3250924 (2002)). H83 in HAL is proposed to bind and orientate the imidazole moiety of L-histidine at the active site and to stabilize an enzyme-bound cationic intermediate, whereas the carboxylate group of E414 may act as a base in catalysis.

The high-resolution three-dimensional protein crystal structure of PAL may be used in methods involving protein engineering to improve the biochemical and biophysical properties of PAL, and to increase the *in vivo* therapeutic effectiveness of PAL. In addition, the structure provides information regarding which regions of the structure are the most flexible (to remove and generate a more compact and stable form of PAL), which residues are located near the active site (to mutate in order to enhance activity and/or minimize the size of the protein as well as to provide information for structure-based inhibitor design), and which surface locations are close to immunogenic (*e.g.* linear epitopes identified in mapping studies) and/or proteolytic sensitive sites (from protease mapping studies), allowing for the introduction of site-specific mutants for direct disruption of problem sites or, alternatively, for surface pegylation or other chemical derivatization to protect sensitive sites present in native PAL.

D. USES OF THE STRUCTURE COORDINATES OF PAL

The high-resolution three-dimensional crystal structure of PAL may be used in computerized methods for selecting regions of the protein for mutation, modification, or combined mutation and modification. For example, the commercially available program GETAREA calculates surface-exposure for amino acid residues based upon X-ray crystallographic coordinates.

The high-resolution three-dimensional crystal structure can further be used in in-silico methods to design ligands for the active site of the enzyme. For example, commercially available software programs for docking and designing structure-based small-molecules can be used to design PAL inhibitors (Billett, *et al.*, Biochim Biophys Acta 524(1):219-230 (1978); Janas, *et al.*, Acta Biochim Pol. 32(2):131-143 (1985); Zon, *et al.*, Phytochemistry 59(1): 9-21 (2002); Alunni, *et al.*, Arch Biochem Biophys. 412(2):170-175 (2003)).

Structure-based PAL Engineering

The elucidation of a reliable three-dimensional structure or structural model for a specific macromolecule permits rational design to become a productive method for optimization of specific structure and/or function of said macromolecule (Penning, *et al.*, Chem Rev. 101(10): 3027-3046 (2001)). Optimization approaches include but are not limited to re-engineering of coenzyme specificity (Bocanegra, *et al.*, Biochemistry 32(11):2737-2740 (1993)), improvement of protein stabilities (Malakauskas, *et al.*, Nat Struct Biol. 5(6): 470-475 (1998); Jiang, *et al.*, Protein Sci. 10(7):1454-1465 (2001); Luo, Protein Sci. 11(5):1218-1226 (2002); Filikov, *et al.*, Protein Sci. 11(6):1452-1461 (2002); O'Fagain, Enz Microb Technol. 33:137–149 (2003); Cammett, *et al.*, J Mol Biol. 327(1):285-297 (2003)), redesign of substrate specificities (Hedstrom, *et al.*, Science 255(5049):1249-1253 (1992); Failla, *et al.*, Fold Des. 1(1):35-42 (1996); Malashkevich, *et al.*, Nat Struct Biol. 2(7):548-553 (1995); Wilks, *et al.*, Biochemistry 31(34):7802-7806 (1992); Feil, *et al.*, Protein Eng. 10(3):255-262 (1997); Whittle, *et al.*, J Biol Chem. 276(24):21500-21505 (2001)), alteration of ligand or receptor binding specificities (Cunningham, *et al.*, Proc Natl Acad Sci USA 88(8):3407-3411 (1991); Reddy, *et al.*, Nat Biotechnol. 14(13):1696-1699 (1996); Doyle, *et al.*, Curr Opin Chem Biol. 4(1):60-63 (2000)), and re-engineering of biological activities (Chen, *et al.*, Proc. Natl. Acad. Sci. USA 90(12): 5618-5622 (1993); Sarkar, *et al.*, Nat Biotechnol. 20:908-913 (2002); Blatt, *et al.*, J Interferon Cytokine Res. 16(7):489-499 (1996)).

Structure-based engineering can be used to generate PAL variants including rational mutants such as truncations, deletions, insertions, splice variants, point mutations, substitutions, chimeras, loop re-engineered mutants, loop swapping mutants, surface veneering mutants, as well as stochastically-derived mutants (including directed evolution-derived mutants, alone or in combination with rationally developed mutants). In addition, PAL variants can be made comprising PAL mutants wherein mutations have been introduced for site-specific pegylation and/or other chemical derivatizations. The PAL mutants for use

in such methods includes any PAL variant having substantially the same functional activity as wild-type R. toruloides PAL, including variants, fragments, and chemical derivatives of the parent PAL protein.

Directed Evolution Protein Optimization

Directed evolution methods randomly mutate gene(s) of interest to explore more completely larger regions of protein mutational space. Numerous directed evolution methods exist, including error-prone PCR and random insertion and deletion (RID) mutagenesis to introduce diversity throughout a DNA sequence, and more focused or "directed" diversity-generating methods such as site-saturation mutagenesis and other oligonucleotide-based mutagenesis methods (Brannigan, *et al.*, (2002) *ibid.*). In addition, DNA sequence recombinational methods have been used to combine advantageous sites of mutation and simultaneously remove deleterious mutations, producing novel DNA sequences (*e.g.*, methods of DNA shuffling, StEP, RACHITT, ITCHY). Finally, structure-based directed evolution techniques have been used to redesign proteins for therapeutic advantage (structure-based combinatorial engineering, SCOPE, and protein design automation, PDA, methods). The SCOPE method is based on a semi-rational protein engineering approach that uses protein structure information coupled with DNA manipulation techniques to design and create multiple crossover protein variant libraries from non-homologous genes (O'Maille, *et al.*, J Mol Biol. 321(4):677-691 (2002)). In PDA, a computational pre-screening of mutational space allows the inclusion of only mutations compatible with a specific protein fold, thus reducing the number of sequence variants to a size amenable to experimental screening (U.S. Patent No, 6,403,312; Dahiyat, *et al.*, Proc Natl Acad Sci USA 94(19):10172-10177 (1997); Dahiyat, *et al.*, Protein Sci. 6(6):1333-1337 (1997); Hayes, *et al.*, Proc Natl Acad Sci USA 99(25):15926-15931 (2002) Orencia, *et al.*, Nature Struct Biol. 8:238-242 (2001).

A large number of examples exist where the use of directed evolution (with coupling to an effective selection or screening protocol) has led to improved catalytic function and biophysical properties (*e.g.*, reduced immunogenicity, increased stability), starting from an initial enzyme species and mutating that species for altered and/or improved function (Vasserot, *et al.*, Drug Discovery Today 8(3):118-126 (2003)). For example, successful mutants have been obtained using directed evolution and other "random" mutagenesis methods on a number of different proteins (Triose-phosphate isomerase, Hermes, *et al.*, Proc Natl Acad Sci USA 87(2):696-700 (1990); Beta-lactamase, Stemmer,

W.P., Nature 370(6488):389-391 (1994), Orencia, M.C., *et al.*, Nature Struct Biol 8:238-242 (2001), Voigt, C. A., *et al.*, (2002) *ibid.*; para-nitrobenzyl esterase, Moore, *et al.*, Nature Biotechnol. 14:458-467 (1996); Galactosidase to fucosidase, Zhang, *et al.*, Proc Natl Acad Sci USA 94(9):4504-4509 (1997); Aspartate aminotransferase, Yano, *et al.*, Proc Natl Acad Sci USA 95(10):5511-5515 (1998); Green fluorescent protein, Crameri, *et al.*, Nat Biotechnol 14(3):315-319 (1996); Horseradish peroxidase, Lin, *et al.*, Biotechnol Prog 15: 467-471 (1999); Cytochrome P450, Joo, *et al.*, Nature 399(6737):670-673 (1999); Biphenyl dioxygenase, Kumamaru, *et al.*, Nat Biotechnol 16(7):663-666 (1998); Arsenate detoxification pathway, Crameri, *et al.*, Nat Biotechnol 15(5):436-438 (1997); Cephalosporinase, Crameri, *et al.*, Nature 391(6664):288-291 (1998); various proteins, Shao, *et al.*, Curr Opin Struct Biol 6(4):513-518 (1996), various proteins, Skandalis, *et al.*, Chem Biol 4:889-898 (1997); Subtilisin, Cunningham, *et al.*, Protein Eng. 1(4):319-325 (1987); Nitrilase, DeSantis, *et al.*, J Am Chem Soc. 125(38):11476-11477 (2003); Alpha-aspartyl dipeptidase, Kong, *et al.*, Biochem Biophys Res Commun. 289(1):137-142 (2001); Aspartate aminotransferase, Rothman, *et al.*, Protein Science 13(3):763-772 (2004); L-aspartase, Wang, *et al.*, Biochem Biophys Res Commun. 276(1):346-349 (2000); and lactate dehydrogenase, Wilks, *et al.*, Biochemistry 31(34):7802-7806 (1992).

These studies have repeatedly demonstrated the utility of applying "random" mutagenesis techniques to the development of improved enzyme variants with increased stability, activity, and resistance to degradative pathways. Structural analysis of evolved protein clones leads to insight on the molecular changes that are involved with the improved physical and chemical properties that are obtained (Orencia, *et al.*, in Advances in Protein Chemistry: Evolutionary protein design, F.H. Arnold, Editor, Academic Press: San Diego, pp. 227-259 (2001)). The rewards to be gained with directed evolution techniques are especially evident in light of the repeated occurrence of beneficial mutations that involve non-active site residues, with some sites of mutation located over 15-20 Å from enzyme active site regions having beneficial effects (Oue, *et al.*, J. Biol. Chem. 274(4):2344-2349 (1999)). Directed evolution and other random mutagenesis techniques, coupled to selection and screening procedures, can be used to develop more proteolytically stable and chemically robust forms of PAL to be used in industrial applications or, alternatively, for enzyme substitution therapy, *e.g.*, for PKU.

E. MODIFIED PAL

Previous experiments have described modified forms of PAL, such as PAL mutants (Schuster, *et al.*, FEBS Lett. 349(2):252-254 (1994); Schuster, *et al.*, Proc Natl Acad Sci USA 92(18):8433–8437 (1995); Langer, *et al.*, Biochemistry 36:10867-10871 (1997); El-Batal, *et al.*, Acta Microbiol Pol. 49(1):51-61 (2000); Röther, *et al.*, Eur. J. Biochem. 269:3065-3075 (2002)) and HAL mutants (Taylor, *et al.*, J. Biol. Chem. 269(44):27473-27477 (1994); Baedeker, *et al.*, Eur. J. Biochem. 269(6):1790-1797 (2002)).

Optimization of PAL Kinetics – Prokaryotic Mutants with Enhanced Catalytic Activity

The biologically active sites of wild-type PAL according to the invention may be modified as desired to optimize PAL kinetic characteristics. Km, the concentration of substrate that gives half-maximal activity, is intimately associated with the therapeutic efficacy of PAL in maintaining Phe levels within an acceptable range, *i.e.*, 120 μM to 240 μM. Km is the affinity of the enzyme for the substrate. By controlling affinity, one can limit or control the efficacy of any enzyme against substrate at different concentrations. For example, if Km is 1000 μM (Rhodosporidium toruloides), the activity of the enzyme will be reduced to about 12.5% at blood Phe levels of 240 μM and to about 3 % at blood Phe levels of 60 μM. If Km is 240 μM, the activity of the enzyme will be reduced to about 50% at blood Phe levels of 240 μM and to about 12 % at blood Phe levels of 60 μM. If Km is 120 μM, the activity of the enzyme will be reduced to about 70% at blood Phe levels of 240 μM and to about 35 % at blood Phe levels of 60 μM. Optimally, a therapeutic objective would be to have an enzyme with sufficient activity to reduce but also maintain Phe within the optimal range of about 120 μM to about 240 μM. An enzyme with a high Km (*i.e.*, 1000 μM) will lose activity rapidly as Phe levels drop to within normal range and will also require the impractical administration of highly concentrated or large volumes of doses. On the other hand, an enzyme with a very low Km may rapidly deplete Phe levels, which may be fatal for hyperphenylanemias but may be useful in the management of cancer.

In some embodiments, the biologically active modified PAL has a kcat of at least about 0.1 s-1 or greater than about 0.5 s-1. In other embodiments, the biologically active modified PAL has a kcat of at least about 0.2 s-1 or greater than about 1.0 s-1. In other embodiments, the biologically active modified PAL has a Km of between about 10 μM to about 1000 μM. In other embodiments, the biologically active modified PAL has a Km of between about 100 μM to about 1000 μM. In other embodiments, the biologically active modified PAL exhibits enzymatic activity that is from about two-fold to about 1000-fold times greater that that of the wild-type. In other embodiments, the biologically active

modified PAL exhibits enzymatic activity that is from about 10% to about 100% higher than that of the wild-type. Such biological active modified PAL proteins may be formed using methods well known in the art, such as by site-directed mutagenesis.

All active site residues in HAL were shown to be present in EncP except for H83 and E414, which are replaced with valine and glutamine residues, respectively (Xiang, L., *et al.*, J. Biol. Chem. 277:32505-32509 (2002)). The role of H83 in HAL in binding and orientating the imidazole moiety of L-histidine at the active site and in stabilizing an enzyme-bound cationic intermediate was investigated (Xiang, *et al.*, J. Bacteriology 187(12):4286-4289 (2005); Xiang, *et al.*, J. Bacteriology 188(14):5331 (2006)). It was proposed that the carboxylate group of E414 may act as a base in catalysis. In the study, EncP mutants were generated by site-directed mutagenesis to assess the contribution of V83 to cinnamic acid formation by EncP. Replacement of valine with histidine generated a mutant, V83H, which was characterized by a loss in PAL activity. Replacement of the valine with alanine resulted in a mutant, V83A, which was more active than the wild-type EncP V83A, had a slightly lower affinity to L-phenylalanine with a Km of 120 μM versus 23 μM for the wild-type enzyme. However, in comparison with wild-type EncP, V83A had a higher kcat higher and was more active than the wild-type enzyme.

Specific Protein Variants: Variants Having Reduced Immunogenicity

A number of strategies are currently used to reduce protein immunogenicity. In some embodiments, modifications that are introduced to minimize the immune response do not destroy the structure, function, or stability of the macromolecule. Effective strategies used include increasing human sequence content (chimeras and/or other 'humanization' approaches), improving solution properties, removing antibody epitopes, introducing chemical derivatization (such as pegylation), and/or identifying and removing MHC agretopes. For an injected therapeutic, *in vivo* immunoreactivity can be addressed by performing epitope mapping followed by rational mutagenesis to modify and/or otherwise mutate these sites of immunogenicity, alone and in combination with site-specific pegylation (Hershfield, *et al.*, Proc. Natl. Acad. Sci. USA 88:7185-7189 (1991); Leong, *et al.*, Cytokine 16(3):106-119 (2001); Lee, *et al.*, Pharm. Res. 20(5):818-825 (2003)) or other chemical derivatization methods to reduce protein immunoreactivity to an acceptable level. Modification of antigenic surface protein regions reduces immunogenicity (Chirino, *et al.*, Drug Discov. Today 9(2):82-90 (2004)). One method of improvement involves the construction of smaller sized proteins that retain catalytic activity (*e.g.*, an absorbance assay

is used for activity measurement). A second method of improvement, protein engineering coupled to ELISA screening, can also be used to identify mutants with reduced immunoreactivity. Another method introduces point mutations for additional surface Lys sites for pegylation derivatization, a method shown to reduce immunogenicity with the test enzyme purine nucleoside phosphorylase (Hershfield, *et al.* (1991) *ibid.*). An alternative pathway uses mutation of residues located in protein epitope regions to remove immunogenic sites (Yeung, *et al.*, J Immunol. 172(11):6658-6665 (2004)). In an approach that is analogous to antibody humanization, homologous loop regions and/or residues from human antibodies are substituted into the corresponding loop regions of a homologous protein.

Improving solution properties of proteins may increase specific enzyme activity and/or reduce immunogenicity. One solution property typical of bacterially expressed recombinant proteins is the formation of protein aggregates due, for example, to inter-chain disulfide bind formation, hydrophobic interactions and/or divalent cations (Chi, *et al.*, Pharm Res 20(9):1325-1336 (2003)). Aggregation of recombinantly expressed proteins can enhance the immune response (Hermeling, *et al.*, Pharm Res 21(6):897-903 (2994); Schllekens, Nephrol Dial Transplant 20(suppl 6):vi3-9 (2005)). One method of improvement involves substituting surface cysteine residues with other amino acid residues (*e.g.*, serine) to minimize the possibility of formation of inter-chain disulfide bonds. For example, substitution of two surface cysteine residues with serine residues reduced the aggregation of chorismate lyase with minor effects on enzyme activity (Holden, *et al.*, Biochim Biophys Acta 1594(1):160-167 (2002)).

Removal of protein therapeutic proteolytic processing sites can also provide a reduction in immunogenicity by preventing proteasomal processing, thereby preventing clipping and processing into peptide fragments for antigen presenting cell binding. A similar phenomenon has been observed in the alteration of flanking regions for class II MHC determinants, preventing display to autoreactive T cells (Maverakis, *et al.*, Proc Natl Acad Sci, USA 100(9):5342-5347 (2003)).

Epitope Mapping

Protein therapeutic epitopes can be calculated using a number of algorithms or experimentally determined with *in vitro* or *in vivo* approaches. Computer programs such as "Peptide Companion" and "Protean" in the Lasergene program suite from DNAStar are commonly used to estimate surface epitope regions of a protein based on the chemical

composition and conformation of a protein. Immunogenic regions in a protein sequence are those regions of highest calculated hydrophilicity, based on a hydrophilicity index, and antigenicity, based on the amphipathicity and other conformational parameters of calculated surface protein regions. Alternatively, agretopes in a protein sequence can be located based on computer-modeled predictions of potential HLA binding (Robinson, *et al.*, Nucleic Acids Res. 31(1):311-314 (2003); De Groot, *et al.*, Novartis Found. Symp. 254:57-72 (2003)). In addition, epitopes can be identified using *in vitro* biochemical (Tangri, *et al.*, Curr. Med. Chem. 9(24):2191-2199 (2002)) and *in vitro* cell-based methods (Stickler, *et al.*, J Immunother. 23(6):654-660 (2000); Stickler, *et al.*, J Immunol Methods 281(1-2):95-108 (2003)). For protein engineering, the relative reduction in immunogenicity can be monitored using assays similar to the *in vitro* cell-based assay of Stickler, *et al.* (Toxicol Sci. 77(2):280-289 (2004)).

Pepscan analysis (epitope mapping) involves use of a library of overlapping peptides covering surface regions of the enzyme sequence and probing with rabbit polyclonal antibodies raised against overlapping peptides covering the entire enzyme protein sequence, and thereby revealing linear epitopes present in the enzyme. Based on the three-dimensional structure of the enzyme, the experimentally identified sites of antigenicity are mutated by either randomly mutating epitope region residues to remove the epitope recognition sites or using site-specific mutation to introduce Lys or Cys residues on the surface near these epitope sites to provide locations for pegylation to cover and protect these sites from immunogenic recognition. ELISA screening of these potentially non-immunogenic enzyme mutants (or pegylated forms of these enzyme mutants) provides *in vitro* identification of subsets of enzyme mutants that display decreased immunoreactivity.

Surface Residue Identification and Mutation

The surface-exposed residues in or near the immunogenic regions of the enzyme can be identified. These locations will be a subset of the total number of solvent-accessible surface locations present in the protein, dependent upon proximity to the surface as well as proximity to regions of immunogenicity/antigenicity. The three-dimensional structure of the protein, determined using X-ray crystallography, NMR, or homology modeling, can be used in commercially available software programs to calculate macromolecule solvent-accessible surface area. The output provided using the program GETAREA 1.1 (Fraczkiewicz, *et al.*, J. Comp. Chem. 19:319-333 (1998)) gives a reliable estimate of surface accessibility for R. toruloides PAL. GETAREA and PARAREA and

similar programs calculate solvent-accessible surface area using continuum methods with a parametric approach based on the Gauss-Bonnet theorem. PARAREA finds potential intersection points in the Gauss-Bonnet path using all atom pairs in the neighbor list of each atom, whereas GETAREA more efficiently calculates the solvent-exposed vertices using intersection half-spaces defined by planes of two-sphere intersections.

In native PAL, GETAREA identified twelve surface-exposed Lys residues, distributed throughout the tetrameric PAL protein surface, as well as one partially exposed surface Cys residue (Cys140). These positions are directly available for pegylation derivatization. Further, the three-dimensional structure of PAL can be used to identify additional surface residues available for site-directed mutagenesis. These additional sites can be mutated using standard protein engineering techniques to generate more favorable PAL mutants with reduced immunogenicity, improved proteolytic resistance, and/or improved stability/activity.

Strategies for mutation of the protein to provide protein mutants with improved properties, such as reduced immunogenicity are known in the art. One popular route mutates every non-alanine residue in an epitope to Ala, and mutates every alanine residue in an epitope to Gly. Other methods remove charged and hydrophobic residues from epitope regions, either by mutation or deletion. Additional mutation strategies introduce site-specific mutations followed by site-specific chemical derivatization.

Truncations can also be used for the production of protein improvements. Bioinformatics analysis of PAL relative to histidine ammonia-lyase (HAL) has suggested truncation mutants (residues 23-716 and 1-564). In addition, analysis of the PAL 3-D structure provides an alternative region for truncation, based on the absence of a C-terminal domain region in the structure of the highly homologous HAL protein. C-terminal domain deletion mutants, including mutants where the corresponding loop region from HAL is fused into the PAL sequence to substitute for the C-terminal domain protruding region of the PAL structure (calculated to have major epitope regions), form smaller, more robust, and predicted less immunogenic variants of PAL.

In another method, protein engineering using rational mutagenesis in combination with directed evolution can be used to obtain mutant forms of PAL. For example, implementation of rational design along with experimental and combinatorial design has improved the activity of compstatin (Morikis, *et al.*, Biochem. Soc. Trans.

32(1):28-32 (2004)). The combination of rational mutagenesis and directed evolution has also shown promise (Bornscheuer, *et al.*, Curr. Opin. Chem. Biol. 5(2):137-143 (2001); Dwyer, *et al.*, Science 304(5679):1967-1971 (2004)).

Mutant forms of PAL that display less than optimal enzymatic activity can be evolved for activity using the protein engineering method of directed molecular evolution, wherein a random mutagenesis step is followed by a selection procedure for mutants that are active, producing a new protein mutant pool of only those clones that have activity. For example, if a specific mutant PAL (that we have designed to introduce a surface lysine residue near an epitope-containing region of the structure) is inactive, performing directed evolution on this mutant (mutating the inactive mutant and selecting for transformants with activity) generates a new mutant pool containing this beneficial mutation (the surface lysine site) plus a subset of additional random mutations located throughout the structure that restore activity to each active clone. In addition, the directed evolution technique of molecular breeding that can cross different species of the same protein or similar sequences of different proteins (Crameri, *et al.* Nature, 391:288-291 (1998); Minshull, *et al.*, Curr. Opin. Chem. Biol. 3(3):284-290 (1999)) can substitute the sequence of human histidine ammonia-lyase to replace some of the most immunogenic regions of PAL with a human sequence that won't be recognized by the immune system.

Mutant forms of PAL can be obtained by making hybrid protein variants based on semi-rational approaches thereby allowing for a more directed level of protein design relative to pure directed evolution-based methods, as well as providing for a more random level of mutational manipulation for those regions of a protein structure identified using such biased protein engineering approaches. For example, the PDA method generates computationally pre-screened libraries of protein variants, allowing for more rapid optimization of protein properties over pure random, or non-biased protein engineering methods (U.S. Patent No. 6,403,312; Dahiyat, Curr Opin Biotechnol. 10(4):387-390 (1999); Filikov, *et al.*, (2002) *ibid.*; Hayes, *et al.*, (2002) *ibid.*; Luo, P., *et al.*, (2002) *ibid.*). Similarly, the SCOPE method constructs hybrid enzyme variants based on "equivalent" subdomains of structure, allowing the creation of multiple crossover protein variant libraries starting from non-homologous genes (O'Maille, *et al.*, (2002) *ibid.*). In a similar fashion, the method developed by Voigt, *et al.*, constructs hybrids based upon the application of a computational algorithm that identifies fragments of proteins, or 'schemas,' that can be recombined without disrupting the integrity of a protein's three-dimensional structure (Voigt, *et al.*, (2002) *ibid.*).

Computational analysis can also identify conserved core regions of structurally and functionally important positions in a protein fold, based on secondary structural element assignments (Mizuguchi, *et al.*, Bioinformatics 16(12):1111-1119 (2000)). Mutant forms of PAL can be obtained following the procedures of any of the art methods.

HAL as an Alternative Enzyme Substitute

The structure of encP from Streptomyces maritimus has been homology modeled based on the structure of P. putida HAL. Although smaller than PAL, the structure of S. maritimus is more similar to human PAL, making encP a possibly less immunogenic form of an ammonia-lyase to use for PAL protein engineering. An alternative method for obtaining non-immunogenic PAL variants involves mutation of S. maritimus encP or other prokaryotic PAL to replace immunogenic sequences by human histidine ammonia-lyase sequences (HAL, Suchi, *et al.*, Biochim. Biophys. Acta 1216(2):293-295 (1993)), using mutation followed by selection for encP or other prokaryotic mutants with PAL activity, to obtain a "humanized" encP or other prokaryotic PAL protein that can be further pegylated following the steps of mutation and selection for activity.

Specific Protein Variants: Variants Having Reduced Protease Sensitivity

A number of strategies are currently used to reduce protein protease susceptibility. In some embodiments, the modifications introduced to minimize proteolytic sensitivity do not destroy the structure, function, or stability of the macromolecule. Effective strategies include providing an enzyme with an active-site stabilizing species such as a competitive inhibitor (Gilbert, *et al.*, (1981) *ibid.*; U.S. Patent Nos. 6,548,644; 6,451,986; 6,433,158; 6,461,849), chemically modifying the amino groups in susceptible Lys and/or Arg sites to block trypsin binding sites, mutating chymotrypsin-sensitive Tyr, Phe, and/or Trp residues to smaller neutral amino acids to abolish cleavage susceptibility, ligate or couple PAL to other protective macromolecules (such as Fc antibody domains or the botulinum neurotoxin nontoxic components), and/or introducing Lys or Cys sites near protease susceptible sites for subsequent chemical derivatization with PEG or other chemically protective and stabilizing groups.

F. CHEMICALLY MODIFIED PAL VARIANTS

Macromolecule chemical modification can be performed in a non-specific fashion (leading to mixtures of derivatized species) or in a site-specific fashion (based on

wild-type macromolecule reactivity-directed derivatization and/or site-selective modification using a combination of site-directed mutagenesis and chemical modification) or, alternatively, using expressed protein ligation methods (Hofmann, *et al.*, Curr Opin Biotechnol. 13(4):297-303 (2002)). In some embodiments, chemical modification is used to reduce immunogenicity. Pegylation is a demonstrated method to reduce immunogenicity of proteins (Bhadra, *et al.*, Pharmazie 57(1):5-29 (2002)), but glycosylation and other chemical derivatization procedures, using modification with phosphorylation, amidation, carboxylation, acetylation, methylation, creation of acid-addition salts, amides, esters, and N-acyl derivatives are also possible (Davis, Science 303:480-482 (2004)).

Pegylated Proteins

A series of different pegylation reactions on PAL, using a range of PEG chemical reagent to PAL protein ratios, will provide PEG-PAL derivatives for each modification method. The optimal degree of pegylation can be determined based upon the residual activity obtained for each derivatized PAL species using the absorbance assay in combination with PAGE and native gel analysis to determine the extent of PEG derivatization. After initial ranges of optimal modification are determined, comparative kinetic analysis (including Vmax and Km determinations, binding constants of substrates, proteolytic stability, pH dependence of activity, temperature-dependence of activity) and immunoreactivity of optimal PEG-PAL species can be determined by ELISA, immunoprecipitation, and Western blot. Protein engineering can also be used to generate the most favorable PAL mutant for pegylation using the optimal derivatization conditions; by minimizing the size of the PAL protein and only modifying the most antigenic regions of the PAL surface, cost of PEG modification will be reduced while at the same time retaining the maximum amount of enzymatic activity and minimum amount of immunogenicity. Similarly, site-specific pegylation can be used to provide enzyme derivatives.

Other chemical modifications such as phosphorylation or other chemical modification of Lys, Arg, and Cys residues can be used to mask immunogenic regions and/or proteolytic sensitive regions. Such chemical modifications include the polymer addition method of Bednarsaki and the Altus Corporation cross-linking method for improving PAL stability, reducing immunogenicity, and improving protease resistance are representative examples. Bednarsaki demonstrated that polymer addition improves protein temperature stability (Wang, *et al.*, J. Am. Chem. Soc. 114(1):378-380 (1992)), and Altus Corporation has found that glutaraldehyde cross-linking improves enzyme stability.

To discover if the *in vivo* therapeutic half-life of a protein such as PAL would benefit from pegylation, a variety of different PEG:PAL conjugates are synthesized, characterized *in vitro* and tested *in vivo* for L-Phe reduction. In order to both optimize the potential effects of pegylation and to identify the preferred sites of PEG attachment, a design strategy is employed wherein polymer length, conformation, and the degree of PEG attachment is varied.

Methods for preparing the pegylated PAL of the present invention generally comprise the steps of (a) reacting PAL with polyethylene glycol under conditions whereby PAL becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). Because the specific sites of PAL modification might significantly alter the intrinsic activity of the conjugate, different types and amounts of PEG were explored. The chemistry used for pegylation of PAL was the acylation of the primary amines of PAL using the NHS-ester of methoxy-PEG (O-[(N-Succinimidyloxycarbonyl)-methyl]-O'-methylpolyethylene glycol). Acylation with methoxy-PEG-NHS or methoxy-PEG-SPA results in an amide linkage that eliminates the charge from the original primary amine.

The present methods provide for a substantially homogenous mixture of polymer:protein conjugate. "Substantially homogenous" as used herein means that only polymer:protein conjugate molecules are observed. The polymer:protein conjugate has biological activity and the present "substantially homogenous" pegylated PAL preparations provided herein are those which are homogenous enough to display the advantages of a homogenous preparation, *e.g.*, ease in clinical application in predictability of lot to lot pharmacokinetics.

The polymer molecules contemplated for use in the pegylation approaches described herein may be selected from among water-soluble polymers or a mixture thereof. The water-soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, monomethoxy-polyethylene glycol, dextran, poly-(N-vinyl pyrrolidone), propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (*e.g.*, glycerol), HPMA, Fleximer.TM., and polyvinyl alcohol, mono-(C1-C10)alkoxy-PEG, aryloxy-PEG, tresyl monomethoxy PEG, PEG propionaldehyde, bis-succinimidyl carbonate PEG, cellulose, or other carbohydrate-based polymers. The polymer selected should be water-soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer may be branched or unbranched. In some embodiments, for

therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable.

In some embodiments, a water-soluble polymer for use herein is polyethylene glycol, abbreviated PEG. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

The proportion of polyethylene glycol molecules to protein molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by the molecular weight of the polyethylene glycol selected and on the number of available reactive groups (typically ε amino groups) present. As relates to molecular weight, in general, the higher the molecular weight of the polymer used, the fewer number of polymer molecules which may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. The present invention contemplates several different linear PEG polymer lengths including but not limited to 5 kDa and 20 kDa, conjugates of two-armed branched PEG polymers, including but not limited to 10 kDa and 40 kDa. In some embodiments, for the PEGylation reactions contemplated herein, the average molecular weight is about 2 kDa to about 100 kDa (the term "about" indicating +/-1 kDa). In other embodiments, the average molecular weight is about 5 kDa to about 40 kDa. The ratio of water-soluble polymer to PAL will generally range from 1:1 for monoPEG, 2:1 for diPEG, *etc.*

Examples 6 through 8 describe the effects of pegylated and nonpegylated forms of lysine mutant R91K PAL from *Rhodosporidium toruloides,* PAL produced by the cyanobacterium *Nostoc punctiforme (*NpPAL), and PAL produced by the cyanobacterium *Anabaena variabilis* (AvPAL) on phenylalanine levels in the ENU2 or $BTBR^{enu2}$ mouse over a 12 day-period. This animal model is a homozygous mutant at the phenylalanine hydroxylase gene (PAH) locus resulting in an animal with severe hyperphenylalanemia. Thus, the high plasma phenylalanine (Phe) levels make this animal the appropriate model for evaluating the ability of phenylalanine ammonia-lyase (PAL) to reduce plasma Phe. The effects of pegylated and nonpegylated forms of lysine mutant R91K PAL and NpPAL on phenylalanine levels in this PKU animal model were also evaluated over a 90-day period. The results showed that pegylated forms of NpPAL and AvPAL resulted in greater reduction

in phenylalanine in the PKU animal model in comparison with unpegylated NpPAL and AvPAL, respectively. Further, such effects of pegylated NpPAL to reduce elevated phenylalanine levels were maintained over a ten-week period. These studies show that pegylation of PAL from the cyanobacteria, *Nostoc punctiforme* and *Anabaena variabilis*, is essential in reducing phenylalanine levels in the PKU affected mice. The findings suggest that a pegylated variant of bacterial PAL may serve as an effective therapeutic in the management of PKU.

The invention contemplates pegylated PAL variants with reduced immunogenicity. One embodiment is a pegylated form of Nostoc punctiforme PAL (NpPAL) variant with reduced immunogenicity. Another embodiment is a pegylated form of Anabaena variabilis PAL (AvPAL) variant with reduced immunogenicity. Specific embodiments contemplate NpPAL or AvPAL variants in which pegylation is achieved by reacting the NpPAL or AvPAL variant with a water-soluble polymer, *e.g.*, polyethylene glycol (PEG). In some embodiments, pegylation is achieved by reacting the NpPAL or AvPAL variant once with PEG at a ratio of at least 1:1, at least 1:1.5, at least 1:2, at least 1:3, or at least 1:4 PAL:PEG. In one embodiment, the PAL variant is an AvPAL variant, and the pegylation is achieved using a PAL:PEG ratio of 1:3. Methods for preparing the pegylated PAL variants of the present invention are described above.

The invention also contemplates re-pegylating the pegylated PAL variants for reduced immunogenicity. In some embodiments, the pegylation is achieved by reacting the NpPAL or AvPAL variant twice or more with PEG, each pegylation at a ratio of at least 1:1, at least 1:1.5, at least 1:2, at least 1:3., or at least 1:4 PAL:PEG. In some embodiments, the pegylation is achieved by reacting the NpPAL or AvPAL variant twice, three times, four times, five times, or more, with PEG, each pegylation at a ratio of at least 1:1, at least 1:1.5, at least 1:2, at least 1:3., or at least 1:4 PAL:PEG. In another embodiment, the PAL variant is an AvPAL variant, and the pegylation is achieved using a PAL:PEG ratio of 1:3 for both the first and second pegylation reactions.

Methods for preparing the re-pegylated PAL variants of the present invention generally comprise the steps of (a) reacting a PAL variant with polyethylene glycol under conditions whereby the PAL variant becomes attached to one or more PEG groups, (b) obtaining the reaction product(s), (c) reacting the pegylated PAL variant with polyethylene glycol under conditions whereby the pegylated PAL variant becomes attached to one or more PEG groups at positions not occupied by PEG from step (a), and (d) obtaining the reaction

product(s). Because the specific sites of PAL modification might significantly alter the intrinsic activity of the PAL vatiant, different types and amounts of PEG were explored. The chemistry used for pegylation of PAL was the acylation of the primary amines of PAL using the NHS-ester of methoxy-PEG (O-[(N-Succinimidyloxycarbonyl)-methyl]-O'-methylpolyethylene glycol). Acylation with methoxy-PEG-NHS or methoxy-PEG-SPA results in an amide linkage that eliminates the charge from the original primary amine.

G. Selection and Screening Assays

For production and screening of active PAL or HAL variants, initial mutant clone expression can utilize any of the known vector expression systems, such as the His-tag vector expression system, facilitating a high-throughput metal chelate purification step for protein variant isolation. A three-tier screening system can be used to identify favorable protein variants. Initial positive clone identification can use transformation and selection for growth in a phenylalanine auxotrophic *E. coli* strain. A second round of screening can utilize the OD290 absorbance measurement (Hodgins, Biochem. Biophys. Res. Commun., 32:246-253 (1968)) amenable to high-throughput processing. Finally, screening for proteolysis resistance (using incubation in the presence of protease cocktail) or, alternatively, immunogenicity reduction (using competitive ELISA measurements), can be used to identify favorable protein variants.

H. Therapeutic Uses and Administration of Optimized PAL Proteins

1. Various Forms of Hyperphenylalanemia (HPA)

The present invention is directed to the treatment of a variety of HPA patient populations with methods that comprise the use of PAL compositions, either alone or in combination with other therapeutic regimens, for managing HPA and/or PKU. In particular, it is contemplated that PAL compositions may be used to treat that patient population with phenylalanine concentrations that are low enough that dietary intervention is not normally used (*i.e.*, patients with mild HPA), patients with moderate PKU, patients with classic or severe PKU, and any subpopulations thereof. Such patients that are amenable to treatment with PAL compositions to ameliorate the effects of mild HPA include pregnant women and

infants with serum concentrations of less than 200 μM. The various patient populations, and their different therapeutic needs, are discussed in further detail in the present section.

Certain embodiments of the present invention are directed to treating classic severe PKU by administering to the subject a protein-restricted diet in combination with a composition comprising PAL or a biologically active variant, mutant, or fragment thereof, wherein the combined administration of the protein-restricted diet and PAL is effective to lower the phenylalanine concentration in the plasma of said subject as compared to said concentration in the absence of said combined administration. In addition, the invention also contemplates treating a pregnant female that has HPA by administering to the female a protein-restricted diet in combination with PAL or a biologically active derivative thereof, such that the combined administration of the protein-restricted diet and PAL is effective to lower the phenylalanine concentration in the plasma of the pregnant woman as compared to such a concentration in the absence of said combined administration. In specific embodiments, therapy is contemplated for a patient who manifests Phe levels greater than 420 μM.

Other embodiments of the invention entail administering a PAL composition to any individual that has HPA, characterized by a plasma Phe concentration greater than 180 μM prior to the administration of PAL, in an amount effective to produce a decrease in such a plasma Phe concentration of the patient. The methods of the invention will be useful in treating an infant having PKU characterized by an elevated Phe concentrations of between greater than 300 μM with PAL compositions described herein. By "infant" the present application refers to a patient that is between the ages of 0 to about 36 months.

Characteristics of Severe Classical PKU and Methods of Treatment Thereof

Severe PKU manifests in a plasma Phe concentration greater than 1200 μM and may be found to be as high as 4800 μM. Patients that have this disorder must be treated with a Phe-free diet in order to bring their plasma Phe concentrations down to a level that is clinically acceptable (typically, less than 600 μM or less than 300 μM). These patients are only able to tolerate a maximum of between 250-350 mg dietary Phe per day (Spaapen *et al.*, Mol. Genet Metab. 78:93-99 (2003)). As such, these patients are started on a Phe-restricted formula diet between 7-10 days after birth and are burdened with this dietary restriction for the remainder their lifespan. Any alleviation of the strict dietary restrictions that these individuals are encumbered with would be beneficial.

The tests used for the diagnosis of individuals with classical Phe are described in further detail below. These tests have revealed that patients with classical severe PKU require a low phenylalanine diet (Lucke *et al.*, Pediatr. Neurol. 28:228-230 (2003)). Thus, it is contemplated that the methods of the invention will entail determining that the patient is suffering from classical PKU by monitoring the plasma Phe concentration of the individual. The patient is then treated by administering prokaryotic PAL compositions alone or a combined regimen of a low protein diet and PAL such that there is produced at least a 25% decrease in the plasma Phe concentrations of the patient. In some embodiments, the method will produce a 30% decrease in the plasma Phe concentration. In other embodiments, the method will produce a 40%, 50%, 60%, 70%, 80%, 90% or greater decrease in the plasma Phe concentration of the individual (for example, where a patient with severe classical PKU has a Phe concentration of 4800 μM a 90% decrease in the Phe concentration will produce a plasma Phe concentration of 480 μM, a concentration that is sufficiently low to require little dietary restriction). Of course, it should be understood that the treatment methods of the present invention (whether for treating severe classical PKU or any other HPA described herein), should attempt to lower the plasma Phe concentrations of the patient to levels as close to a range of about 120 μM to about 360 μM ± 15 μM as possible, or to an optimal range of about 120 μM to about 240 μM.

In some embodiments, the plasma Phe concentrations of the classical PKU patient being treated is reduced from any amount of unrestricted plasma Phe concentration that is greater than 1000 μM to any plasma Phe level that is less than 600 μM. Of course, even if the combined treatment with PAL and the protein-restricted diet produces a lesser decrease in plasma Phe concentration, *e.g.*, to a level of between 800 μM to about 1200 μM, this will be viewed as a clinically useful outcome of the therapy because patients that have a plasma Phe concentration in this range can manage the disease by simply restricting the amount of protein in the diet as opposed to eating a Phe-restricted formula, thereby resulting in a marked improvement in the quality of life of the individual, as well as leading to greater patient compliance with the dietary restriction.

Any increase in the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering the PAL therapy, the patient will be able to increase his/her intake of dietary Phe from 250-350 mg/day to 350-400 mg/day (*i.e.*, the Phe tolerance phenotype of the patient is altered from that of a classic

PKU patient to a moderate PKU patient). Of course, it would be desirable that the therapeutic intervention taught herein would allow the patient to increase his/her intake of dietary Phe from 250-350 mg/day to 400-600 mg/day (*i.e.*, the Phe tolerance phenotype of the patient is altered from that of a classic PKU patient to a mild PKU patient), or in some cases, to allow the patient to have an intake of greater than 600 mg Phe/day (*i.e.*, normal dietary intake).

Characteristics of BH4-non-responsive PKU Patients and Methods of Treatment Thereof

A second group of patients that can be treated with the methods of the present invention are those individuals that have been determined to have an elevated plasma Phe concentrations *i.e.*, any concentration that is greater than 200 μM, but have been diagnosed to be non-responsive to BH4 therapy (as determined by the BH4 loading test described below). Such patients may include those individuals that have mild PKU (*i.e.*, plasma Phe concentrations of up to 600 μM), individuals that have moderate PKU (*i.e.*, plasma Phe concentrations of between 600 μM to about 1200 μM), as well as patients that have classic severe PKU (*i.e.*, plasma Phe concentrations that are greater than 1200 μM).

The patients that are non-responsive to BH4 therapy are given PAL in combination with a reduced amount of protein in their diet in order to decrease the plasma Phe concentrations of the patient. The methods of the present invention are such that the administration of PAL produces a greater decrease in the plasma Phe concentrations of the patient as compared to the decrease that is produced with the same dietary protocol administered in the absence of PAL therapy. The dietary restrictions may be a diet that restricts the Phe intake by providing a synthetic medical protein formula that has a diminished amount of Phe or alternatively, the dietary restriction may be one which simply requires that the patient limit his/her overall protein intake but nevertheless allows the patient to eat normal foodstuffs in limited quantities.

The therapeutic outcomes discussed for classical PKU patients are incorporated into the present section by reference. The therapeutic outcomes for patients with moderate PKU (*i.e.*, patients that has an unrestricted plasma Phe concentration of 600 μM to 1200 μM) include at least a 25% decrease in the plasma Phe concentrations of the patient. In some embodiments, the method will produce a 30% decrease in the plasma Phe concentration. In other embodiments, the method will produce a 40%, 50%, 60%, 70%, 80%, 90% or greater decrease in the plasma Phe concentration of the individual (for example,

where a patient with moderate classical PKU has a Phe concentration of 1000 μM, a 90% decrease in the Phe concentration will produce a plasma Phe concentration of 100 μM, a concentration that is sufficiently low to require little or no dietary restriction).

In some embodiments, the plasma Phe concentrations of the moderate PKU patient being treated is reduced from any amount of unrestricted plasma Phe concentration that is between 600 μM to 1200 μM to any plasma Phe level that is less than 300 μM. In one embodiment, treatment with PAL (either alone or in combination with a dietary restriction) produces a decrease in plasma Phe concentration, *e.g.*, to a level of between 200 μM to about 400 μM, which will be viewed as a clinically useful outcome of the therapy because patients that have a plasma Phe concentration in this range can manage the disease by simply restricting the amount of protein in the diet as opposed to eating a Phe-restricted formula. Indeed, in many studies, it is taught that such patients may even eat a normal diet.

Any increase in the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering the PAL therapy (either alone or in combination with other therapeutic intervention), the patient will be able to increase his/her intake of dietary Phe from 350-400 mg/day to 400-600 mg/day (*i.e.*, the Phe tolerance phenotype of the patient is altered from that of a moderate PKU patient to a mild PKU patient). Of course, it would be desirable that the therapeutic intervention taught herein would allow the patient to increase his/her intake of dietary Phe from 350-400 mg/day to have an intake of greater than 600 mg Phe/day (*i.e.*, normal dietary intake).

Even if the patient being treated is one who manifests only mild PKU, *i.e.*, has a dietary allowance of 400-600 mg Phe intake/day) will benefit from the PAL-based therapies of the present invention because it is desirable to produce a normalized plasma Phe concentration that is as close to 360 μM ± 15 μM as possible. For such patients, an advantageous therapeutic outcome will include at least a 25% decrease in the plasma Phe concentrations of the patient. In one embodiment, the method will produce a 30% decrease in the plasma Phe concentration. In another embodiment, the method will produce a 40%, 50%, 60%, or greater decrease in the plasma Phe concentration of the individual (for example, where a patient with mild PKU has a Phe concentration of 600 μM, a 60% decrease in the Phe concentration will produce a plasma Phe concentration of 360 μM, *i.e.*, an acceptable, normal concentration of plasma Phe).

In some embodiments, the plasma Phe concentrations of the mild PKU patient being treated is reduced from any amount of non-restricted plasma Phe concentration that is between 400 μM to 600 μM to any plasma Phe level that is less than 100 μM. Of course, even if the treatment with PAL (either alone or in combination with a dietary restriction) produces a lesser decrease in plasma Phe concentration, *e.g.*, to a level of between 200 μM to about 400 μM, this will be viewed as a clinically useful outcome of the therapy.

Any increase the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering PAL therapy (either alone or in combination with other therapeutic intervention), the patient will be able to increase his/her intake of dietary Phe from 400-600 mg/day (*i.e.*, the Phe tolerance phenotype of the patient is altered from that of a mild PKU patient to a mild HPA patient) to allow the patient to have an intake of greater than 600 mg Phe/day (*i.e.*, normal dietary intake).

Furthermore, even if the patient is one who only manifests the symptoms of non PKU HPA, *i.e.*, has an elevated plasma Phe concentration of up to 600 μM, but is otherwise allowed to eat a normal protein diet will benefit from PAL therapy of the invention because it has been shown that elevated Phe concentrations have significant effects on the IQ of such individuals. Moreover, as discussed below, PAL therapeutic intervention of subjects with special needs, *e.g.*, pregnant women and infants, is particularly important even if that patient's plasma Phe levels are within the perceived "safe" level of less than 200 μM.

Maternal PKU and Methods of Treatment Thereof

Metabolic control of plasma Phe levels in PKU women planning conception and those who are pregnant is important because of the serious consequences to the fetus exposed to even moderately elevated Phe levels *in utero*, regardless of the PAH status of the fetus. Therapeutic control of plasma Phe concentration is especially important in the first trimester of pregnancy, as failure to achieve adequate control will result in disorders including microcephaly, mental deficiency and congenital heart disease.

For example, the NIH Consensus Statement (vol 17 #3, October 2000) on phenylketonuria reported that exposure of a fetus to maternal Phe levels of 3-10 mg/dL produced a 24% incidence of microcephaly, whilst those exposed to greater than 20 mg/dL (*i.e.*, greater than 1200 μM) had a 73% incidence of microcephaly. Likewise congenital heart disease was found in over 10% of children exposed to maternal Phe levels that were greater

than 20 mg/dL. Importantly, it has been noted that levels of Phe greater than 6 mg/dL significantly decrease the IQ of the child. Thus, it is imperative to ensure that the plasma Phe concentration of women with all forms of phenylketonuria, even those manifesting the mildest HPA, must be tightly controlled in order to avoid the risk of maternal PKU syndrome. However, the acceptable target levels for the plasma Phe concentrations of PKU women that have been used in U.S. clinics have ranged between 10 mg/dL and 15 mg/dL, which are much higher than the 2-6 mg/dL levels recommended for pregnant women or the 1-4 mg/dL that are used in British and German clinics to diminish the risks of developing maternal PKU syndrome.

Another important consideration for pregnant women is their overall protein intake. During pregnancy, it is important that women eat sufficient protein because it has been suggested that a low protein diet during pregnancy will result in retarded renal development and subsequent reduction in the number of nephrons and potentially leads to hypertension in adulthood. (D'Agostino, N. Engl. J. Med. 348(17)1723-1724, (2003)). The following table provides exemplary guidelines for the recommended total dietary protein intake for various individuals.

Table 1: United States Guidelines for Dietary Protein Requirements

	Age	Recommended Total Protein Intake (g)
Infant	6 months or less	13
	6 months-1 year	14
	1-3 years	16
Children	4-6 years	24
	7-10 years	28
Males	11-14 years	45
	15-18 years	59
	19-24	58
	25-50	63
	51+	63
Females	11-14 years	46
	15-18 years	44
	19-24	46
	25-50	50
	51+	50
Pregnant		60
Lactating		65

As can be seen from the above exemplary guidelines, in the United States, the recommended protein intake for women of child-bearing age (*e.g.*, less than 51) is from about 44 to 50 g/day, whereas pregnant women require are recommended an intake of about 60 g/day. In Canada and the United Kingdom, the recommended protein intake for pregnant women is in the order of about 70 g/day and 52 g/day. Thus, the need to ensure that the plasma Phe concentration levels of pregnant women are tightly controlled is further complicated by the fact that this group of PKU patient requires more protein than non-pregnant PKU females of comparable age.

In view of the above, it is contemplated that PAL therapies of the present invention will be particularly useful in pregnant women. It is contemplated that a woman suffering from any form of HPA who is pregnant or is contemplating pregnancy will be placed on a course of PAL therapy to ensure that her plasma Phe concentration levels are maintained as close to 180 μM to about 360 μM as possible. Such a course of therapy would allow that woman to increase her level of normal protein intake.

The discussion of levels of plasma Phe concentrations and the degrees to which such Phe concentrations should be decreased discussed herein above are incorporated into the present section for pregnant women.

Managing PKU in Infants and Methods of Treatment Thereof

As discussed herein throughout, it has been determined that an elevation in the plasma Phe concentration in infants (ages zero to 3 years old) results in significant drop in IQ of the child. However, as has been discussed elsewhere in the specification, patients that have elevated plasma Phe concentrations of anywhere up to 400 μM do not normally receive any dietary intervention. Thus, infants at the age of zero to 3 years in age suffer from significant deleterious effects from the present therapies. The instant application contemplates treating any infant having an unrestricted plasma Phe concentration that is greater than 360 μM ± 15 μM with a therapeutic composition that comprises PAL in order to produce a beneficial decrease the plasma Phe concentration of that subject.

In some embodiments, the infant is aged between zero and 3 years of age and has an unrestricted plasma Phe concentration of about 1200 μM prior to the administration of PAL and said administration decreases the plasma Phe concentration. In one embodiment, the plasma Phe concentration is decreased to from greater than 1800 to about 1500 μM, about 1200 μM, about 1100 μM, about 1000 μM, about 900 μM, about 800 μM, about 700 μM,

about 600 μM, about 550 μM, about 500 μM, about 450 μM, about 400 μM, about 350 μM, about 300 μM, about 275 μM, about 250 μM upon administration. In other embodiments, the infant is aged between zero and 3 years of age and has an unrestricted plasma Phe concentration of greater than 1200 μM and, this plasma Phe concentration is decreased to about 800 μM, to about 500 μM, or to about 360 μM, upon administration of PAL, either alone or in combination with diet. Those of skill in the art would understand that the invention contemplates treating infants with unrestricted plasma Phe concentrations of greater than 360 μM with PAL to produce decreases in such plasma Phe concentrations. The discussion of therapeutic reductions of plasma Phe concentrations above is incorporated herein by reference. Further, any decrease over 10% of the initial unrestricted plasma Phe concentration will be considered a therapeutic outcome for the therapeutic regimens for the infants. It should be understood that the PAL therapies may be combined with dietary restrictions to effect the therapeutic decrease in plasma Phe concentrations in such infants.

Table 2 lists a number of disease conditions wherein administration of therapeutically effective amounts of PAL would be beneficial. Parenteral, oral, or other standard routes of administration and dosage can be determined using standard methods.

Table 2: Exemplary Disease Conditions Amenable to PAL Protein Therapy

Phenylketonuria
Hyperphenylalanemia
Tyrosinemia
Cancer

2. COMPOSITIONS FOR USE IN THE TREATMENT

The present invention contemplates therapeutic intervention of PKU/HPA. Such intervention is based initially on the use of PAL, which may be used alone or in combination with dietary restrictions. Further PAL and/or dietary restrictions may further be combined with other therapeutic compositions that are designed, for example, to combat other manifestations of PKU, such as for example, large neutral amino acids to prevent Phe accumulation in the brain (see Koch, *et al.*, Mol. Genet. Metabol. 79:110-113 (2003)) or tyrosine supplementation. The present section provides a discussion of the compositions that may be used in the treatments contemplated herein.

PAL Compositions

In general, the present invention contemplates pharmaceutical compositions comprising effective amounts of protein or derivative products of the invention together with pharmaceutically acceptable diluents, stabilizers, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Polysorbate 20, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimerosol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol); see, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition (1990, Mack Publishing Co., Easton, Pa.) pages 1435:1712, which are herein incorporated by reference. An effective amount of active ingredient is a therapeutically, prophylactically, or diagnostically effective amount, which can be readily determined by a person skilled in the art by taking into consideration such factors as body weight, age, and therapeutic goal.

The PEG:PAL compositions of the present invention may also include a buffering agent to maintain the pH of the solution within a desired range. Exemplary agents include sodium acetate, sodium phosphate, and sodium citrate. Mixtures of these buffering agents may also be used. The amount of buffering agent useful in the composition depends largely on the particular buffer used and the pH of the solution. For example, acetate is a more efficient buffer at pH 5 than pH 6 so less acetate may be used in a solution at pH 5 than at pH 6. In one embodiment, the pH range for the compositions of the present invention is pH 3.0-7.5.

The compositions of the present invention may further include an isotonicity-adjusting agent to render the solution isotonic and more compatible for injection. An example is sodium chloride within a concentration range of 0-150 mM.

As used herein, and when contemplating PEG:PAL conjugates, the term "therapeutically effective amount" refers to an amount, which gives a decrease in serum L-phenylalanine that provides benefit to a patient. The amount will vary from one individual to another and will depend upon a number of factors, including the overall physical condition of the patient. The amount of PAL used for therapy gives an acceptable rate of serum L-phenylalanine decrease and maintains this value at a beneficial level (usually at least about 30% and typically in a range of 10% to 50%). A therapeutically effective amount of the present compositions may be readily ascertained by one skilled in the art using publicly available materials and procedures.

The invention provides for administering PEG:PAL conjugates less frequently than native PAL. The dosing frequency will vary depending upon the condition being treated, but in general will be about one time per week. It is understood that the dosing frequencies actually used may vary somewhat from the frequencies disclosed herein due to variations in responses by different individuals to the PEG:PAL conjugates; the term "about" is intended to reflect such variations.

The present invention may thus be used to reduce serum L-phenylalanine levels. Most commonly, serum L-phenylalanine levels are increased due to hyperphenylalaninemia. Among the conditions treatable by the present invention include hyperphenylalaninemia associated with phenylketonuria. Also treatable are conditions that may lead to increased serum L-tyrosine levels such as found in tyrosinemia. In addition, numerous cancer-related conditions, where depletion of serum L-phenylalanine and/or serum L-tyrosine levels would be beneficial, may also be treated with the PEG:PAL conjugates of the invention.

In one embodiment, PEG:PAL conjugates prepared in accordance with the present invention are administered by injection intraperitoneally, subcutaneously, or intramuscularly. However, it would be clear to one skilled in the art that other routes of delivery could also be effectively utilized using the compositions of the present invention.

The methods described herein use pharmaceutical compositions comprising the molecules described above, together with one or more pharmaceutically acceptable excipients or vehicles, and optionally other therapeutic and/or prophylactic ingredients. Such excipients include liquids such as water, saline, glycerol, polyethylene glycol, hyaluronic acid, ethanol, cyclodextrins, modified cyclodextrins (*i.e.*, sufobutyl ether cyclodextrins), *etc.* Suitable excipients for non-liquid formulations are also known to those of skill in the art.

Pharmaceutically acceptable salts can be used in the compositions of the present invention and include, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients and salts is available in *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990).

Additionally, auxiliary substances, such as wetting or emulsifying agents, biological buffering substances, surfactants, and the like, may be present in such vehicles. A

biological buffer can be virtually any solution which is pharmacologically acceptable and which provides the formulation with the desired pH, *i.e.*, a pH in the physiologically acceptable range. Examples of buffer solutions include saline, phosphate buffered saline, Tris buffered saline, Hank's buffered saline, and the like.

Depending on the intended mode of administration, the pharmaceutical compositions may be in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, suspensions, creams, ointments, lotions or the like, and in some embodiments, in unit dosage form suitable for single administration of a precise dosage. The compositions will include an effective amount of the selected drug in combination with a pharmaceutically acceptable carrier and, in addition, may include other pharmaceutical agents, adjuvants, diluents, buffers, *etc.*

In general, compounds or conjugates of this invention will be administered as pharmaceutical formulations including those suitable for oral (including buccal and sub-lingual), rectal, nasal, topical, pulmonary, vaginal or parenteral (including intramuscular, intraarterial, intrathecal, subcutaneous and intravenous) administration or in a form suitable for administration by inhalation or insufflation. One exemplary manner of administration is intravenous using a convenient daily dosage regimen, which can be adjusted according to the degree of affliction.

For solid compositions, conventional nontoxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate, and the like. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, *etc.*, an active compound or conjugate as described herein and optional pharmaceutical adjuvants in an excipient, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents, tonicifying agents, and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, triethanolamine oleate, *etc.* Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, referenced above.

For oral administration, the composition will generally take the form of a tablet, capsule, or softgel capsule, or may be an aqueous or nonaqueous solution, suspension or syrup. Tablets and capsules are some of the oral administration forms. Tablets and capsules for oral use will generally include one or more commonly used carriers such as lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. When liquid suspensions are used, the active agent may be combined with emulsifying and suspending agents. If desired, flavoring, coloring and/or sweetening agents may be added as well. Other optional components for incorporation into an oral formulation herein include, but are not limited to, preservatives, suspending agents, thickening agents, and the like.

Parenteral formulations can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solubilization or suspension in liquid prior to injection, or as emulsions. In one embodiment, sterile injectable suspensions are formulated according to techniques known in the art using suitable carriers, dispersing or wetting agents and suspending agents. The sterile injectable formulation may also be a sterile injectable solution or a suspension in a nontoxic parenterally acceptable diluent or solvent. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils, fatty esters or polyols are conventionally employed as solvents or suspending media. In addition, parenteral administration may involve the use of a slow release or sustained release system such that a constant level of dosage is maintained.

The PEG:PAL conjugates identified described above can be administered to a patient at therapeutically effective doses to treat or ameliorate cardiovascular disease. The toxicity and therapeutic efficacy of such conjugates can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, such as, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50} /ED_{50}. Conjugates exhibiting large therapeutic indices are employed in some embodiments.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. In some embodiments, the dosage lies within a range of circulating concentrations that include the ED_{50} with little or minimal toxicity. The dosage can vary within this range depending upon the dosage form employed

and the route of administration utilized. The therapeutically effective dose can be determined from cell culture assays, and from animal models.

Dietary Protein

In addition to administering PAL and related analogs to HPA/PKU patients, it is contemplated that the dietary protein of the patients also may be restricted or modified. Those of skill in the art are aware of various commercially available protein formulas for use in the treatment of PKU. Such formulas include MAXIMAID, PHENEX 1, PHENEX 2 (Ross Laboratories, Liverpool, UK), LOFENALAC, PHENYL-FREE (Mead-Johnson), and the like.

Those of skill in the art may use the referenced protein formulas, which are generally free of Phe concentrations. The protein formulas often are supplemented with amino acids that are deficient in PKU patients. Such amino acids include, for example, L-tyrosine, and L-glutamine. It has been suggested that it may be desirable to supplement the diet of PKU patients with valine, isoleucine and leucine (see U.S. Patent No. 4,252,822). In certain clinical manifestations, the toxic effects of PKU are caused by Phe blocking the brain uptake of other amino acids such as tyrosine and tryptophan. It has been found that supplementing the diet of a PKU patient with excess of such large neutral amino acids blocks Phe uptake into the brain and lowers brain Phe levels. Thus, it is contemplated that for the methods of the present invention, the dietary regimen may further be supplemented with compositions that comprise one or more of these amino acids (Koch, *et al.*, Mol. Genet. Metabol. 79:110-113 (2003)).

Further, as it is known that L-carnitine and taurine, which are normally found in human milk and other foodstuffs of animal origin, also should be supplied in addition to the protein restriction. In certain embodiments, the L-carnitine may be supplied as 20 mg/100 g of protein supplement, and the taurine may be supplied as 40 mg/100 g protein supplement in order to help supply amounts of these factors normally found in human milk and foods of animal origin.

In addition, those of skill in the art are referred to the 2000 National Academy of Sciences-National Research Council Dietary Reference Intakes for a further listing of other components, such as essential vitamins and minerals that should be supplied to the patient to ensure that other supplements are being provided despite the dietary protein restriction.

Referring to the discussion above regarding total protein amounts and desirable plasma Phe concentrations, one of skill in the art will be able to determine the amount of dietary protein restriction that is required and thus adjust the diet of the patient accordingly. Taking for example, a male of about 11-14 years of age, that individual should receive 45 g protein/day. In the event that the individual is one that has severe classic PKU, his unrestricted plasma Phe concentration will likely be greater than 1200 μM, and most, if not all of the dietary protein source for that individual is likely to be from a powdered protein supplement, which may lower his plasma Phe concentrations to less than 600 μM. By administering PAL to that subject, a therapeutic outcome would be one which produces greater decrease in the plasma Phe concentrations of patient or alternatively, the therapeutic outcome is one in which the individual's plasma Phe concentrations is lowered to a similar degree, but that individual is able to tolerate protein from a normal diet rather than from a dietary formula.

Similarly, for a male of about 11-14 years of age who has moderate PKU, it may be possible using the methods of the present invention to give him the allotted 45 g protein/day through a normal protein intake rather than a restricted formula. Determining whether the methods of the invention are effective will entail determining the plasma Phe concentrations of the patient on a regular basis to ensure that the plasma Phe concentrations remain below at least 400 μM. Tests for determining such concentrations are described below. In some embodiments, concentrations of less than or about 360 μM are achieved.

3. IDENTIFYING AND MONITORING PATIENT POPULATIONS

As discussed herein throughout, it will be necessary in various embodiments of the present invention to determine whether a given patient is responsive to PAL therapy, and to determine the phenylalanine concentrations of the patient both initially to identify the class of PKU patient being treated and during an ongoing therapeutic regimen to monitor the efficacy of the regimen. Exemplary such methods are described herein below.

BH4 Loading Test

The BH4 loading test allows discrimination between patients that have HPA due to a deficit in BH4 or through a deficiency in PAH.

The simplest BH4 loading test is one in which exogenous BH4 is administered and the effects of the administration on lowering of plasma Phe concentrations is determined. Intravenous loading of 2 mg/kg BH4 was initially proposed by Danks, *et al.*, Lancet 1:1236

(1976), as BH4 of greater purity has become available it has become possible to perform the test using an oral administration of BH4 in amounts of about 2.5 mg/kg body weight. Ultimately, a standardized approach was proposed by Niederwieser *et al.* in which a 7.5 mg/kg single oral dose of BH4 is administered (Niederwieser, *et al.*, Eur. J. Pediatr. 138:441 (1982)), although some laboratories do still use upwards of 20 mg BH4/kg body weight.

In order for the simple BH4 loading test to produce reliable results, the blood Phe levels of the patient need to be higher than 400 μM. Therefore, it is often customary for the patient to be removed from the PKU diet for 2 days prior to performing the loading test. A BH4 test kit is available and distributed by Dr. Schircks Laboratories (Jona, Switzerland). This kit recommends a dosage of 20 mg BH4/kg body weight about 30 minutes after intake of a normal meal.

Determination of Phe Concentrations

There are numerous methods for determining the presence of Phe in blood (Shaw *et al.*, Analytical Methods in Phenylketonuria-Clinical Biochemistry, In Bickett *et al.* Eds., Phenylketonuria and Some Other Inborn Errors of Amino Acid Metabolism, Stuttgart, Georg Thiem Verlag, 47-56 (1971)). Typically, phenylalanine and tyrosine concentrations are determined from the serum of a patient using a fluorometric assay. This assay relies on the formation of fluorescent substance when phenylalanine is heated with ninhydrin in the presence of leucylalanine (McCaman, *et al.*, J. Lab. Clin. Med. 59:885-890 (1962)).

The most popular method for determining Phe concentrations is the Guthrie test in which discs are punctured from filter paper that has been saturated with a blood sample from the patient. The uniform discs are incubated in a tray of agar that has been seeded with Bacillus subtilis and contains a specific inhibitor of Bacillus subtilis growth. As the phenylalanine transfers from the uniform discs onto the agar, the Phe reverse the inhibition of bacterial growth thereby yielding an area of bacterial growth that can be correlated to phenylalanine concentration by comparison to similar assays performed using discs containing known amounts of Phe.

Other methods of quantifying Phe concentration include HPLC, mass spectrometry, thin layer chromatography and the like. Such methods can be used to determine the plasma Phe concentration of a patient before the therapy and to monitor the Phe concentration during the therapeutic regimen to determine the efficacy thereof.

It is contemplated that the plasma Phe levels of the patients will be monitored at convenient intervals (*e.g.*, daily, every other day or weekly) throughout the time course of the therapeutic regimen. By monitoring the plasma Phe levels with such regularity, the clinician will be able to assess the efficacy of the treatment and adjust the PAL and/or dietary protein requirements accordingly.

4. COMBINATION THERAPY

Certain methods of the invention involve the combined use of PAL and dietary protein restriction to effect a therapeutic outcome in patients with various forms of HPA. To achieve the appropriate therapeutic outcome in the combination therapies contemplated herein, one would generally administer to the subject the PAL composition and the dietary restriction in a combined amount effective to produce the desired therapeutic outcome (*i.e.*, a lowering of plasma Phe concentration and/or the ability to tolerate greater amounts of Phe/protein intake without producing a concomitant increase in plasma Phe concentrations). This process may involve administering the PAL composition and the dietary protein therapeutic composition at the same time. This may be achieved by administering a single composition or pharmacological protein formulation that includes all of the dietary protein requirements and also includes the PAL within said protein formulation. Alternatively, the dietary protein (supplement or normal protein meal) is taken at about the same time as a pharmacological formulation (tablet, injection or drink) of PAL. PAL also may be formulated into a protein bar or other foodstuff such as brownies, pancakes, cake, suitable for ingestion.

In other alternatives, PAL treatment may precede or follow the dietary protein therapy by intervals ranging from minutes to hours. In embodiments where the protein and the PAL compositions are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that PAL will still be able to exert an advantageously effect on the patient. In such instances, it is contemplated that one would administer the PAL within about 2-6 hours (before or after) of the dietary protein intake, with a delay time of only about 1 hour in some embodiments. In certain embodiments, it is contemplated that PAL therapy will be a continuous therapy where a daily dose of PAL is administered to the patient indefinitely. In other situations, *e.g.*, in pregnant women having only the milder forms of PKU and HPA, it may be that PAL therapy is only continued for as long as the woman is pregnant and/or breast feeding.

Further, in addition to therapies based solely on the delivery of PAL and dietary protein regulation, the methods of the present invention also contemplate combination therapy with a third composition that specifically targets one or more of the symptoms of HPA. For example, it is known that the deficit in tyrosine caused by HPA results in a deficiency in neurotransmitters dopamine and serotonin. Thus, in the context of the present invention, it is contemplated that PAL and dietary protein based methods could be further combined with administration of L-dopa, carbidopa and 5-hydroxytryptophan neurotransmitters to correct the defects that result from decreased amounts of tyrosine in the diet.

As the administration of PAL would not generate tyrosine (unlike administration of PAH), such treatment will still result in tyrosine being an essential amino acid for such patients. Therefore dietary supplementation with tyrosine may be desirable for patients receiving PAL in combination with the BH4 therapy.

I. SCREENING AND CHARACTERIZATION OF PAL HOMOLOGS

Another aspect of the invention is a screening assay for identifying bacterial PAL that can prevent, ameliorate, or reduce enhanced levels of phenylalanine by contacting a cell containing elevated levels of phenylalanine with the bacterial PAL and determining whether the bacterial PAL reduces such elevated levels of phenylalanine. In certain embodiments, the method is a high throughput assay. In one embodiment, complete genomes of the bacterial species are sequenced and screened for the presence of PAL homologs using a bioinformatics approach. In another embodiment, genes with homology to encP are identified and BLAST analysis of the related protein sequence is conducted to determine percent identity. Primary sequence alignments of such proteins are assessed for the presence of conserved residues essential for PAL activity. Position 83 (HAL from P. putida) of all HAL enzymes is a histidine whereas it is an aliphatic amino acid, for example, leucine or valine in enzymes with PAL activity. Analysis at position 83 will elucidate whether HAL annoted enzymes may be mislabeled and have actually demonstrate PAL activity. For example, position 413 is a glutamine (Q) in most HAL enzymes and is followed at position 414 by a glutamic acid (E) in all HAL enzymes. By comparison, position 414 is a glutamine (Q) followed at position 415 by an aspartic acid (D) or an asparagine (N) in most PAL enzymes identified so far. Representative annotated HAL enzymes which are determined by

this bioinformatic method to be most likely mislabeled as prokaryotic PAL enzymes include HAL enzymes from Rhodobacter sphaeroides 2.4.1 (ZP 00005404), from Rubrobacter xylanophilus DSM9941 (ZP 00188602), from Photorhhabdus luminescens subsp. LaumondiiTT01 (NP 930421), from Thermoanaerobacter tengcongensis (AA051415).

In a further embodiment, PAL catalytic activity of the protein product of such homologs is confirmed. In another embodiment, the genomes of PAL homologs are PCR amplified and cloned into pHIS8 or a similar vector. The putative PAL proteins are heterologously expressed in *E. coli* as His-tagged proteins, purified and assayed for PAL activity. Assays for PAL activity may include but are not limited to ability to convert phenylalanine to cinnamate *in vitro*, inability to convert tyrosine to p-coumarate *in vitro,* assays to test for HAL activity and assays to characterize the kinetics of the enzymes.

J. PRODUCTION OF PROKARYOTIC PAL

Another aspect of the invention is a method of producing prokaryotic PAL. In one embodiment, recombinant PAL is over-expressed as an N-terminal octahistidyl-tagged fusion protein in a vector, for example, *E. coli* BL21(DE3)/pLysS (Invitrogen) with an inducible promoter such as with IPTG (isopropyl-beta-D-thiogalactopyranoside). In another embodiment, recombinant PAL is over-expressed in *E. coli* BL21(DE3)/pLysS cells without an N-terminal tag. Seed culture for a bioreactor/fermenter is grown from a glycerol stock in shake flasks. Such seed culture is then used to spike into a controlled bioreactor in fed-batch mode. Glucose is supplemented and pH is controlled with base (NH4OH) and agitation is up to 1200 rpm. O_2 feed keeps dissolved oxygen to greater than 20%. The cells are grown at a temperature of 30°C until reaching and OD_{600} of 70-100 (~22-25 hrs) and then induced with 0.4 mM IPTG. The temperature is reduced to 22 to 26°C and grown until activity change is < 0.1 IU/ml (approximately 40-48 hrs and an OD_{600} typically of 200). Cell culture media is typically defined and composed of yeast extract protein, peptone-tryptone, glucose, glycerol, casamino acids, trace salts and phosphate buffering salts. The recombinant PAL product is produced intra-cellularly and not secreted. The bacteria are harvested by continuous centrifugation (Alfa-Laval, Carr, Ceba, or equivalent).

K. PURIFICATION OF PROKARYOTIC PAL

A further aspect of the present invention features a method to purify bacterial PAL or a biologically active fragment, mutant or analog thereof. According to a first embodiment, a transformed cell mass is grown and ruptured leaving crude recombinant enzyme. Exogenous materials are normally separated from the crude bulk to prevent fouling of the columns. Chromatographic purification is conducted using one or several chromatographic resins. Subsequently, the purified protein is formulated into a buffer designed to provide stable activity over an extended period of time. In another embodiment, the method to purify the bacterial PAL comprises: (a) lysis of the bacteria containing recombinant PAL using a pressure homogenizer (but potentially by other physical means such as glass bead lysis); (b) heat treatment; (c) clarification of this lysate using a second continuous centrifugation step and/or depth filtration (as with Cuono Zeta Plus or Maximizer, Pall Filtron, or Millipore Millistak or Opticao filters); (d) passage through a charcoal filtration step (as with Millipore Millistak 40AC); (e) passage through a final filtration step (as with a Sartorious Sartopore 0.2 μm filter); (f) passage over a butyl hydrophobic interaction chromatography (as in Toyopearl Butyl 650M from Tosoh Biosciences); (g) passage over a Q ion exchange column (as in a Macroprep High Q from BioRad); and (h) recovery of final product by buffer exchange with tangential flow filtration (as with a Sartorious Hydrosart or PES 100 kDa membrane). Those skilled in the art readily appreciate that one or more of the chromatography steps may be omitted or substituted, or that the order of the chromatography steps may be changed within the scope of the present invention. Finally, appropriate sterilizing steps may be performed as desired.

Having now generally described the invention, the same may be more readily understood through the following reference to the following examples. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, *etc.*), but some experimental error and deviation should, of course, be allowed for.

EXAMPLE 1

Cloning of *Nostoc punctiforme* and *Anabaena variabilis* PAL

DNA Manipulations

N. punctiforme genomic DNA was purchased from ATCC (29133D) and the PAL gene (ZP_00105927) was PCR-amplified from primers 5'-CACTGTCATATGAATATAACATCTCTACAACAGAACAT-3' (SEQ ID NO:12) and 5'-GACAGTGGCGGCCGCTCACGTTGACTTTAAGCTCGAAAAAATATG-3' (SEQ ID NO:13). The resulting PCR product was digested with NdeI and NotI and the 1.7 kb fragment was ligated into pET-28a(+) and pET-30a(+) (Novagen) for N-His tagged and untagged, respectively.

A. variabilis cells were purchased from ATCC (29413). Genomic DNA was extracted (Qiagen) and the PAL gene (YP_324488) was amplified by SOE-PCR to remove an NheI site. Primer 1 (5'-CACTGTGCTAGCATGAAGACACTATCTCAAGCACAAAG-3') (SEQ ID NO:14) and primer 2 (5'-GGAAATTTCCTCCATGATAGCTGGCTTGGTTATCAACATCAATTAGTGG -3') (SEQ ID NO:15) were used to amplify nucleotides 1-1190 and primer 3 (5'-CCACTAATTGATGTTGATAACCAAGCCAGCTATCATGGAGGAAATTTCC-3') (SEQ ID NO:16) and primer 4 (5'-CACTGTGCGGCCGCTTAATGCAAGCAGGGTAAGATATCTTG-3') (SEQ ID NO:17) were used to amplify nucleotides 1142-1771. These two PCR products were combined to amplify the full-length gene with primers 1 and 4. The resulting PCR product was digested with NheI, blunted with Klenow (NEB), then digested with NotI. The 1.7 kb fragment was ligated into pET-28a(+) and pET-30a(+) (Novagen).

Bacterial Strains and Culture Conditions

E. coli BL21(DE3) cells (Stratagene) were transformed with pGro7 (TaKaRa) and competent BL21(DE3)pGro7 cells were prepared by the Inoue Method (Sambrook and Russell, *Molecular Cloning: A Laboratory Manual,* 3rd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001)). These cells were transformed with pET-28-NpPAL and cultured in 25 ml LB with 50 mg/L kanamycin and 20 mg/L chloramphenicol overnight at 37°C. Twenty milliliters of this culture was seeded into 1 L of LB medium with kanamycin, chloramphenicol, and 500 mg/L L-arabinose and grown at 37°C. At an OD_{600} of 0.6, the culture was chilled on ice. After 5 minutes the culture was induced with 0.3 mM IPTG and grown for 16 hours at 20°C. Cells were harvested by centrifugation.

BL21(DE3)pLysS cells (Stratagene) were transformed with AvPAL and cultured identically to NpPAL without the arabinose induction.

EXAMPLE 2

Purification of NpPAL and AvPAL

The cultures were centrifuged in a bench-top centrifuge at 5,000 *g* for 20 minutes and the supernatant discarded. The cell pellets were typically frozen at –70°C prior to further processing. Upon thawing, the cell pellets were suspended to approximately 80 optical density units (600 nm) in TBS (25 mM Tris, 150 mM NaCl, pH 7.8). The cells were lysed by two passes through an APV pressure homogenizer at 12-14,000 psi. The crude lysate was then heat-treated at 55°C for 2 hours. The lysate is centrifuged at 10,000 *g* for 30 minutes and the supernatant retained and filtered with a 0.2 μm vacuum filter (Corning).

The PAL was purified from the clarified lysate by passage sequentially over a butyl 650M column (Tosoh BioSciences) and a MacroPrep High Q column (BioRad). The eluted product showed a high level of purity by both SDS PAGE and reverse phase HPLC.

EXAMPLE 3

Generation of Pegylated PAL Variants

Protein Pegylation

Pegylation uses modifications of literature methods (Hershfield, *et al.*, (1991) *ibid.*; U.S. Patent No. 6,057,292; Lu, *et al.*, Biochemistry 40(44):13288-13301 (2001); Nektar Therapeutics, 2003 catalog). Activated PEGs include both the linear PEG succinimidyl succinates (mPEG-SPA, MW 5 kDa or MW 20 kDa) and the branched PEG hydrosuccinimides ($mPEG_2$-NHS ester, MW 10 kDa or MW 40 kDa), which are both capped on one end with a methoxy group and available from Nektar Therapeutics; experimental determination of optimal pegylated proteins is normally required (Veronese, F.M., *et al.*, J. Bioactive Compatible Polymers 12:196-207 (1997)). Optimal pegylation conditions are determined using different ratios of PAL:PEG (taking into account the molar ratio of protein along with the number of lysines per protein monomer), different pHs, different buffers, various temperatures and incubation times. High PAL protein:PEG derivatization ratios are necessary since native PAL has a large number of lysines (29 and 18 per *Rhodosporidium toruloides* (Rt) and *Anabaena variabilis* (Av) monomer, respectively) and because un-

modified PAL displays immunoreactivity upon repeated injection in mice and since naked (wild-type) PAL is quickly inactivated upon exposure to proteases. Pegylation reactions are stopped by freezing at –20°C, and the samples will be analyzed by SDS-PAGE, MALDI-TOF mass spectroscopy, activity assessment, proteolytic sensitivity, and immunoreactivity.

Prior to activity, proteolysis, and immune assessment, and in order to remove excess unreacted PEG, reactions are dialyzed against pH 8.5, 0.05 M potassium phosphate buffer overnight at 4°C with stirring using Tube-O-Dialyzers (GenoTechnology). After protein concentration is determined using the NI protein assay kit (Geno Technology), PAL activity measurements will be performed on underivatized and PEG derivatized PAL samples using standard reaction conditions, as previously described. Following *in vitro* characterization, *in vivo* trials will be conducted with the most promising pegylated therapeutic candidates using the PKU mouse model.

Characterization

Protein concentration is determined using the PAL extinction coefficient (0.5 and 0.83 mg $mL^{-1}cm^{-1}$ for RtPAL and AvPAL, respectively) at 280 nm for non-modified protein samples and for pegylated protein samples the concentration is calculated using the NI Protein Assay (GenoTechnology) that includes sample processing to remove non-protein contaminants that might interfere with accurate protein concentration determination.

PEG-PAL products are characterized with MALDI-TOF MS to determine the number of PEG molecules attached to each PAL monomer, as well as characterized using activity assessment and SDS-PAGE and native gel analysis, to assure retention of activity, complete derivatization, and no loss of tetrameric PAL formation, respectively. For PAL and PEG-PAL samples, MALDI-TOF mass spectroscopic analysis requires the use of 0.5 M urea or 0.025 M guanidine-HCl to improve subunit dissociation and the reproducibility of species detection.

PAL Activity Assay

The PAL activity assay is conducted using a Cary UV spectrophotometer (Cary 50) in the kinetics mode. The activity of PAL with L-phenylalanine substrate is assayed at room temperature (25°C) by measuring the production of trans-cinnamate monitored by the absorbance increase at 290 nm (Hodgins, (1968) *ibid.*). The molar extinction coefficient of trans-cinnamic acid at 290 nm is 10.2381 liter $M^{-1}cm^{-1}$. Reaction mixtures contain 22.5 mM phenylalanine in 100 mM Tris-HCl buffer, pH 8.5. For standard

measurements the final enzyme concentration is 0.0035 mg/mL, but for kinetic studies the enzyme concentration in the assay is adjusted so that the slope at 290 nm per min is in the range of 0.005 to 0.02. Activity data is expressed as specific activity ($\mu mol \times min^{-1} mg^{-1}$). One unit of PAL is defined as that amount of enzyme that produces 1 μmol of trans-cinnamic acid per minute at room temperature.

EXAMPLE 4

Test of *In vitro* Half-Life and Immunogenicity

After biochemical characterization, the most promising PEG-PAL candidates are screened for immunoreactivity against antibodies raised by PKU mice injected with native PAL (non-pegylated) using three different and complementary techniques (Western blot, ELISA, and immunoprecipitation (IP)).

For Western blot analysis, PAL anti-serum (from mice injected with native PAL) is used in a dilution 1:10,000. As a negative control the serum from buffer treated-mice is also used in the same dilution. The secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG (Promega), is diluted to 1:5,000 and color is developed using the AP substrate Western Blue (Promega). The ELISA test is performed using Nunc/Immuno Maxisorp plates (Nalge Nunc International) following standard procedures using 1 mg/mL of PAL in PBS and blocking with PBS, 0.05% Tween-20, 2% BSA. The mouse antisera (from native PAL exposed mice) is diluted 1:10,000 in EB block solution (PBS, 0.05% Tween-20, 2% BSA), and a HRP-goat anti-mouse IgG is used as secondary antibody with TMB used for detection at 450 nm.

Immunoprecipitation is used to test for PAL antibody binding. Protein samples (PAL or pegylated PAL) are incubated in TTBS buffer (Tris buffered saline with 0.1% Tween) and PAL activity is measured before adding the antibody sample. Each sample is incubated with 8-fold excess of positive control anti-PAL serum and a duplicate negative control reaction using non-immune mouse serum. After incubation, protein G Sepharose 4 (50%, v/v) is added in excess, taking into account the mouse IgG binding capacity of the beads, and the samples are incubated again at 4°C overnight with rotation. Supernatants are recovered by centrifugation and the PAL activity of each sample is assayed on the supernatants. The bead pellets are not discarded, so that further analysis by Western blot can be performed. To confirm that antibody-bead binding has occurred, Western blot is used to

detect the PAL antigen on the beads. Beads that have been recovered by centrifugation after the PAL binding step are washed several times with TTBS and TBS buffers. Following these rinses, SDS-PAGE loading buffer is added to the beads and the samples are heated at 95°C for 5 minutes. Samples are then analyzed by Western blot using PAL anti-serum. Enzyme variants showing poor antibody binding have corresponding little PAL in the pelleted bead fractions as detected by Western blot and show higher activities remaining in the supernatant as compared to native un-modified PAL which displays high antibody binding.

EXAMPLE 5

Test of Protease Sensitivity

Protease mapping studies on native PAL from R. toruloides have indicated primary sites of proteolytic sensitivity. Removal of such sites may reduce or eliminate proteolytic sensitivity and contribute to the development of an effective PKU enzyme substitute. However, elimination of such sites for proteolytic sensitivity may result in the reduction or loss of enzyme activity.

After protein engineering has created improved PAL (and PEG-PAL) mutants that retain activity, screening for protease resistance using incubation with a trypsin/chymotrypsin protease cocktail, followed by monitoring for retention of activity (*via* OD_{290} measurement) and reduced protein cleavage (*via* PAGE gel analysis) allows for the identification of mutants with appropriate *in vitro* properties to be used for *in vivo* testing.

Proteolytic stability will be assessed using incubation with a protease cocktail that approximates the intestinal environment and contains 2.3 mM trypsin, 3.5 mM chymotrypsin, 3.05 mM carboxypeptidase A, and 3.65 mM carboxypeptidase B. Proteolysis testing will involve enzymatic incubations, adding proteases to the PAL solutions, to determine the degree of protease sensitivity for the different protein variants being examined (native or mutant protein with or without pegylation or other chemical modification), including time courses of activity retention and stability retention after protease exposure. SDS-PAGE and MALDI-TOF mass spectrometric mapping experiments will be used to determine the location of any protease sensitive sites (Kriwacki, R.W., *et al.*, J. Biomol. Tech. 9(3):5-15 (1980)). These mapping results will be important to determine primary sites of protease susceptibility (such as the two primary sites already identified), so that all major

sensitivity sites can be removed using pegylation protection and/or mutation to remove and/or protect susceptible regions from the PAL architecture.

EXAMPLE 6

Generation of PEGylated NpPAL and AvPAL

In general, PEGylation for both NpPAL and AvPAL involves mixing the protein with SUNBRIGHT ME-200HS 20 kDa NHS-activated PEG (NOF).

Protocol for PEGylation, standard "HC" method using NHS-activated 20 kDa linear PEG:

1) The protein was evaluated for the presence of endotoxin.

A protein solution (0.1 mL) was diluted in 0.9 ml fresh MQ water and tested with a hand-held Charles River apparatus (EndoPTS) for endotoxin at the 0.5 EU/ml sensitivity level. If endotoxin was greater than 0.5 EU/ml, then endotoxin was reduced initially by Mustang E filtration, followed by Sterogene Etox resin, and optionally by further chromatographic purification. Reduction was limited but sufficiently useful by passage over DEAE FF (Amersham) at pH 7.8.

2) Concentration and buffer exchange of protein:

The protein was concentrated to greater than 25 mg/ml but less than or equal to 75 mg/ml and buffer exchanged to 50 mM KPO_4, pH 8.5. If a spin filter was used to prepare this concentration, the filter was first tested for endotoxin by spinning at reduced speed and time (3000 rpm, 3 minutes) with buffer alone, then testing the retained buffer for endotoxin in the same way as the protein in step 1. The buffer batch record/recipe for 50 mM KPO4, pH 8.5 consisted of water (QS to 1 L), potassium phosphate dibasic (8.4913 g/L of 48.75 mM), and potassium phosphate monobasic (0.17011 g/L of 1.25 mM). The solution was filtered through a 0.2 μm filter and stored at room temperature. The concentrated product was slowly filtered (1-2 mL/min) through a Mustang E filter acrodisc. A sample diluted and blanked with sterile TBS, pH 7.5 was measured at A280 to determine protein concentration. The extinction coefficient was 0.83 for NpPAL and 0.75 for AvPAL.

3) PEGylation of NpPAL and AvPAL:

PEG normally stored at –80°C was warmed to room temperature. KPO4 buffer was added to PEG to resuspend by vortexing at maximum speed, and shaking tube

hard in hand to ensure all large chunks were suspended. The protein was added to the well-suspended PEG solution within one minute of having first wetted the PEG and mixed by very gentle inversion. Tubes wrapped in aluminum foil were placed on the axis of a rocker and rocked very gently at room temperature for 3 hours. The tubes were filled with TBS (pH 7.5) and sterile filtered. The suspensions were either formulated immediately or stored at 4°C until ready for formulation.

4) Formulation

The formulation buffer recipe/batch record consisted of water (QS to 1 L), Tris-Base (3.2 mM), Tris-HCl (16.8 mM), and sodium chloride; the buffer solution was filtered through a 0.2 μm filter and stored at room temperature. The buffer solution was subjected to tangential flow filtration using a Vivalow 50 (smaller lots) or Vivalfow 200 (larger lots) with an 100 MWCO regenerated cellulose membrane. The solution was flushed with MQ water, 0.1 N NaOH, and 200 mL water again. The solution was equilibrated with TBS, pH 7.5 at 50 mL/min cross-flow. The pH of the permeate was determined to ensure a pH of 7.5.

The solution was buffer exchanged by first diluting with TBS approximately 3-fold and returning to original volume at least four times. Cross-flow was typically 180-200 mL/min for both Vivaflow 50 and 200.

The final product was filtered through Mustang E. The presence of endotoxin was evaluated after diluting 0.1 mL with 1.9 ml sterile fresh water. If endotoxin was greater than 1 EU/mL, reduction was conducted with Sterogene Etox gel. Formulated and sterile PEGylated NpPAL or AvPAL were sealed in vials and placed at –70°C until ready for *in vivo* studies.

EXAMPLE 7

Effect of *Nostoc punctiforme* PAL (NpPAL) and its PEGylated Form in PKU Affected Mice

The objective of this study was to determine plasma phenylalanine (Phe) levels following subcutaneous administrations of NpPAL or pegylated NpPAL.

The effects of (1) a lysine mutant R91K PAL from *Rhodosporidium toruloides* and its pegylated form (1:3 PA::PEG) (PEG from Nippon Oil and Fat, NOF) at a dose of 3.0

IU/mL; (2) NpPAL and its pegylated form (1:3 PAL:PEG NOF) at doses ranging from 0.6 IU/mL to 3.0 IU/mL; and (3) vehicle were tested in homozygous ENU2 mice (also known as $BTBR^{enu2}$) mice. R91K PAL was previously shown to have improved activity relative to wild-type *R. toruloides* PAL. R91K is located in the helix spanning Asp86 to Leu101, near the surface of the protein. All solutions had less than 1.0 EU/mL endotoxin and were stored at –75-80°C. The ENU2 or $BTBR^{enu2}$ mouse is homozygous mutant at the phenylalanine hydroxylase gene (PAH) locus resulting in an animal with severe hyperphenylalanemia. The high plasma phenylalanine (Phe) levels make this animal the appropriate model for evaluating the ability of phenylalanine ammonia-lyase (PAL) to reduce plasma Phe.

Experimental Design

Table 3: Group Designations and Dose Levels

Group #	N	Administered	Dose Level (IU/mouse)	Test Article Activity (IU/mL)	Dose Volume (mL)
1	2	R91K	1.0	3.0	0.33
2	2	R91K 1:3 PAL:PEG NOF	1.0	3.0	0.33
3	5	npPAL	0.2	0.6	0.33
4	5	npPAL	0.4	1.2	0.33
5	5	npPAL	0.6	1.8	0.33
6	5	npPAL	1.0	3.0	0.33
7	5	npPAL 1:3 PAL:PEG NOF	0.2	0.46	0.43
8	5	npPAL 1:3 PAL:PEG NOF	0.4	0.92	0.43
9	5	npPAL 1:3 PAL:PEG NOF	0.6	1.38	0.43
10	5	npPAL 1:3 PAL:PEG NOF	1.0	2.30	0.43
11	2	Vehicle	0.0	0.0	0.43

The solutions were administered *via* subcutaneous bolus between the shoulder blades on day 1, in the right abdomen on day 4, and in the left abdomen on day 8. The solutions were administered using a 25-gauge needle and 1 or 3cc syringe into the subcutaneous space. Each animal received 0.2, 0.4, 0.6 or 1.0 IU of test article in the dose volumes described in the table above. The animals were lightly anesthetized by inhalation of

halothane (1-3% mg/kg) and also restrained by hand. Dose administration was performed at approximately the same time on each dose administration day.

Animals were observed daily for morbidity, mortality and general health. In particular, the injection site was observed for signs of redness or edema. Body Weights were measured and recorded on Days –3, 8 and 12 and used as a clinical parameter but not used to determine dose volume. Blood collections (P-plasma, S-serum) were performed on Day –3 (P,S), Day 2 (P), Day 4 Pre-dose (P), Day 5 (P), Day 8 Pre-dose (P,S), Day 9 (P,S), Day 12 (P), and Day 23 (S). Approximately 50-100 µL of whole blood was collected at each time point (for 25-50 µL of plasma or serum). Blood was collected from the tail vein. Plasma or serum was collected as indicated in the schedule above. The tail vein was punctured and the blood droplets were collected using RAM Scientific Green-Top Capillary Blood Collection Tubes (#07 7250). For serum collection, blood droplets were collected into a tube with no additives. Plasma/serum were harvested and transferred into a storage tube to be stored at either –20°C (plasma) or –80°C (serum).

Results

The results for NpPAL short-term dosing studies demonstrate the efficacy of the PEGylated enzymes in lowering excessive phenylalanine levels. Notably, unPEGylated NpPAL did not lower phenylalanine levels after any injection, even before there could be any immunogenic response. Therefore, PEGylation is an absolute requirement for NpPAL to be a potential treatment for PKU (FIGURE 5).

EXAMPLE 8

Effect of *Anabaena variabilis* PAL (AvPAL) and its PEGylated Form in PKU Affected Mice

The objective of this study was to determine plasma phenylalanine (Phe) levels following subcutaneous administrations of AvPAL or pegylated AvPAL.

The effects of (1) a lysine mutant R91K PAL from *Rhodosporidium toruloides* in its pegylated form (1:3 PAL::PEG) (PEG from Nippon Oil and Fat, NOF) at a dose of 3.0 IU/mL; (2) AvPAL and its pegylated form (1:3 PAL:PEG NOF) at doses of 0.6 IU/mL and 3.0 IU/mL; and (3) vehicle were tested in homozygous ENU2 mice (also known as BTBR^{enu2}) mice. R91K PAL was previously shown to have improved activity relative to wild-type *R. toruloides* PAL. R91K is located in the helix spanning Asp86 to Leu101, near

the surface of the protein. All solutions had less than 1.0 EU/mL endotoxin and were stored at -75-80°C. The ENU2 or BTBR^{enu2} mouse is homozygous mutant at the phenylalanine hydroxylase gene (PAH) locus resulting in an animal with severe hyperphenylalanemia. The high plasma phenylalanine (Phe) levels make this animal the appropriate model for evaluating the ability of phenylalanine ammonia-lyase (PAL) to reduce plasma Phe.

Experimental Design

Table 4: Group Designations and Dose levels

Group #	N	Administered	Dose Level (IU/mouse)	Test Article Activity (IU/mL)	Dose Volume (mL)	Route	Dose Frequency
1	3	Vehicle	0	0	0.33	SC	Days 1, 4, 8
2	5	avPAL	0.2	0.6	0.33		
3	5	avPAL	1.0	3.0	0.33		
4	5	avPAL PEG	0.2	0.6	0.33		
5	6	avPAL PEG	1.0	3.0	0.33		
6	5	R91K PEG	1.0	3.0	0.33		

The solutions were administered *via* subcutaneous bolus in the animal's rear back on days 1, 4, and 8. The solutions were administered using a 25-gauge needle and 1 or 3cc syringe into the subcutaneous space. Each animal received 0.2, 0.4, 0.6 or 1.0 IU of test article in the dose volumes described in the table above. The animals were lightly anesthetized by inhalation of halothane (1-3% mg/kg) and also restrained by hand. Dose administration was performed at approximately the same time on each dose administration day.

Animals were observed daily for morbidity, mortality and general health. In particular, the injection site was observed for signs of redness or edema. Body Weights were measured and recorded on Days –3, 8 and 12 and used as a clinical parameter but not used to determine dose volume. Blood collections (P-plasma, S-serum) were performed on Day –3 (P,S), Day 2 (P), Day 4 Pre-dose (P), Day 5 (P), Day 8 Pre-dose (P,S), Day 9 (P,S), Day 12 (P), and Day 22 (S). Approximately 50-100 µL of whole blood was collected at each time point (for 25-50 µL of plasma or serum). Blood was collected from the tail vein. Plasma or serum was collected as indicated in the schedule above. The tail vein was punctured and the blood droplets were collected using RAM Scientific Green-Top Capillary Blood Collection Tubes (#07 7250). For serum collection, blood droplets were collected into a tube with no

additives. Plasma/serum were harvested and transferred into a storage tube to be stored at either –20°C (plasma) or –80°C (serum).

Results

The results for AvPAL short-term dosing studies demonstrate the efficacy of the PEGylated enzymes in lowering excessive phenylalanine levels. Notably, unPEGylated AvPAL did not lower phenylalanine levels after any injection, even before there could be any immunogenic response. Therefore, PEGylation was shown to be an absolute requirement for AvPAL to be a potential treatment for PKU (FIGURE 6).

EXAMPLE 9

Chronic (90 day) Tolerance Study of UnPEGylated and PEGylated R91K PAL and PEGylated NpPAL in PKU Affected Mice

The objective of this study was to evaluate the pharmacodynamic parameters and immunogenicity effects of chronic (90 day) dosing with pegylated and unpegylated R91K PAL and pegylated NpPAL in ENU/2 mice, a disease model of phenylketonuria.

Two batches of each test article (R91K and its PEGylated form R91KPAL: PEG 1:3, npPAL and its PEGylated form npPAL:PEG 1:3 NOF, and vehicle Tris-HCl) were produced, one used for the first half of the study and the second for the second half of the study. Test solutions were stored at -70°C to -80°C. A total of 55 (n= 3-7 per dose group) homozygous ENU2 mice (aka $BTBR^{enu2}$) were used, and under no circumstances were non-naïve (mice previously injected with PAL) animals used.

Experimental Design

Table 5: Group Designations and Dose Levels

Group Number	Group Descriptors	Administered	Number of Doses	N
1	Vehicle control, s.i.d.	Tris NaCl	90	3
2	b.i.w. [a]	R91K	26	3
3	b.i.w. post week 3 [b]	R91K	22	3
4	Escalating Dose, s.i.d.	R91K	90	7
5	Decreasing Dose, s.i.d	R91K	90	7
6	b.i.w. [a]	R91K 1:3 NOF	26	6
7	b.i.w. post week 3 [b]	R91K 1:3 NOF	22	6
8	Escalating Dose, s.i.d.	R91K 1:3 NOF	90	7

9	Decreasing Dose, s.i.d	R91K 1:3 NOF	90	7
10	Bacterial PAL, s.i.w. [c]	npPAL 1:3 NOF	13	6

b.i.w.: twice per week s.i.d.: once per day s.i.w.: once per week

[a] Dose day 1 and 4 on a 7 day schedule (example, every Monday and Thursday)

[b] Groups 3 and 7 will be dosed on day 1 and 8, and starting on day 22 will follow the same 7 day schedule as groups 2 and 6.

[c] Dose day 1 on a 7 day schedule (example, every Monday)

Table 6: Dose Levels

		Dose Days				
		1 – 14	15 – 28	29 – 42	43 – 56	57 – 90
Group Numbers	**Group Descriptors**	**Dose level in IU/mouse/day**				
1	Vehicle control	0	0	0	0	0
2, 3, 6 and 7	Static dose, b.i.w.	1.0	1.0	1.0	1.0	1.0
4 and 8	Escalating Dose, s.i.d.	0.2	0.4	0.6	0.8	1.0
5 and 9	Decreasing Dose, s.i.d.	3.0	2.0	1.0	0.8	0.6
10	Bacterial PAL, s.i.w.	1.0	1.0	1.0	1.0	1.0

Dose administration was performed once daily for groups 1, 4, 5, 8 and 9. Groups 2 and 6 (b.i.w.) were dosed twice per week on days 1 and 4, *e.g.*, every Monday and Thursday. Groups 3 and 7 (b.i.w. post week 3) were dosed on days 1 and 8 and then again on day 22, after which the schedule matched that of groups 2 and 6 (every Monday and Thursday). Group 10 was dosed once weekly. The test solutions were administered by subcutaneous injection (bolus) using a 25 or 26-gauge needle and a 1 cc syringe into the subcutaneous space between the shoulder blades. Each animal received a predetermined dose volume calculated using the Test solution activity (IU/mL) and the dose level (IU/mouse). Dose administration was performed at approximately the same time on each dose administration day.

Animals were observed daily for morbidity, mortality and general health. In particular, the injection site was observed for signs of redness or edema. Body Weights were measured and recorded once weekly, (day –3, 5, 12, …, *e.g.*, every Friday) and at study termination. If there was a death or unscheduled termination due to moribund state, body weight was recorded at that time. Approximately 100 µL of whole blood were collected at

each time point (for 25 μL of plasma and 25 μL of serum). Blood was collected *via* tail vein puncture from conscious animals. The blood droplets for plasma were collected using RAM Scientific Green-Top Capillary Blood Collection Tubes (#07 7250). For serum collection, blood was collected into a tube with no additives. Blood samples were stored on wet ice for no more than 2 hours before separation by centrifugation into plasma/serum and stored at –70 to -80°C until assessment in assays. Blood was collected for plasma and serum for the determination of both phenylalanine and antibody titers on the following days: -3 (pre-study start), 9, 17, 24, 31, 38, 45, 52, 59, 66, 73, 80, 87 and 91. On day 89, blood and plasma was also be collected from Group 1, 5, 9 and 10 animals. Single timepoints each week may not be providing an accurate view of blood Phe response to dosing. Additional timepoints showed whether or not Phe levels are varying between doses. The day 9 sample was of vital importance to study integrity since it marks the time when the PD effect was lost in all dosing regimens attempted to date. A final 100 μL blood sample was collected.

Results

The long-term dosing study shown in FIGURE 7 shows continued efficacy after numerous weekly injections over a ten-week period (with NpPAL-PEG).

EXAMPLE 10

Generation of AvPAL Variants (Cysteine Mutants)

Amino acid substitutions were made in the AvPAL polypeptide to reduce aggregation that occurs in bacterially expressed, recombinant proteins. Protein aggregation may reduce enzyme activity and/or increase immunogenicity *in vivo*. One such form of aggregation occurs as a result of formation of inter-chain disulfide bonds. To minimize this possibility, various AvPAL cysteine residues, alone or in combination, were replaced with serine residues.

The AvPAL polypeptide has 6 cysteine residues, at positions 64, 235, 318, 424, 503 and 565 (SEQ ID NO:4). The following AvPAL single cysteine mutants were generated: AvPAL_C64S (SEQ ID NO:7), AvPAL_C318S (SEQ ID NO:8), AvPAL_C503S (SEQ ID NO:9), and AvPAL_C565S (SEQ ID NO:10). An AvPAL double cysteine mutant, AvPAL_S565SC503S (SEQ ID NO:11), was also generated. FIGURE 8 shows the amino acid sequences of these AvPAL cysteine mutants.

Cloning

The AvPAL gene was amplified from *Anabaena variabilis* genomic DNA (ATCC 29413-U, Qiagen DNeasy Kit) with forward primer AvarPALfor (5'-CACTGTCATATGAAGACACTATCTCAAGCACAAAG-3') (SEQ ID NO:18) and reverse primer AvarPALrev (5'-CACTGTCTCGAGATGCAAGCAGGGTAAGATATCTTG-3') (SEQ ID NO:19). The resulting PCR product was treated with Taq and then ligated into pCR2.1 TOPO TA (Invitrogen). The resulting plasmid was named 1p40.

A 5' NheI site was added and an internal NheI site was removed by SOE-PCR. The upstream AvPAL fragment was amplified from 1p40 with forward primer N-Nhe-AvPAL (5'-CACTGTGCTAGCATGAAGACACTATCTCAAGCACAAAG-3') (SEQ ID NO:20) and reverse primer Nhe-AvPALrev (5'-GGAAATTTCCTCCATGATAGCTGGCTTGGTTATCAACATCAATTAGTGG-3') (SEQ ID NO:21), and the downstream AvPAL fragment was amplified from 1p40 with forward primer Nhe-AvPALfor (5'-CCACTAATTGATGTTGATAACCAAGCCAGCTATCATGGAGGAAATTTCC-3') (SEQ ID NO:22) and reverse primer AvPALrev-r (5'-ACAGTGGCGGCCGCTTAATGCAAGCAGGGTAAGATATCTTG-3') (SEQ ID NO:23). In a single PCR reaction, the two PCR products were annealed and extended with DNA polymerase to produce the full-length AvPAL gene, and then amplified with primers N-Nhe-AvPAL and AvPALrev-r. The resulting PCR product was digested with NheI, blunted with Klenow, digested with NotI, and ligated into the pET28a+ vector (prepared by digestion with NdeI, blunting with Klenow, and digestion with NotI). The resulting plasmid was named 3p86-23.

New restriction sites were added by PCR. AvPAL was amplified from plasmid 3p86-23 with forward primer AvEcoRIfor (5'-CACTGTGAATTCATGAAGACACTATCTCAAGCACAAAG-3') (SEQ ID NO:24) and reverse primer AvSmaIrev (5'-CACTGTCCCGGGTTAATGCAAGCAGGGTAAGATATCT-3') (SEQ ID NO:25). The resulting PCR product was digested with EcoRI and SmaI and ligated into EcoRI- and SmaI-digested pIBX7 vector. The resulting plasmid was named 7p56 Av3.

Cysteine Mutants

Two cysteine codons in the AvPAL gene, corresponding to positions 503 and 565 of the AvPAL polypeptide, were substituted with serine codons by site-directed

mutagenesis (QuickChange XL II, Stratagene). The cysteine codon at position 503 was changed to a serine codon in plasmid 7p56 Av3 by PCR with forward primer Av_C503S (5'-GTCATTACGATGCACGCGCCTCTCTATCACCTGCAACTGAG-3') (SEQ ID NO:26) and reverse primer Av_C503Srev (5'-CTCAGTTGCAGGTGATAGAGAGGCGCGTGCATCGTAATGAC-3') (SEQ ID NO:27). The serine codon is underlined and the G to C mutation in the coding strand (C to G mutation in the non-coding strand) is indicated in bold. The resulting plasmid was named j282. The cysteine codon at position 565 was changed to a serine codon in plasmid j282 with forward primer Av_C565S (5'-CAGTTCAAGATATCTTACCCTCCTTGCATTAACCCGGGCTGC-3') (SEQ ID NO:28) and reverse primer Av_C565Srev (5'-GCAGCCCGGGTTAATGCAAGGAGGGTAAGATATCTTGAACTG-3') (SEQ ID NO:29). The serine codon is underlined and the G to C mutation in the coding strand (C to G mutation in the non-coding strand) is indicated in bold. The resulting plasmid was named j298a.

Cysteine codons in the AvPAL gene at positions 64, 318 and 565 of the AvPAL polypeptide were similarly substituted with serine codons using the following primer pairs: C64S, forward primer Av_C64S (5'-GCAGGGTATTCAGGCATCTTCTGATTACATTAATAATGCTGTTG-3') (SEQ ID NO:30) and reverse primer Av_C64Srev (5'-CAACAGCATTATTAATGTAATCAGAAGATGCCTGAATACCCTGC-3') (SEQ ID NO:31); C318S, forward primer Av_C318S (5'-CAAGATCGTTACTCACTCCGATCCCTTCCCCAGTATTTGGGGC-3') (SEQ ID NO:32) and reverse primer Av_C318Srev (5'-GCCCCAAATACTGGGGAAGGGATCGGAGTGAGTAACGATCTTG-3') (SEQ ID NO:33); and C565S, forward primer Av_C565S (SEQ ID NO:28) and reverse primer Av_C565Srev (SEQ ID NO:29). The serine codons are underlined, and the G to C mutations in the coding strands and the C to G mutations in the non-coding strands are indicated in bold.

EXAMPLE 11

In vitro Enzyme Activity of AvPAL Variants (Cysteine Mutants)

The purpose of this study was to determine the effect of serine substitution of the various cysteine residues in the AvPAL polypeptide on *in vitro* phenylalanine ammonia-lyase (PAL) enzyme activity.

AvPAL variants (*i.e.*, cysteine mutants) were cloned as described in EXAMPLE 10. The AvPAL cysteine mutant expression plasmids were transformed into bacteria and the AvPAL cysteine mutant polypeptides were expressed as described in EXAMPLE 1 and purified as described in EXAMPLE 2.

The wild-type (WT) AvPAL and AvPAL cysteine mutants were tested for *in vitro* PAL enzyme activity as described in EXAMPLE 3. Table 7 shows that compared to unpegylated WT AvPAL, the *in vitro* PAL specific activity of the purified, unpegylated AvPAL cysteine mutant proteins was reduced by serine substitution of the cysteine residue at position 64 (AvPAL_C64S), but was not adversely affected by serine substitution of the cysteine residues at either of positions 503 or 565, or at both positions 503 and 565 (AvPAL_C503S, AvPAL_C565S, and AvPAL_C565SC503S, respectively).

Table 7: Specific Activity of AvPAL Cysteine Mutants

AvPAL Protein	PEGylation	Specific Activity (U/mg)
WT AvPAL	-	1.7
AvPAL_C503S	-	1.9
AvPAL_C64S	-	1.3
AvPAL_C565S E1	-	2.0
AvPAL_C565S E2	-	2.1
AvPAL_C565SC503S	-	2.2
WT AvPAL	+	1.1
AvPAL_C565SC503S	+	1.1

To determine whether the introduction of the serine residues had any effect on enzymatic activity of pegylated AvPAL proteins, the WT AvPAL and double cysteine mutant, AvPAL_C565SC503S, were pegylated as described in EXAMPLE 6. Table 7 shows that the *in vitro* PAL specific activity of the pegylated AvPAL protein was not adversely affected by serine substitution of the cysteine residues at both positions 503 and 565.

EXAMPLE 12

In vitro Biochemical Characterization of AvPAL Variants (Cysteine Mutants)

The purpose of this study was to determine the effect of serine substitution of the various cysteine residues in the AvPAL polypeptide on: (1) accelerated stability; (2) aggregate formation; and (3) site-specific pegylation.

Accelerated Stability

The effect of serine substitution of cysteine residues in AvPAL on *in vitro* stability was determined by storing the purified AvPAL cysteine mutants, either pegylated or un-pegylated, for various time periods at 37°C, and then measuring the *in vitro* PAL specific activity of these proteins as described in EXAMPLE 3.

Wild-type AvPAL and AvPAL cysteine mutants, either upegylated or pegylated, were prepared as described in EXAMPLE 11.

As shown in FIGURE 9A, the specific activities of the unpegylated AvPAL proteins were stable for at least 5 days at 37°C, and were not adversely affected by serine substitution of the cysteine residues at position 565, or at both positions 503 and 565. Similarly, as shown in FIGURE 9B, the specific activities of the pegylated AvPAL proteins were stable for at least 6 days at 37°C. The single cysteine AvPAL mutant, AvPAL_C565S, showed somewhat reduced stability compared to wild-type AvPAL and the double cysteine AvPAL mutant, AvPAL_C565SC503S, after 6 days at 37°C.

Aggregate Formation

The effect of serine substitution of cysteine residues in AvPAL on formation of protein aggregates in solution was determined by separating the purified, unpegylated wild-type AvPAL and AvPAL cysteine mutants by either denaturing and native gel electrophoresis or by SEC-HPLC.

The purified AvPAL preparations were separated by gel electrophoresis under either denaturing conditions (4-12% NuPAGE Bis-Tris) or native conditions (8% Tris-Gly, pH 8.3). The separated AvPAL proteins were stained with Coomassie Blue.

The purified AvPAL preparations were separated by SEC-HPLC. AvPAL proteins were loaded onto a TSK gel column (G3000SWxl, 7.8mm x 30 cm, 5 µm (Tosoh Bioscience, LLC)) in 20 mM Na-phosphate, 300 mM NaCl, pH 6.9, and eluted at a flow rate of 0.5 mL/min. The separated AvPAL proteins were analyzed on an Agilent series 1100 spectrometer.

Aggregates were present in the wild-type AvPAL preparation and in the AvPAL_C503S and AvPAL_C64S preparations, but not in the AvPAL_C565S and AvPAL_C565SC503S preparations, as judged by either gel electrophoresis (FIGURE 10A) or SEC-HPLC (FIGURE 10B).

Site-specific Pegylation

The effect of serine substitution of cysteine residues in AvPAL on site-specific pegylation was determined by pegylating the wild-type AvPAL and double cysteine mutant AvPAL_C503SC565S as described in EXAMPLE 6, and then comparing the relative pegylation at the AvPAL lysine residues: K2, K10, K32, K115, K145, K195, K301, K335, K413, K419, K493, K494 and K522.

Approximately 100 μg (10 μL at 10 μg/μL) of unpegylated or pegylated AvPAL proteins were denatured in 8 M urea. The denatured proteins were then digested in a 100 μL reaction volume with trypsin at pH 8.2 overnight (~20 hours) at 37°C. The trypsin-digested proteins were reduced by treatment with 1 μL of 1 M DTT for 1 hour at 37°C, followed by quenching with 3 μL 15% TFA. Digested proteins were separated on a C18 reverse-phase column. Percent pegylation of each of the pegylated AvPAL peptides was calculated by subtractive peptide mapping of the corresponding unpegylated peptide.

As shown in FIGURE 11, at a ratio of AvPAL protein:PEG of 1:3, there was no striking difference in the percent pegylation of any of the lysine (K) residues with the possible exception of K419, in which the percent pegylation of the double cysteine mutant C565SC503S was lower compared to wild-type AvPAL. However, the results obtained using the double cysteine mutant at increasing AvPAL protein:PEG ratios, in which no dose-response relationship was observed, taken together with the relatively small percent pegylation, indicates that the observed differences at K1419 are not likely to be meaningful. Thus, serine substitution of cysteine residues at positions 503 and 565 does not appear to affect site-specific pegylation of AvPAL.

EXAMPLE 13

Mechanism of Aggregation of AvPAL Proteins

Studies were performed to investigate the mechanism of aggregation of bacterially expressed AvPAL proteins.

Concentrating the purified AvPAL preparations, and incubating the concentrated protein solutions for 2 hours at 37°C, accelerated aggregation of purified AvPAL proteins in solution. Aggregation was detected by separating the AvPAL proteins by SEC-HPLC. To determine whether disulfide cross-linking was responsible for the aggregation, 50 mM dithiotheitol (DTT) was added to the concentrated protein solution, followed by incubation for 2 hours at 37°C.

AvPAL proteins expressed in bacteria were purified as described in EXAMPLE 2, and concentrated using a spin filter (Millipore Biomax –10K NMWL). Proteins were spun at about 15,000 *g* for a few minutes in an Eppendorf Centrifuge 5415C. For cysteine mutants that tend to aggregate (*e.g.*, AvPAL_C503S and AvPAL_C64S), proteins were concentrated to about 20 mg/mL and incubated for 2 hours at 37°C. For cysteine mutants that are resistant to aggregation (*e.g.*, AvPAL_C565S and AvPAL_C565SC503S), proteins were concentrated to about 40 mg/ml and incubated for 2 hours at 37°C.

As shown in Table 8, preparations of purified AvPAL cysteine mutants AvPAL_C64S and AvPAL_C503S formed aggregates upon incubation for 2 hours at 37°C. As expected, this aggregation was exacerbated when the AvPAL proteins were concentrated prior to incubation for 2 hours at 37°C. The aggregation could be blocked by exposure of the concentrated proteins to DTT, indicating that the aggregation is due to disulfide cross-linking. In contrast, the preparations of purified AvPAL cysteine mutants AvPAL_C565S and AvPAL_C565SC503S did not form aggregates upon incubation for 2 hours at 37°C, indicating that the cysteine residue at position 565 is involved in aggregation of AvPAL *via* disulfide cross-linking.

Table 8: Disulfide Cross-link Related Aggregation of AvPAL Cysteine Mutants

AvPAL Protein	Treatment	Aggregate Formation
AvPAL_C503S	37°C/2 h	+
AvPAL_C64S	37°C/2 h	+
AvPAL_C565S E1	37°C/2 h	-
AvPAL_C565S E2	37°C/2 h	-
AvPAL_C565SC503S	37°C/2 h	-
AvPAL_C503S	Concentrate + 37°C/2 h	++
AvPAL_C64S	Concentrate + 37°C/2 h	++
AvPAL_C565S E1	Concentrate + 37°C/2 h	-
AvPAL_C565S E2	Concentrate + 37°C/2 h	-
AvPAL_C565SC503S	Concentrate + 37°C/2 h	-
AvPAL_C503S	Conc. + DTT + 37°C/2 h	-

AvPAL_C64S	Conc. + DTT + 37°C/2 h	-
AvPAL_C565S E1	Conc. + DTT + 37°C/2 h	-
AvPAL_C565S E2	Conc. + DTT + 37°C/2 h	-
AvPAL_C565SC503S	Conc. + DTT + 37°C/2 h	-

To determine which cysteine residues exist as free sulfhydryls, a purified AvPAL preparation was denatured in the presence of 8 M urea, alkylated by iodoacetamide, digested with trypsin, and analyzed by LC/MS. All of the AvPAL cysteine residues were labeled by iodoacetamide, indicating that all of the cysteine residues of bacterially expressed AvPAL exist as free sulfhydryls.

To determine which cysteine residues are present on the surface of the native protein, a purified AvPAL preparation was first treated with N-ethylmaleimide (NEM), then denatured in the presence of 8 M urea, alkylated by iodoacetamide, digested with trypsin, and analyzed by LC/MS. The cysteine residues at positions 235 and 424 were not alkylated by NEM, and the cysteine residue at position 318 was only partially alkylated by NEM, indicating that the cysteine residues at positions 64, 503 and 565 are on the surface of native AvPAL and the cysteine residue at position 318 is partially exposed on the surface of native AvPAL.

To determine which cysteine residues are involved in the inter-chain disulfide cross-linking, 67 µL of a 0.7 mg/mL solution of purified, unpegylated wild-type AvPAL preparation was denatured in 8M urea for 1 hour at 37°C, and then digested in a 100 µL reaction volume with trypsin at pH 8.2 overnight (~17.5 hours) at 25°C. The trypsin-digested proteins were separated and analyzed by mass spectrometry, in which peptides corresponding to the predicted disulfide pairs were identified and quantitated as total ion counts (TIC).

Table 9 shows that disulfide pairs were detected for C503-C503, C503-C565, C565-C318 and C565-C565. The cysteine residues at position 565, and to a lesser extent at position 503, were found in disulfide pairs in the purified AvPAL preparation.

Table 9: Aggregate Disulfide Pairs

Disulfide Pair	Results (TIC/1000)
C64-C318	n.d.[#]
C64-C64	n.d.
C64-C503	n.d.
C64-C565	n.d.
C503-C318	n.d.
C503-C503	11
C503-C565	112

C565-C318	13
C565-C565	37
C318-C318	n.d.

#not detected

Studies were performed to determine whether additional mechanisms besides disulfide cross-linking might be involved in AvPAL protein aggregation.

Purified AvPAL preparations were incubated with either 0.05% Tween or 10 mM EDTA, and then separated by SEC-HPLC as described in EXAMPLE 12. Tween reduces protein aggregation due to hydrophobic interactions, and EDTA reduces protein aggregation due to the presence of divalent cations. As shown in FIGURE 12, exposure to 0.05% Tween or 10 mM EDTA had no effect on AvPAL protein aggregation. The additional peak at 10 minutes in the 10 mM EDTA treated AvPAL is due to absorbance of EDTA at 210 nm.

To further investigate the role of disulfide cross-linking in AvPAL protein aggregation, purified AvPAL was reduced by treatment with DTT and then desalted prior to separation by SEC-HPLC. As shown in FIGURE 13A, AvPAL protein aggregation was minimized by treatment with DTT, and aggregates re-formed following incubation for 18 hours at 37°C. In contrast, as shown in FIGURE 13B, aggregates did not re-form once the AvPAL surface cysteines were modified (*i.e.*, alkylated) by treatment with N-methylmaleimide (NEM) after DTT exposure, but before desalting and incubation for 18 hours at 37°C.

Based on the above, aggregation of bacterially expressed AvPAL appears to be due solely to formation of inter-chain disulfide bonds, and not due to hydrophobic effects or presence of divalent cations. The cysteine residues at positions 565 and 503 are involved in formation of inter-chain disulfide bonds in AvPAL preparations.

EXAMPLE 14

Effects of AvPAL Variants (Cysteine Mutants) and their PEGylated Forms in Mice

The purpose of these studies was to determine the effect of serine substitution of the cysteine residues at positions 503 and 565 in the AvPAL polypeptide on *in vivo* phenylalanine (Phe) levels in mice.

The pegylated forms of the AvPAL double cysteine mutant AvPAL_C565SC503S were tested for *in vivo* activity in homozygous ENU2 (also known as $BTBR^{enu2}$) mice basically as described in EXAMPLE 8. The ENU2 mouse is homozygous mutant at the PAH locus resulting in an animal with severe HPA. The high plasma Phe levels make this animal the appropriate model for evaluating the ability of PAL to reduce plasma Phe.

In the first study, the AvPAL double cysteine mutant AvPAL_C565SC503S was tested at various doses. ENU2 mice (males and females) were divided into 5 dose groups: 4 test groups (n=4) and one vehicle group (n=2). Each mouse was given 8 weekly s.c. doses of vehicle, low dose pegylated double cysteine mutant AvPAL (0.25 IU), mid dose pegylated double cysteine mutant AvPAL (1.0 IU), high dose pegylated double cysteine mutant AvPAL (4.0 IU), or pegylated wild-type AvPAL (4.0 IU). Plasma was collected pre-dose and at 48 hours post-dose (up to day 57) and analyzed for Phe levels. Serum was also collected pre-dose and at 48 hours post-dose (up to day 57) for analysis of anti-AvPAL antibody levels. Mice were also weighed once per week beginning 2 days prior to the first dose (up to day 40).

Two mice died during the study, one vehicle-treated mouse and one low dose pegylated double cysteine mutant AvPAL-treated mouse. As shown in FIGURE 14, a dose-dependent reduction in Phe levels was observed in plasma 48 hours after each s.c. injection of pegylated double cysteine mutant AvPAL. At equivalent doses, there was no difference in plasma Phe levels between mice treated with pegylated wild-type AvPAL or pegylated double cysteine mutant AvPAL. As shown in FIGURE 15, there was also no significant difference in body weights between mice treated with vehicle, pegylated wild-type AvPAL, or pegylated double cysteine mutant AvPAL. It is likely that no significant differences in body weights were observed because the both male and female mice were used in the study.

The anti-AvPAL antibody titers in these mice were analyzed with an indirect ELISA assay. In this assay, microtiter plates were coated with AvPAL, blocked, and then exposed to appropriately diluted sera from each mouse bleed. AvPAL, which was bound to the surface of microtiter plates, was recognized and bound by AvPAL-specific antibodies present in the serum samples. Detectably labeled goat anti-mouse IgG antibodies detected the bound anti-AvPAL antibodies. Serum samples were initially diluted 1:50, and analyzed in comparison to the "cutpoint," which came from pooled mouse serum diluted 1:50. The samples with signal lower than the cutpoint were reported as <50, or "Negative." The rest of

the samples, deemed "Positive," were further diluted in 1:3 series titering to a dilution in which the signal dropped to below the cutpoint. The highest dilution factor that gave a positive signal (*i.e.*, higher than the cutpoint) was reported as the titer of that sample. During this titer series, a 3- fold change of titer may not reflect a significant difference of the antibody detected because the difference could be the result of a minimal change of signal at the cutpoint level.

As shown in Table 10, the anti-AvPAL antibody titers were lower in mice treated with the pegylated double cysteine mutant AvPAL as compared to mice treated with an equivalent dose (4.0 IU) of pegylated wild-type AvPAL. Although no clear dose response was observed, mice treated with the high dose (4.0 IU) of pegylated double cysteine mutant AvPAL had higher anti-AvPAL antibody titers than mice treated with the low dose (0.25 IU) of pegylated double cysteine mutant AvPAL.

Table 10: Anti-AvPAL IgG Titers

Pegylated AvPAL Protein	Sample	Pre	D 8	D 15	D 22	D 29
AvPAL_C565SC503S (0.25 IU)	S 2 03	<50	50	<50	50	50
	S 2 04	<50	50	50	50	450
	S 2 05	<50	<50	<50	<50	<50
	S 2 06	<50	<50	50	50	50
AvPAL_C565SC503S (1.0 IU)	S 3 07	<50	<50	<50	<50	<50
	S 3 08	<50	<50	<50	<50	<50
	S 3 09	<50	<50	<50	<50	50
	S 3 10	<50	<50	<50	<50	<50
AvPAL_C565SC503S (4.0 IU)	S 4 11	<50	<50	<50	50	50
	S 4 12	<50	<50	50	50	50
	S 4 13	50	50	50	450	150
	S 4 14	<50	<50	<50	<50	<50
WT AvPAL (4.0 IU)	S 5 15	<50	<50	150	150	450
	S 5 16	<50	50	150	150	450
	S 5 17	<50	<50	150	4050	12150
	S 5 18	<50	50	150	450	150

Pegylated AvPAL Protein	Sample	Pre	D 36	D 43	D 50	D 57
AvPAL_C565SC503S (0.25 IU)	S 2 03	<50	<50	150	150	<50
	S 2 04	<50	>1350	>1350	>1350	4050
	S 2 05	<50	N/A*	N/A	N/A	N/A
	S 2 06	<50	<50	50	50	<50
AvPAL_C565SC503S (1.0 IU)	S 3 07	<50	50	150	>1350	150
	S 3 08	<50	<50	<50	<50	<50
	S 3 09	<50	450	>1350	>1350	450
	S 3 10	<50	<50	50	50	<50
AvPAL_C565SC503S (4.0 IU)	S 4 11	<50	50	150	50	50
	S 4 12	<50	150	150	150	50
	S 4 13	50	450	450	450	150
	S 4 14	<50	150	150	150	150

Pegylated AvPAL Protein	Sample	Pre	D 36	D 43	D 50	D 57
WT AvPAL (4.0 IU)	S 5 15	<50	450	1350	1350	150
	S 5 16	<50	150	450	450	150
	S 5 17	<50	4050	4050	4050	450
	S 5 18	<50	50	50	50	<50

*No sample/data not available

The reduced anti-AvPAL IgG titers in mice administered 4.0 IU of pegylated double cysteine mutant AvPAL as compared to 4.0 IU pegylated wild-type AvPAL were maintained throughout the study.

In the second study, the AvPAL double cysteine mutant AvPAL_C565SC503S was tested at different pegylation ratios. Male ENU2 mice were divided into 5 dose groups: 4 test groups (n=4) and one vehicle group (n=2). Each mouse was given 8 weekly s.c. doses of vehicle, low dose pegylated double cysteine mutant AvPAL (4 IU and 1:1.6 AvPAL:PEG ratio), mid dose pegylated double cysteine mutant AvPAL (4 IU and 1:2.4 AvPAL:PEG ratio), high dose pegylated double cysteine mutant AvPAL (4 IU and 1:3 AvPAL:PEG ratio), or pegylated wild-type AvPAL (4 IU and 1:3 AvPAL:PEG ratio). Plasma was collected pre-dose and at 4 days post-dose (to day 61) and analyzed for Phe levels. Serum was also collected pre-dose at 4 days post-dose (up to day 57) for analysis of anti-AvPAL antibody levels. Mice were also weighed once per week beginning 2 days prior to the first dose (to day 40).

One vehicle-treated mouse died during the study. As shown in FIGURE 16, a PEG ratio-dependent reduction in Phe levels was observed in plasma 4 days after each s.c. injection of pegylated double cysteine mutant AvPAL. At equivalent PEG ratios, there was no difference in plasma Phe levels between mice treated with pegylated wild-type AvPAL or pegylated double cysteine mutant AvPAL. As shown in FIGURE 17, the body weights of mice treated with pegylated wild-type AvPAL or pegylated double cysteine mutant AvPAL were significantly higher than vehicle-treated mice.

The anti-AvPAL antibody titers in these mice were analyzed with the indirect ELISA assay described above.

As shown in Table 11, the anti-AvPAL antibody titers were lower in mice treated with the pegylated double cysteine mutant AvPAL as compared to mice treated with an equivalent dose of pegylated wild-type AvPAL having the same ratio (1:3) of AvPAL to PEG. An inverse dose response was observed between the anti-AvPAL antibody titers and

the ratio of AvPAL to PEG, consistent with the expectation that pegylation of proteins, such as AvPAL, is associated with reduced immunogenicity *in vivo*.

Table 11: Anti-AvPAL IgG Titers

PEGylated AvPAL Protein	Sample	Pre	D 15	D 28	D 43	D 64
WT AvPAL (PEG 1:3 NOF)	S 1 01	<50	450	12150	4050	>1350
	S 1 06	<50	450	450	450	4050
	S 1 10	<50	50	50	150	450
	S 1 17	<50	150	450	450	1350
AvPAL_C565SC503S (PEG 1:1.6)	S 2 02	50	450	12150	1350	1350
	S 2 07	<50	1350	12150	12150	36450
	S 2 11	<50	450	1350	12150	12150
	S 2 18	50	150	36450	26.57M	>36450
AvPAL_C565SC503S (PEG 1:2.4)	S 3 03	<50	50	150	450	4050
	S 3 08	<50	50	50	50	450
	S 3 12	<50	50	150	450	4050
	S 3 13	<50	50	450	1350	4050
AvPAL_C565SC503S (PEG 1:3)	S 4 04	<50	50	50	450	450
	S 4 09	50	50	50	150	450
	S 4 14	<50	<50	50	450	1350
	S 4 16	<50	<50	150	50	450
Vehicle	S 5 05	<50	<50	<50	<50	N/A*
	S 5 15	<50	<50	<50	<50	<50

*N/A: no serum sample for this timepoint

The above results show that the pegylated double cysteine mutant AvPAL AvPAL_C565SC503S has *in vivo* PAL enzyme activity that is comparable to the pegylated wild-type AvPAL. Because unpegylated wild-type AvPAL had no detectable *in vivo* PAL enzyme activity (see EXAMPLE 8), it is concluded that AvPAL variants, including the pegylated double cysteine mutant AvPAL, AvPAL_C565SC503S, and the pegylated wild-type AvPAL have greater phenylalanine converting activity than the wild-type AvPAL.

The above results also show that the pegylated AvPAL variant, which has reduced protein aggregation *in vitro* due to cysteine to serine substitutions at both positions 503 and 565, has reduced immunogenicity compared to the pegylated wild-type AvPAL. Because pegylation itself is associated with reduced immunogenicity, it is concluded that AvPAL variants have reduced immunogenicity *in vivo* as compared to wild-type AvPAL.

EXAMPLE 15

Generation of Re-Pegylated Forms of AvPAL Variants

Studies were performed to determine whether the pegylated forms of AvPAL variants could be subjected to re-pegylation to increase the amount of pegylation, *i.e.*,

percentage of PEG molecules on AvPAL lysine residues, and, if so, whether the re-pegylated forms of AvPAL variants retain enzymatic activity *in vitro* and have any improved property *in vivo*, namely, reduced immunogenicity.

Pegylation of AvPAL Variants

The double cysteine mutant AvPAL, AvPAL_C565SC503S, was pegylated basically as described in EXAMPLE 6 at a different ratios of PAL:PEG (*i.e.*, AvPAL_C565SC503S:SUNBRIGHT ME-200HS 20 kDa NHS-activated PEG (NOF)).

Briefly, the standard reaction mixture contained: 7 mg/ml (corresponding to 2 mM lysine residues) AvPAL_C565SC503S and 2, 3, 4 or 6 mM mPEG20K-NHS (methoxypolyethyleneglycol-carboxymethyl-N-hydroxysuccinimide ester, 20 kDa, SUNBRIGHT ME-200HS, NOF) in 50 mM potassium phosphate buffer pH 8.5, corresponding to the 1:1, 1:1.5, 1:2 or 1:3 molar ratio, respectively, of total AvPAL lysine residues to PEG molecules (see Table 12). After a 3 hour incubation at room temperature, the reaction mixture was buffer-exchanged into 200 mM potassium phosphate, pH 8.5, and concentrated to greater than 20 mg/ml using tangential flow filtration (Vivaflow 50 or 200, polyethersulfone or regenerated cellulose membrane, 100 kDa MWCO, Sartorius) in preparation for the subsequent re-pegylation step.

Table 12: Pegylation and Re-pegylation Conditions for AvPAL

PEGylation			Re-PEGylation		
AvPAL (mg/ml)	**Total Lysine residues (mM)**	**mPEG20K-NHS (mM)**	**rAvPAL-PEG (mg/ml)**	**Total Lysine residues (mM)**	**mPEG20K-NHS (mM)**
7	2	6	-	-	-
7	2	6	7	2	6
7	2	6	3.5	1	3
7	2	6	7	2	2
7	2	6	7	2	4
7	2	6	7	2	6
7	2	6	10.5	3	6
7	2	2	-	-	-
7	2	2	7	2	2
7	2	2	7	2	6
7	2	2	10.5	3	6
7	2	2	14	4	6
7	2	3	7	2	6
7	2	4	7	2	6

Re-pegylation of Pegylated AvPAL Variants

AvPAL_C565SC503S, pegylated at different PAL:PEG ratios, was re-pegylated basically as described in EXAMPLE 6 using various concentrations of pegylated enzyme, and different amounts and different ratios of lysine residues in the AvPAL enzyme and molecules of SUNBRIGHT ME-200HS 20 kDa NHS-activated PEG (NOF).

For each re-pegylation reaction, the starting material was a pegylated AvPAL_C565SC503S (rAvPAL-PEG) that was concentrated to greater than 20 mg/mL in 200 mM potassium phosphate, pH 8.5. mPEG20K-NHS was weighed out separately for each re-pegylation reaction, and re-suspended in a minimal volume of water. rAvPAL-PEG, diluted in the buffer as necessary, was added directly to the re-suspended mPEG20K-NHS solution to reach the final concentration indicated in Table 12. The re-pegylation reactions were allowed to proceed for 3 hours at room temperature, and then quenched by diluting two-fold with 25 mM TrisHCl, pH 7.3. The tangential flow filtration method (Vivaflow 50 or 200, polyethersulfone or regenerated cellulose membrane, 100 kDa MWCO, Sartorius) was used to buffer-exchange and concentrate the re-pegylated rAvPAL-PEG samples in 10 mM Tris HCl, 135 mM NaCl, 1 mM L-Phe pH 7.3. The final re-pegylated rAvPAL-PEG samples were further concentrated, as needed, using centrifugal concentration.

In Vitro Characterization Methods

Total Protein Assay: The concentrations of pegylated and re-pegylated rAvPAL-PEG samples were determined by the bicinchoninic acid (BCA) assay, using a commercially available assay kit from Pierce (cat#23227) with a bovine serum albumin as a standard. Briefly, 25μL of samples and standards were incubated with 200 μL of the BCA reagent at 37°C for 30 min. Absorbance at 562 nm was read in the SPECTRAmax PLUS microplate spectrophotometer (Molecular Devices Corporation).

rAvPAL Activity Assay: The specific activities of pegylated and re-pegylated rAvPAL-PEG samples were determined based on the established PAL activity assay protocol as described in EXAMPLE 3. Briefly, upon adding protein samples (25-50 μL) to the 1 mL assay reaction, the increase in OD_{290} is monitored as L-Phe is converted to *trans*-cinnamic acid. When necessary, samples were diluted in the assay dilution buffer (10 mM TrisHCl, 135 mM NaCl, pH7.3) prior to the assay. Each data point represented an averaged value of three independent measurements.

Capillary Gel Electrophoresis (CGE): CGE analyses were performed using the ProteomeLab SDS-MW Analysis Kit from Beckman-Coulter (cat # 390953). Pegylated and re-pegylated rAvPAL-PEG and rAvPAL-PEG-RP1 samples were heated at 95°C for 5 minutes in the presence of SDS and β-mercaptoethanol, and then loaded onto capillaries (PA800 CE, 23 cm fused silica) in the P/ACE MDQ Capillary Electrophoresis System from Beckman-Coulter. CGE profiles display the amount of UV absorbance at 214 nm. Each CGE sample contained a 10 kDa migration marker as a reference for analyses. Capillaries were re-conditioned between rAvPAL-PEG samples.

TNBS Assay: A trinitrobenzenesulfonic acid (TNBS) assay was used to provide an estimate of the free amine groups present in the pegylated and re-pegylated rAvPAL-PEG samples.

Peptide Mapping: Tryptic peptide mapping of rAvPAL-PEG and rAvPAL-PEG- samples was performed as described in EXAMPLE 10 to determine the percent pegylation at the lysine residues on the AvPAL variant proteins. The amount of pegylation, *i.e.*, percentage of PEG molecules on AvPAL lysine residues, K10, K32, K115, K145, K195, K301, K335, K413, K493/K494 and K522, of the re-pegylated AvPAL_C565SC503S was determined.

In Vitro Characterization of Pegylated and Re-Pegylated AvPAL Variants

rAvPAL Enzyme Activity: As shown in FIGURE 18 (top), pegylation or re-pegylation caused a temporary decrease in rAvPAL enzyme activity *in vitro*. This appeared to be due to reversible pegylation at specific tyrosine residues near the catalytic active-site. Compared to single pegylation, re-pegylation appeared to enhance the effect of the pegylation reactions on rAvPAL enzyme activity, *i.e.*, to temporarily reduce its specific activity, which gradually recovered over time. A higher concentration of PEG in the pegylation reaction correlated with a greater temporary reduction in specific activity. However, after a few

weeks at 4°C, the specific activity of the re-pegylated rAvPAL-PEG, first generated using a variety of PAL:PEG ratios and concentrations of enzyme and PEG molecules (the mM concentrations of lysine residues in AvPAL and PEG are indicated), reached at least about 80% of the singly pegylated AvPAL-PEG generated using a PAL:PEG ratio of 1:3 (2 mM lysine residues:6 mM PEG, filled circles). Furthermore, the pegylated and re-pegylated AvPAL-PEG enzymes underwent comparable rates of activity recovery after pegylation.

As shown in FIGURE 18 (bottom), a similar trend was observed when the pegylated rAvPAL-PEG was first generated using a PAL:PEG ratio of 1:1 (2 mM lysine residues:2 mM PEG, filled squares). Overall, although re-pegylation temporarily affected AvPAL activity, the resultant specific activities of re-pegylated rAvPAL-PEG were within acceptable ranges compared to the singly pegylated rAvPAL-PEG generated at a PAL:PEG ratio of 1:3.

CGE Analysis of Pegylated and Re-pegylated rAvPAL: CGE analyses indicated that re-pegylation increased the overall extent of pegylation, relative to single pegylation. As shown in FIGURE 19A, re-pegylation resulted in decreased electrophoretic mobility of rAvPAL-PEG, implying an increased molecular size due to attachment of additional PEG molecules. Furthermore, the degree of PEGylation correlated well with the concentration of PEG used during the re-pegylation step. As shown in FIGURE 19B, irrespective of the reaction conditions during the first pegylation step, 6 mM or higher concentration of mPEG20K-NHS was equally effective in increasing pegylation of the rAVPAL-PEG. In summary, compared to the singly pegylated rAvPAL, re-pegylated rAvPAL-PEG clearly showed reduced CGE mobility and, hence, increased molecular mass.

Free Amines in rAvPAL-PEG upon Re-pegylation: The TNBS assay was performed to estimate the amount of free amine groups present in re-pegylated rAVPAL-PEG, relative to the singly pegylated rAvPAL-PEG. As shown in Table 13, re-pegylation in the presence of higher PEG concentrations, such as 6 mM, most significantly decreased the amount of free amines present on the rAvPAL protein, indicating an overall increase in pegylation. Under the conditions used here, most of the free amines in non-pegylated rAvPAL that are available for chemical modification are effectively pegylated after the re-pegylation.

Table 13: Free Amines Present in Re-pegylated rAvPAL-PEG

	[AvPAL] µM	[Free amines] µM	Free amines / rAvPAL monomer	moles PEG / mole rAvPAL
rAvPAL	13.9	204.0	14.6	-
2 mM Lys: 6 mM PEG	23.5	66.8	2.8	11.8
2:6 and 1:3	22.9	42.3	1.8	12.8
2:6 and 2:2	33.1	118.1	3.6	11.0
2:6 and 2:4	32.9	96.3	2.9	11.7
2:6 and 2:6	26.3	16.8	0.6	14.0
2:6 and 3:6	30.5	37.2	1.2	13.4

Percent Pegylation of Lysine Residues upon Re-pegylation: As shown in FIGURE 20, tryptic peptide mapping indicated that re-pegylation improved the percent pegylation of lysine residues on rAvPAL-PEG. Except for the lysine residue at position 10 of AvPAL, which was already 100% pegylated during the single pegylation step, the remaining un-conjugated lysine residues were targeted for pegylation upon re-pegylation. Most notably, the pegylation at the lysine residue at position 301 of AvPAL increased by as much as 500% upon re-pegylation. Re-pegylation conditions in the presence of higher concentrations of activated mPEG20K-NHS, such as 6 mM, appeared to be more effective in augmenting the extent of pegylation in rAvPAL-PEG than lower PEG concentrations, such as 2 mM.

EXAMPLE 16

Effect of Re-Pegylating an AvPAL-PEG Variant in Mice

The re-pegylated AvPAL_C565SC503S (rAvPAL-PEG-RP1), generated using a PAL:PEG ratio of 1:3 in the both the first and second pegylation reactions as described in EXAMPLE 15, was compared to the singly pegylated AvPAL_C565SC503S (rAvPAL-PEG), generated using a PAL:PEG ratio of 1:3 as described in EXAMPLES 6 and 15, with respect to their ability to reduce plasma Phe levels in a mouse model of PKU and to induce an antibody response *in vivo*.

The specific activity of the two pegylated and re-pegylated AvPAL variants enzymes was measured as described in EXAMPLE 3. rAvPAL-PEG-RP1 was found to have a specific activity (1.17 U/mg), which was about 20% lower than the specific activity of rAvPAL-PEG (1.47 U/mg).

The pegylated and re-pegylated forms of AvPAL_C565SC503S, rAvPAL-PEG and rAvPAL-PEG-RP1, respectively, were tested for *in vivo* activity in homozygous

ENU2 (also known as BTBR^{enu2}) mice basically as described in EXAMPLE 8. The study design is shown below in Table 14.

Table 14: Design of *In Vivo* Study to Compare PEGylated and Re-PEGylated Forms of an AvPAL Variant

Group #	N	Test Article	Dosing period (week)	Dose (mg/kg)	Nominal Test Article Activity (IU/mL)	Dose Volume (mL/kg)	Total Number of Doses and Dose Frequency
1	4	rAvPAL-PEG	Weeks 1 - 8	80	12	10	14 once weekly administration (day 1 of a 7 week)
			Weeks 9 - 14	20	3		
2	4	rAvPAL-PEG-RP1	Weeks 1 - 8	80	12		
			Weeks 9 - 14	20	3		
3	4	rAvPAL-PEG	Weeks 1 - 8	80	12		
		rAvPAL-PEG-RP1	Weeks 9 - 14	20	3		

Two groups of mice, groups 1 and 2, were administered subcutaneously a high (80 mg/kg) weekly dose of either rAvPAL-PEG or rAvPAL-PEG-RP1, respectively, for 8 weeks, followed by a low (20 mg/kg) weekly dose of rAvPAL-PEG or rAvPAL-PEG-RP1, respectively, for 6 weeks. One group of mice, group 3, was administered the high dose of rAvPAL-PEG followed by the low dose of rAvPAL-PEG-RP1. Plasma was collected pre-dose and at various days post-dose, and analyzed for Phe levels. Plasma Phe levels were measured as described in EXAMPLE 8, and the data obtained is shown in FIGURE 21. Serum was also collected pre-dose and at various days post-dose for analysis of anti-AvPAL antibody levels. Serum anti-rAvPAL IgG titers were measured as described in EXAMPLE 14, and the data obtained is shown in Table 15.

As shown in FIGURE 21, for all 3 groups, 7-8 weeks of weekly 80 mg/kg subcutaneous dosing of rAvPAL-PEG (DP, filled circles or filled inverted triangles) or rAvPAL-PEG-RP1 (2xPEG, open circles) resulted in stabilization of plasma Phe levels to <200 μM. After stabilization, a decrease to weekly 20 mg/kg subcutaneous dosing with rAvPAL-PEG resulted in 4 days of plasma Phe levels to <200 μM. The plasma Phe levels in ENU2 mice treated with 20 mg/kg rAvPAL-PEG (DP, filled circles) were lower than mice treated with 20 mg/kg rAvPAL-PEG-RP1 (2xPEG, open circles or filled inverted triangles), possibly due to the greater specific activity of the singly pegylated rAvPAL-PEG in comparison with that of the doubly pegylated rAvPAL-PEG-RP1.

As shown in Table 15, the anti-AvPAL IgG titers were lower in ENU2 mice injected with rAvPAL-PEG-RP1 (group 2) than those injected with rAvPAL-PEG (groups 1

and 3). This indicated that re-pegylating the AvPAL variant to have an increased amount of pegylation, in particular with 20 kDa linear PEG molecules, was associated with a decreased immunogenicity *in vivo*.

Table 15: Anti-AvPAL IgG Titers

Pegylated AvPAL Protein	Sample	D -2	D 14	D 26	D 42	D 68
rAvPAL-PEG	1-1	<50	50	150	150	150
	1-2	<50	50	1350	450	150
	1-3	150	450	4050	1350	1350
	1-4	150	1350	450	450	1350
rAvPAL-PEG-RP1	2-1	<50	<50	50	150	50
	2-2	150	50	<50	50	<50
	2-3	<50	<50	<50	50	<50
	2-4	<50	<50	<50	<50	<50
rAvPAL-PEG weeks 1-8, then	3-1	50	450	1350	1350	450
rAvPAL-PEG-RP1 weeks 9-14	3-2	50	1350	1350	1350	1350
	3-3	<50	450	4050	1350	1350
	3-4	<50	50	450	150	450

In summary, re-pegylating the AvPAL-PEG variant with 20 kDa linear PEG molecules to increase the percent pegylation at various lysine residues in AvPAL was associated with a slightly decreased enzyme activity *in vitro*, but, importantly, reduced immunogenicity *in vivo*.

EXAMPLE 17

Clinical Evaluation With Prokaryotic PAL Compositions

The following example provides guidance on the parameters to be used for the clinical evaluation of compositions comprising prokaryotic PAL or biologically active variants, mutants, and fragments thereof ("PAL") in the therapeutic methods of the present invention. As discussed herein throughout, PAL will be used in the treatment of HPA including HPA, mild phenylketonuria (PKU) and classic PKU. Clinical trials will be conducted which will provide an assessment of oral or subcutaneous doses of PAL for safety, pharmacokinetics, and initial response of both surrogate and defined clinical endpoints. The trial will be conducted for a minimum, but not necessarily limited to, 24 weeks to collect sufficient safety information for 100 evaluable patients. The initial dose for the trials will vary from about 0.001 to about 1.0 mg/kg/week. In the event that this dose does not produce a reduction in excess plasma phenylalanine (Phe) levels in a patient, or produce a significant direct clinical benefit measured as an ability to increase daily oral Phe intake without

increases in plasma Phe levels, the dose should be increased as necessary, and maintained for an additional minimal period of, but necessarily limited to, 24 weeks to establish safety and to evaluate further efficacy.

Measurements of safety will include adverse events, allergic reactions, complete clinical chemistry panel (kidney and liver function), urinalysis, and CBC with differential. In addition, other parameters including the reduction in levels of blood Phe levels, neuropsychological and cognitive testing, and global assessments also will be monitored. The present example also contemplates the determination of pharmacokinetic parameters of the drug in the circulation, and general distribution and half-life of PAL in blood. It is anticipated that these measures will help relate dose to clinical response.

Methods

Patients who have elevated levels of plasma Phe will undergo a baseline a medical history and physical exam, neuropsychological and cognitive testing, a standard set of clinical laboratory tests (CBC, Panel 20, CH50, UA), levels of urinary pterins, dihydropteridine reductase (DHPR) levels, and a fasting blood (plasma) panel of serum amino acids. The patient will be followed closely with weekly visits to the clinic. Patients will return to the clinic for a complete evaluation one week after completing the treatment period. Should dose escalation be required, the patients will follow the same schedule outlined above. Safety will be monitored throughout the trial.

Diagnosis and Inclusion/Exclusion Criteria

The patient may be male or female, with a documented diagnosis of HPA or mild PKU confirmed by genetic testing and evidence of elevated Phe levels in blood. The study will include HPA or PKU patients who do not accurately follow dietary control. Female patients of childbearing potential must have a negative pregnancy test (urine β-hCG) just prior to each dosing and must be advised to use a medically accepted method of contraception throughout the study. A patient will be excluded from this study if the patient is pregnant or lactating; has received an investigational drug within 30 days prior to study enrollment; or has a medical condition, serious intercurrent illness, or other extenuating circumstance that may significantly decrease study compliance.

Dietary Intervention

Following the initial randomization and two-week treatment period, all study participants will undergo dietary counseling and will follow a standard diet and/or a standard

Phe-restricted diet complemented with Phe-specific medical foods for a total of four to six weeks. Diets will be managed at home and dietary intake will be recorded in daily logs. Analyses of the intakes of nutrients and medical foods and the percent of Recommended Dietary Intakes (RDI) will be compared among the treatment groups.

PAL Safety

PAL therapy will be determined to be safe if no significant acute or chronic drug reactions occur during the course of the study. The longer-term administration of the drug will be determined to be safe if no significant abnormalities are observed in the clinical examinations, clinical labs, or other appropriate studies.

EXAMPLE 18

Clinical Evaluation of a Pegylated AvPAL Variant

The pegylated double cysteine mutant AvPAL, AvPAL_C565SC503S will be clinically evaluated in humans. The objective of the clinical evaluation is to determine the safety, tolerability, pharmacokinetics (PK), and efficacy in PKU patients. Blood phenylalanine (Phe) levels will serve as a clinical endpoint.

Phase 1

Phase 1 is an open label, single dose escalation study in 35 PKU patients of ages between 16 and 50 years. The primary objective is to assess the safety and tolerability of pegylated double cysteine mutant AvPAL, AvPAL_C565SC503S, and the secondary objectives are to evaluate the pegylated enzyme's PK and Phe reduction. Seven cohorts of 5 subjects are administered sequentially with increasing doses of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 mg/kg pegylated AvPAL_C565SC503S. The subjects in each cohort receive a single dose, and then are followed up for a total of 6 weeks. The inclusion criteria is that the blood Phe level at screening and the average blood Phe level over the past 3 years is ≥600 μM.

Phase 2

The Phase 2 study is divided into two parts with no interruption:

- Part 1 – 16 week – 8-week administration, then dose optimization
- Part 2 – 40 week – extension

Phase 2 is a two-part, open label, dose optimization study in 35 subjects with PKU. Part 1 conducted over a period of 16 weeks duration involves the administration of doses of pegylated AvPAL_C565SC503S once weekly for 8 weeks, followed by a period of dose optimization. Each subject's dose is adjusted to attain blood Phe concentrations below 600 μM. The primary objective is to evaluate the safety and tolerability of multiple administration of the pegylated AvPAL_C565SC503S, and the secondary objectives are to evaluate the pegylated enzyme's effects on blood Phe concentrations, immune response, *e.g.*, anti-AvPAL antibody titers, and steady state PK. In part 2, the subjects are administered pegylated AvPAL_C565SC503S for 40 weeks, with individualized optimized dose and frequency of administration.

Phase 3

Once the Phase 1 and 2 studies are complete, additional studies may potentially include a Phase 3 six-month double-blinded study in a greater number of subjects, with additional studies in special populations, such as, for example and not for limitation, non-PKU hyperphenylalanemia (HPA) patients, BH4-responsive PKU patients and non-BH4-responsive PHU patients.

We claim:

1. An *Anabaena variabilis* phenylalanine ammonia-lyase (AvPAL) variant, wherein one or more cysteine residues selected from the group consisting of cysteine residues at positions 503 and 565 has been substituted by a serine residue, and wherein said AvPAL variant has a greater phenylalanine-converting activity and/or a reduced immunogenicity as compared to a wild-type AvPAL (SEQ ID NO:4).

2. The AvPAL variant of claim 1, wherein the cysteine residue at position 503 of AvPAL has been substituted with a serine residue (SEQ ID NO:9).

3. The AvPAL variant of claim 1, wherein the cysteine residue at position 565 of AvPAL has been substituted with a serine residue (SEQ ID NO:10).

4. The AvPAL variant of claim 1, wherein the cysteine residues at positions 503 and 565 have been substituted with serine residues (SEQ ID NO:11).

5. The AvPAL variant of any one of claims 1 to 4, which is pegylated.

6. The AvPAL variant of claim 5, wherein said pegylation is achieved by reacting the AvPAL variant with NHS-activated polyethylene glycol at a ratio of 3 polyethylene glycol per lysine residue of AvPAL variant.

7. An AvPAL variant, wherein the cysteine residue at position 503 of AvPAL has been substituted with a serine residue (SEQ ID NO:9).

8. An AvPAL variant, wherein the cysteine residue at position 565 of AvPAL has been substituted with a serine residue (SEQ ID NO:10).

9. An AvPAL variant, wherein the cysteine residues at positions 503 and 565 have been substituted with serine residues (SEQ ID NO:11).

10. The AvPAL variant of claim 9, which is pegylated.

11. The AvPAL variant of claim 10, wherein said pegylation is achieved by reacting the AvPAL variant with NHS-activated polyethylene glycol at a ratio of at least 1.6 polyethylene glycol per lysine residue of AvPAL variant.

12. The AvPAL variant of claim 10, wherein said pegylation is achieved by reacting the AvPAL variant with NHS-activated polyethylene glycol at a ratio of at least 2.4 polyethylene glycol per lysine residue of AvPAL variant.

13. The AvPAL variant of claim 10, wherein said pegylation is achieved by reacting the AvPAL variant with NHS-activated polyethylene glycol at a ratio of 3 polyethylene glycol per lysine residue of AvPAL variant.

14. The AvPAL variant of claim 10, wherein at least 28 percent of lysine residues at positions 2, 10, 32, 145, 195, 301, 413, 493, and 522 of the AvPAL variant are pegylated.

15. The AvPAL variant of claim 10, wherein at least 51 percent of lysine residues at positions 2, 10, 195, 413, 493, and 522 of the AvPAL are pegylated.

16. The AvPAL variant of claim 10, wherein at least 75 percent of lysine residues at positions 2, 10, 195, 493, and 522 of the AvPAL are pegylated.

17. A pharmaceutical composition, comprising the AvPAL variant of any one of claims 1 to 16 and a pharmaceutically acceptable carrier.

18. Use of the AvPAL variant of any one of claims 1 to 16 in preparation of a medicament for treating a disease in a subject caused all or in part by a deficiency in phenylalanine hydroxylase (PAH).

19. The use of claim 18, wherein the disease is characterized by elevated phenylalanine levels.

20. The use of claim 18 or 19, wherein said subject has about 10% or less of a normal PAH activity.

21. Use of the AvPAL variant of any one of claims 1 to 16 in preparation of a medicament for treating phenylketonuria (PKU) in a subject.

22. The use of claim 21, wherein said PKU is classic severe PKU.

23. The use of any one of claims 18 to 22, wherein the subject has a deficiency in PAH activity.

24. The use of any one of claims 18 to 23, wherein the subject has a mutant PAH.

25. The use of claim 24, wherein said mutant PAH comprises a mutation in the catalytic domain of PAH.

26. The use of claim 25, wherein said mutation comprises one or more mutations selected from the group consisting of F39L, L48S, I65T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241C, R261Q, A300S, L308F, A313T, K320N, A373T, V388M E390G, A395P, P407S, and Y414C.

27. The use of any one of claims 18 to 26, wherein the subject is a pregnant female.

28. The use of any one of claims 18 to 27, wherein the medicament is prepared to be administered to the subject in combination with a protein-restricted diet.

29. The use of any one of claims 18 to 26, wherein the subject is an infant between 0 and 3 years of age.

30. The use of claim 29, wherein the infant has a plasma phenylalanine concentration of between about 360 μM to about 4800 μM.

31. The use of any one of claims 18 to 30, wherein the subject is a human.

32. The use of a composition comprising a AvPAL variant of claim 1 in the preparation of a medicament for treating a pregnant female having

hyperphenylalaninemia (HPA) in combination with a protein-restricted diet, wherein the combined administration of the composition and protein-restricted diet is effective to lower the phenylalanine concentration in the plasma of the subject as compared to said concentration in the absence of said combined administration.

33. The use of a composition comprising a AvPAL variant of claim 1 in the preparation of a medicament for treating an infant having phenylketonuria (PKU), in an amount effective to produce a decrease in the plasma phenylalanine concentration of said infant, wherein said infant is between 0 and 3 years of age and said infant has a plasma phenylalanine concentration of between about 360 μM to about 4800 μM.

SEQUENCE LISTING

<110> BioMarin Pharmaceutical Inc.
Vellard, Michel C
Fitzpatrick, Paul A
Kakkis, Emil D
Wendt, Dan

<120> Compositions of prokaryotic phenylalanine ammonia-lyase and methods of using compositions thereof

<130> 0165 PC10

<150> US 11/451,999
<151> 2006-06-12

<160> 33

<170> PatentIn version 3.4

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<211> 1710
<212> DNA
<213> Nostoc punctiforme

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<210> 2
<211> 569
<212> PRT
<213> Nostoc punctiforme

<400> 2

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Pro Phe Thr Asn Ser Ser Asp Ser Ile Val Thr Val Gly Asp Arg Asn
20 25 30

Leu Thr Ile Asp Glu Val Val Asn Val Ala Arg His Gly Thr Gln Val
35 40 45

Arg Leu Thr Asp Asn Ala Asp Val Ile Arg Gly Val Gln Ala Ser Cys
50 55 60

Asp Tyr Ile Asn Asn Ala Val Glu Thr Ala Gln Pro Ile Tyr Gly Val
65 70 75 80

Thr Ser Gly Phe Gly Gly Met Ala Asp Val Val Ile Ser Arg Glu Gln
85 90 95

Ala Ala Glu Leu Gln Thr Asn Leu Ile Trp Phe Leu Lys Ser Gly Ala
100 105 110

Gly Asn Lys Leu Ser Leu Ala Asp Val Arg Ala Ala Met Leu Leu Arg
115 120 125

Ala Asn Ser His Leu Tyr Gly Ala Ser Gly Ile Arg Leu Glu Leu Ile
130 135 140

Gln Arg Ile Glu Thr Phe Leu Asn Ala Gly Val Thr Pro His Val Tyr
145 150 155 160

Glu Phe Gly Ser Ile Gly Ala Ser Gly Asp Leu Val Pro Leu Ser Tyr
165 170 175

Ile Thr Gly Ala Leu Ile Gly Leu Asp Pro Ser Phe Thr Val Asp Phe

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            180                 185                 190

Asp Gly Lys Glu Met Asp Ala Val Thr Ala Leu Ser Arg Leu Gly Leu
        195                 200                 205

Pro Lys Leu Gln Leu Gln Pro Lys Glu Gly Leu Ala Met Met Asn Gly
    210                 215                 220

Thr Ser Val Met Thr Gly Ile Ala Ala Asn Cys Val Tyr Asp Ala Lys
225                 230                 235                 240

Val Leu Leu Ala Leu Thr Met Gly Val His Ala Leu Ala Ile Gln Gly
                245                 250                 255

Leu Tyr Gly Thr Asn Gln Ser Phe His Pro Phe Ile His Gln Cys Lys
            260                 265                 270

Pro His Pro Gly Gln Leu Trp Thr Ala Asp Gln Met Phe Ser Leu Leu
        275                 280                 285

Lys Asp Ser Ser Leu Val Arg Glu Glu Leu Asp Gly Lys His Glu Tyr
    290                 295                 300

Arg Gly Lys Asp Leu Ile Gln Asp Arg Tyr Ser Leu Arg Cys Leu Ala
305                 310                 315                 320

Gln Phe Ile Gly Pro Ile Val Asp Gly Val Ser Glu Ile Thr Lys Gln
                325                 330                 335

Ile Glu Val Glu Met Asn Ser Val Thr Asp Asn Pro Leu Ile Asp Val
            340                 345                 350

Glu Asn Gln Val Ser Tyr His Gly Gly Asn Phe Leu Gly Gln Tyr Val
        355                 360                 365

Gly Val Thr Met Asp Arg Leu Arg Tyr Tyr Ile Gly Leu Leu Ala Lys
    370                 375                 380

His Ile Asp Val Gln Ile Ala Leu Leu Val Ser Pro Glu Phe Ser Asn
385                 390                 395                 400

Gly Leu Pro Pro Ser Leu Val Gly Asn Ser Asp Arg Lys Val Asn Met
                405                 410                 415

Gly Leu Lys Gly Leu Gln Ile Ser Gly Asn Ser Ile Met Pro Leu Leu
            420                 425                 430

Ser Phe Tyr Gly Asn Ser Leu Ala Asp Arg Phe Pro Thr His Ala Glu
        435                 440                 445

Gln Phe Asn Gln Asn Ile Asn Ser Gln Gly Tyr Ile Ser Ala Asn Leu
    450                 455                 460
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Thr Arg Arg Ser Val Asp Ile Phe Gln Asn Tyr Met Ala Ile Ala Leu
465                 470                 475                 480

Met Phe Gly Val Gln Ala Val Asp Leu Arg Thr Tyr Lys Met Lys Gly
                485                 490                 495

His Tyr Asp Ala Arg Thr Cys Leu Ser Pro Asn Thr Val Gln Leu Tyr
            500                 505                 510

Thr Ala Val Cys Glu Val Val Gly Lys Pro Leu Thr Ser Val Arg Pro
        515                 520                 525

Tyr Ile Trp Asn Asp Asn Glu Gln Cys Leu Asp Glu His Ile Ala Arg
    530                 535                 540

Ile Ser Ala Asp Ile Ala Gly Gly Gly Leu Ile Val Gln Ala Val Glu
545                 550                 555                 560

His Ile Phe Ser Ser Leu Lys Ser Thr
                565
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<210> 3
<211> 1704
<212> DNA
<213> Anabaena variabilis

<400> 3

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gtagcgcgta atggcacctt agtgtcttta accaataaca ctgatatttt gcagggtatt    180

caggcatctt gtgattacat taataatgct gttgaatctg gggaaccaat ttatggagtg    240

acatctggtt ttggcggtat ggccaatgtt gccatatccc gtgaacaagc atctgaactc    300

caaaccaact tagtttggtt cctgaaaaca ggtgcaggga acaaattacc cttggcggat    360

gtgcgcgcag ctatgctctt gcgtgcaaac tctcatatgc gcggtgcatc tggcatcaga    420

ttagaactta tcaagcgtat ggagattttc cttaacgctg gtgtcacacc atatgtgtat    480

gagtttggtt caattggtgc aagtggtgat ttagtgccac tatcctacat tactggttca    540

ctgataggct tagatcccag ttttaaggtt gacttcaacg gtaaagaaat ggatgcgcca    600

acagctctac gtcaactgaa tttgtcaccc ttgacattgt tgccgaagga aggcttggcg    660

atgatgaacg gcacttcagt catgacaggt attgcagcaa actgcgtcta cgatactcaa    720

attttaactg cgatcgctat gggcgttcac gctctagata tccaagcttt aaacggaacc    780

aatcaatcat tccatccatt tatccataat tccaaaccac atcctggtca attatgggca    840

gcagatcaga tgatttcttt gttagccaat tcccagttag ttcgtgatga gttagatggt    900

aaacacgatt atcgtgatca cgagttgatt caagatcgtt actcactccg atgccttccc    960

cagtatttgg ggccaatcgt tgatggaatt tcccagattg ccaaacaaat tgaaatcgaa   1020
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ttattggcta aacacctaga tgtgcagatt gccctcctcg cctcaccaga gtttagcaat 1200

ggactaccac catctttatt aggcaaccga gaacgtaaag tcaatatggg actcaaaggt 1260

ctgcaaatat gcggtaactc aattatgcca ctgttgacct tctatggaaa ttccatcgcc 1320

gatcgctttc ctacccatgc agaacaattt aatcagaaca tcaacagtca aggatacact 1380

tcagcgactc tagcccgccg ttctgtggat atcttccaga attatgtggc gatcgctctg 1440

atgtttggag tccaagctgt tgacctccgc acatataaaa agactggtca ttacgatgca 1500

cgcgcctgtc tatcacctgc aactgagcgc ttatattcag cagtccgcca cgtagttgga 1560

caaaaaccaa cttcagatcg cccatatatt tggaatgata atgagcaagg actggatgag 1620

catattgccc ggatttctgc tgatatcgct gctggtggtg tgattgtgca agcagttcaa 1680

gatatcttac cctgcttgca ttaa 1704

<210> 4
<211> 567
<212> PRT
<213> Anabaena variabilis

<400> 4

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Ser Phe Thr Gly Asn Ser Ser Ala Asn Val Ile Ile Gly Asn Gln Lys
20 25 30

Leu Thr Ile Asn Asp Val Ala Arg Val Ala Arg Asn Gly Thr Leu Val
35 40 45

Ser Leu Thr Asn Asn Thr Asp Ile Leu Gln Gly Ile Gln Ala Ser Cys
50 55 60

Asp Tyr Ile Asn Asn Ala Val Glu Ser Gly Glu Pro Ile Tyr Gly Val
65 70 75 80

Thr Ser Gly Phe Gly Gly Met Ala Asn Val Ala Ile Ser Arg Glu Gln
85 90 95

Ala Ser Glu Leu Gln Thr Asn Leu Val Trp Phe Leu Lys Thr Gly Ala
100 105 110

Gly Asn Lys Leu Pro Leu Ala Asp Val Arg Ala Ala Met Leu Leu Arg
115 120 125

Ala Asn Ser His Met Arg Gly Ala Ser Gly Ile Arg Leu Glu Leu Ile
130 135 140

Lys Arg Met Glu Ile Phe Leu Asn Ala Gly Val Thr Pro Tyr Val Tyr
145 150 155 160

Glu Phe Gly Ser Ile Gly Ala Ser Gly Asp Leu Val Pro Leu Ser Tyr
165 170 175

Ile Thr Gly Ser Leu Ile Gly Leu Asp Pro Ser Phe Lys Val Asp Phe
180 185 190

Asn Gly Lys Glu Met Asp Ala Pro Thr Ala Leu Arg Gln Leu Asn Leu
195 200 205

Ser Pro Leu Thr Leu Leu Pro Lys Glu Gly Leu Ala Met Met Asn Gly
210 215 220

Thr Ser Val Met Thr Gly Ile Ala Ala Asn Cys Val Tyr Asp Thr Gln
225 230 235 240

Ile Leu Thr Ala Ile Ala Met Gly Val His Ala Leu Asp Ile Gln Ala
245 250 255

Leu Asn Gly Thr Asn Gln Ser Phe His Pro Phe Ile His Asn Ser Lys
260 265 270

Pro His Pro Gly Gln Leu Trp Ala Ala Asp Gln Met Ile Ser Leu Leu
275 280 285

Ala Asn Ser Gln Leu Val Arg Asp Glu Leu Asp Gly Lys His Asp Tyr
290 295 300

Arg Asp His Glu Leu Ile Gln Asp Arg Tyr Ser Leu Arg Cys Leu Pro
305 310 315 320

Gln Tyr Leu Gly Pro Ile Val Asp Gly Ile Ser Gln Ile Ala Lys Gln
325 330 335

Ile Glu Ile Glu Ile Asn Ser Val Thr Asp Asn Pro Leu Ile Asp Val
340 345 350

Asp Asn Gln Ala Ser Tyr His Gly Gly Asn Phe Leu Gly Gln Tyr Val
355 360 365

Gly Met Gly Met Asp His Leu Arg Tyr Tyr Ile Gly Leu Leu Ala Lys
370 375 380

His Leu Asp Val Gln Ile Ala Leu Leu Ala Ser Pro Glu Phe Ser Asn
385 390 395 400

Gly Leu Pro Pro Ser Leu Leu Gly Asn Arg Glu Arg Lys Val Asn Met
405 410 415

Gly Leu Lys Gly Leu Gln Ile Cys Gly Asn Ser Ile Met Pro Leu Leu

420 425 430

Thr Phe Tyr Gly Asn Ser Ile Ala Asp Arg Phe Pro Thr His Ala Glu
435 440 445

Gln Phe Asn Gln Asn Ile Asn Ser Gln Gly Tyr Thr Ser Ala Thr Leu
450 455 460

Ala Arg Arg Ser Val Asp Ile Phe Gln Asn Tyr Val Ala Ile Ala Leu
465 470 475 480

Met Phe Gly Val Gln Ala Val Asp Leu Arg Thr Tyr Lys Lys Thr Gly
485 490 495

His Tyr Asp Ala Arg Ala Cys Leu Ser Pro Ala Thr Glu Arg Leu Tyr
500 505 510

Ser Ala Val Arg His Val Val Gly Gln Lys Pro Thr Ser Asp Arg Pro
515 520 525

Tyr Ile Trp Asn Asp Asn Glu Gln Gly Leu Asp Glu His Ile Ala Arg
530 535 540

Ile Ser Ala Asp Ile Ala Ala Gly Gly Val Ile Val Gln Ala Val Gln
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Asp Ile Leu Pro Cys Leu His
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<210> 5
<211> 523
<212> PRT
<213> Streptomyces maritimus

<400> 5

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20 25 30

Arg Ser Arg Val Arg Ala Ser Arg Asp Val Leu Val Lys Phe Val Gln
35 40 45

Asp Glu Arg Val Ile Tyr Gly Val Asn Thr Ser Met Gly Gly Phe Val
50 55 60

Asp His Leu Val Pro Val Ser Gln Ala Arg Gln Leu Gln Glu Asn Leu
65 70 75 80

Ile Asn Ala Val Ala Thr Asn Val Gly Ala Tyr Leu Asp Asp Thr Thr
85 90 95

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Ala Arg Thr Ile Met Leu Ser Arg Ile Val Ser Leu Ala Arg Gly Asn
            100                 105                 110

Ser Ala Ile Thr Pro Ala Asn Leu Asp Lys Leu Val Ala Val Leu Asn
        115                 120                 125

Ala Gly Ile Val Pro Cys Ile Pro Glu Lys Gly Ser Leu Gly Thr Ser
    130                 135                 140

Gly Asp Leu Gly Pro Leu Ala Ala Ile Ala Leu Val Cys Ala Gly Gln
145                 150                 155                 160

Trp Lys Ala Arg Tyr Asn Gly Gln Ile Met Pro Gly Arg Gln Ala Leu
                165                 170                 175

Ser Glu Ala Gly Val Glu Pro Met Glu Leu Ser Tyr Lys Asp Gly Leu
            180                 185                 190

Ala Leu Ile Asn Gly Thr Ser Gly Met Val Gly Leu Gly Thr Met Val
        195                 200                 205

Leu Gln Ala Ala Arg Arg Leu Val Asp Arg Tyr Leu Gln Val Ser Ala
    210                 215                 220

Leu Ser Val Glu Gly Leu Ala Gly Met Thr Lys Pro Phe Asp Pro Arg
225                 230                 235                 240

Val His Gly Val Lys Pro His Arg Gly Gln Arg Gln Val Ala Ser Arg
                245                 250                 255

Leu Trp Glu Gly Leu Ala Asp Ser His Leu Ala Val Asn Glu Leu Asp
            260                 265                 270

Thr Glu Gln Thr Leu Ala Gly Glu Met Gly Thr Val Ala Lys Ala Gly
        275                 280                 285

Ser Leu Ala Ile Glu Asp Ala Tyr Ser Ile Arg Cys Thr Pro Gln Ile
    290                 295                 300

Leu Gly Pro Val Val Asp Val Leu Asp Arg Ile Gly Ala Thr Leu Gln
305                 310                 315                 320

Asp Glu Leu Asn Ser Ser Asn Asp Asn Pro Ile Val Leu Pro Glu Glu
                325                 330                 335

Ala Glu Val Phe His Asn Gly His Phe His Gly Gln Tyr Val Ala Met
            340                 345                 350

Ala Met Asp His Leu Asn Met Ala Leu Ala Thr Val Thr Asn Leu Ala
        355                 360                 365
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Asn Arg Arg Val Asp Arg Phe Leu Asp Lys Ser Asn Ser Asn Gly Leu
370 375 380

Pro Ala Phe Leu Cys Arg Glu Asp Pro Gly Leu Arg Leu Gly Leu Met
385 390 395 400

Gly Gly Gln Phe Met Thr Ala Ser Ile Thr Ala Glu Thr Arg Thr Leu
405 410 415

Thr Ile Pro Met Ser Val Gln Ser Leu Thr Ser Thr Ala Asp Phe Gln
420 425 430

Asp Ile Val Ser Phe Gly Phe Val Ala Ala Arg Arg Ala Arg Glu Val
435 440 445

Leu Thr Asn Ala Ala Tyr Val Val Ala Phe Glu Leu Leu Cys Ala Cys
450 455 460

Gln Ala Val Asp Ile Arg Gly Ala Asp Lys Leu Ser Ser Phe Thr Arg
465 470 475 480

Pro Leu Tyr Glu Arg Thr Arg Lys Ile Val Pro Phe Phe Asp Arg Asp
485 490 495

Glu Thr Ile Thr Asp Tyr Val Glu Lys Leu Ala Ala Asp Leu Ile Ala
500 505 510

Gly Glu Pro Val Asp Ala Ala Val Ala Ala His
515 520

<210> 6
<211> 510
<212> PRT
<213> Pseudomonas putida

<400> 6

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Arg Ala Ile His Ala Ala Pro Val Arg Leu Gln Leu Asp Ala Ser Ala
20 25 30

Ala Pro Ala Ile Asp Ala Ser Val Ala Cys Val Glu Gln Ile Ile Ala
35 40 45

Glu Asp Arg Thr Ala Tyr Gly Ile Asn Thr Gly Phe Gly Leu Leu Ala
50 55 60

Ser Thr Arg Ile Ala Ser His Asp Leu Glu Asn Leu Gln Arg Ser Leu
65 70 75 80

Val Leu Ser His Ala Ala Gly Ile Gly Ala Pro Leu Asp Asp Asp Leu
85 90 95

```
Val Arg Leu Ile Met Val Leu Lys Ile Asn Ser Leu Ser Arg Gly Phe
            100                 105                 110

Ser Gly Ile Arg Arg Lys Val Ile Asp Ala Leu Ile Ala Leu Val Asn
        115                 120                 125

Ala Glu Val Tyr Pro His Ile Pro Leu Lys Gly Ser Val Gly Ala Ser
    130                 135                 140

Gly Asp Leu Ala Pro Leu Ala Thr Met Ser Leu Val Leu Leu Gly Glu
145                 150                 155                 160

Gly Lys Ala Arg Tyr Lys Gly Gln Trp Leu Ser Ala Thr Glu Ala Leu
                165                 170                 175

Ala Val Ala Gly Leu Glu Pro Leu Thr Leu Ala Ala Lys Glu Gly Leu
            180                 185                 190

Ala Leu Leu Asn Gly Thr Gln Ala Ser Thr Ala Tyr Ala Leu Arg Gly
        195                 200                 205

Leu Phe Tyr Ala Glu Asp Leu Tyr Ala Ala Ala Ile Ala Cys Gly Gly
    210                 215                 220

Leu Ser Val Glu Ala Val Leu Gly Ser Arg Ser Pro Phe Asp Ala Arg
225                 230                 235                 240

Ile His Glu Ala Arg Gly Gln Arg Gly Gln Ile Asp Thr Ala Ala Cys
                245                 250                 255

Phe Arg Asp Leu Leu Gly Asp Ser Ser Glu Val Ser Leu Ser His Lys
            260                 265                 270

Asn Cys Asp Lys Val Gln Asp Pro Tyr Ser Leu Arg Cys Gln Pro Gln
        275                 280                 285

Val Met Gly Ala Cys Leu Thr Gln Leu Arg Gln Ala Ala Glu Val Leu
    290                 295                 300

Gly Ile Glu Ala Asn Ala Val Ser Asp Asn Pro Leu Val Phe Ala Ala
305                 310                 315                 320

Glu Gly Asp Val Ile Ser Gly Gly Asn Phe His Ala Glu Pro Val Ala
                325                 330                 335

Met Ala Ala Asp Asn Leu Ala Leu Ala Ile Ala Glu Ile Gly Ser Leu
            340                 345                 350

Ser Glu Arg Arg Ile Ser Leu Met Met Asp Lys His Met Ser Gln Leu
        355                 360                 365
```

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Pro Pro Phe Leu Val Glu Asn Gly Gly Val Asn Ser Gly Phe Met Ile
    370                 375                 380

Ala Gln Val Thr Ala Ala Ala Leu Ala Ser Glu Asn Lys Ala Leu Ser
385                 390                 395                 400

His Pro His Ser Val Asp Ser Leu Pro Thr Ser Ala Asn Gln Glu Asp
                405                 410                 415

His Val Ser Met Ala Pro Ala Ala Gly Lys Arg Leu Trp Glu Met Ala
            420                 425                 430

Glu Asn Thr Arg Gly Val Pro Ala Ile Glu Trp Leu Gly Ala Cys Gln
        435                 440                 445

Gly Leu Asp Leu Arg Lys Gly Leu Lys Thr Ser Ala Lys Leu Glu Lys
    450                 455                 460

Ala Arg Gln Ala Leu Arg Ser Glu Val Ala His Tyr Asp Arg Asp Arg
465                 470                 475                 480

Phe Phe Ala Pro Asp Ile Glu Lys Ala Val Glu Leu Leu Ala Lys Gly
                485                 490                 495

Ser Leu Thr Gly Leu Leu Pro Ala Gly Val Leu Pro Ser Leu
            500                 505                 510
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<210> 7
<211> 567
<212> PRT
<213> Artificial

<220>
<223> Cysteine to serine substitution at position 64 in Anabaena variabilis PAL

<400> 7

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Ser Phe Thr Gly Asn Ser Ser Ala Asn Val Ile Ile Gly Asn Gln Lys
            20                  25                  30

Leu Thr Ile Asn Asp Val Ala Arg Val Ala Arg Asn Gly Thr Leu Val
        35                  40                  45

Ser Leu Thr Asn Asn Thr Asp Ile Leu Gln Gly Ile Gln Ala Ser Ser
    50                  55                  60

Asp Tyr Ile Asn Asn Ala Val Glu Ser Gly Glu Pro Ile Tyr Gly Val
65                  70                  75                  80

Thr Ser Gly Phe Gly Gly Met Ala Asn Val Ala Ile Ser Arg Glu Gln
```

```
                85                  90                  95

Ala Ser Glu Leu Gln Thr Asn Leu Val Trp Phe Leu Lys Thr Gly Ala
            100                 105                 110

Gly Asn Lys Leu Pro Leu Ala Asp Val Arg Ala Ala Met Leu Leu Arg
        115                 120                 125

Ala Asn Ser His Met Arg Gly Ala Ser Gly Ile Arg Leu Glu Leu Ile
    130                 135                 140

Lys Arg Met Glu Ile Phe Leu Asn Ala Gly Val Thr Pro Tyr Val Tyr
145                 150                 155                 160

Glu Phe Gly Ser Ile Gly Ala Ser Gly Asp Leu Val Pro Leu Ser Tyr
                165                 170                 175

Ile Thr Gly Ser Leu Ile Gly Leu Asp Pro Ser Phe Lys Val Asp Phe
            180                 185                 190

Asn Gly Lys Glu Met Asp Ala Pro Thr Ala Leu Arg Gln Leu Asn Leu
        195                 200                 205

Ser Pro Leu Thr Leu Leu Pro Lys Glu Gly Leu Ala Met Met Asn Gly
    210                 215                 220

Thr Ser Val Met Thr Gly Ile Ala Ala Asn Cys Val Tyr Asp Thr Gln
225                 230                 235                 240

Ile Leu Thr Ala Ile Ala Met Gly Val His Ala Leu Asp Ile Gln Ala
                245                 250                 255

Leu Asn Gly Thr Asn Gln Ser Phe His Pro Phe Ile His Asn Ser Lys
            260                 265                 270

Pro His Pro Gly Gln Leu Trp Ala Ala Asp Gln Met Ile Ser Leu Leu
        275                 280                 285

Ala Asn Ser Gln Leu Val Arg Asp Glu Leu Asp Gly Lys His Asp Tyr
    290                 295                 300

Arg Asp His Glu Leu Ile Gln Asp Arg Tyr Ser Leu Arg Cys Leu Pro
305                 310                 315                 320

Gln Tyr Leu Gly Pro Ile Val Asp Gly Ile Ser Gln Ile Ala Lys Gln
                325                 330                 335

Ile Glu Ile Glu Ile Asn Ser Val Thr Asp Asn Pro Leu Ile Asp Val
            340                 345                 350

Asp Asn Gln Ala Ser Tyr His Gly Gly Asn Phe Leu Gly Gln Tyr Val
        355                 360                 365
```

```
Gly Met Gly Met Asp His Leu Arg Tyr Tyr Ile Gly Leu Leu Ala Lys
    370                 375                 380

His Leu Asp Val Gln Ile Ala Leu Leu Ala Ser Pro Glu Phe Ser Asn
385                 390                 395                 400

Gly Leu Pro Pro Ser Leu Leu Gly Asn Arg Glu Arg Lys Val Asn Met
                405                 410                 415

Gly Leu Lys Gly Leu Gln Ile Cys Gly Asn Ser Ile Met Pro Leu Leu
            420                 425                 430

Thr Phe Tyr Gly Asn Ser Ile Ala Asp Arg Phe Pro Thr His Ala Glu
        435                 440                 445

Gln Phe Asn Gln Asn Ile Asn Ser Gln Gly Tyr Thr Ser Ala Thr Leu
    450                 455                 460

Ala Arg Arg Ser Val Asp Ile Phe Gln Asn Tyr Val Ala Ile Ala Leu
465                 470                 475                 480

Met Phe Gly Val Gln Ala Val Asp Leu Arg Thr Tyr Lys Lys Thr Gly
                485                 490                 495

His Tyr Asp Ala Arg Ala Cys Leu Ser Pro Ala Thr Glu Arg Leu Tyr
            500                 505                 510

Ser Ala Val Arg His Val Val Gly Gln Lys Pro Thr Ser Asp Arg Pro
        515                 520                 525

Tyr Ile Trp Asn Asp Asn Glu Gln Gly Leu Asp Glu His Ile Ala Arg
    530                 535                 540

Ile Ser Ala Asp Ile Ala Ala Gly Gly Val Ile Val Gln Ala Val Gln
545                 550                 555                 560

Asp Ile Leu Pro Cys Leu His
                565
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<210> 8
<211> 567
<212> PRT
<213> Artificial

<220>
<223> Cysteine to serine substitution at position 318 in Anabaena variabilis PAL

<400> 8

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Met Lys Thr Leu Ser Gln Ala Gln Ser Lys Thr Ser Ser Gln Gln Phe
1               5                   10                  15
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Ser Phe Thr Gly Asn Ser Ser Ala Asn Val Ile Ile Gly Asn Gln Lys
20 25 30

Leu Thr Ile Asn Asp Val Ala Arg Val Ala Arg Asn Gly Thr Leu Val
35 40 45

Ser Leu Thr Asn Asn Thr Asp Ile Leu Gln Gly Ile Gln Ala Ser Cys
50 55 60

Asp Tyr Ile Asn Asn Ala Val Glu Ser Gly Glu Pro Ile Tyr Gly Val
65 70 75 80

Thr Ser Gly Phe Gly Gly Met Ala Asn Val Ala Ile Ser Arg Glu Gln
85 90 95

Ala Ser Glu Leu Gln Thr Asn Leu Val Trp Phe Leu Lys Thr Gly Ala
100 105 110

Gly Asn Lys Leu Pro Leu Ala Asp Val Arg Ala Ala Met Leu Leu Arg
115 120 125

Ala Asn Ser His Met Arg Gly Ala Ser Gly Ile Arg Leu Glu Leu Ile
130 135 140

Lys Arg Met Glu Ile Phe Leu Asn Ala Gly Val Thr Pro Tyr Val Tyr
145 150 155 160

Glu Phe Gly Ser Ile Gly Ala Ser Gly Asp Leu Val Pro Leu Ser Tyr
165 170 175

Ile Thr Gly Ser Leu Ile Gly Leu Asp Pro Ser Phe Lys Val Asp Phe
180 185 190

Asn Gly Lys Glu Met Asp Ala Pro Thr Ala Leu Arg Gln Leu Asn Leu
195 200 205

Ser Pro Leu Thr Leu Leu Pro Lys Glu Gly Leu Ala Met Met Asn Gly
210 215 220

Thr Ser Val Met Thr Gly Ile Ala Ala Asn Cys Val Tyr Asp Thr Gln
225 230 235 240

Ile Leu Thr Ala Ile Ala Met Gly Val His Ala Leu Asp Ile Gln Ala
245 250 255

Leu Asn Gly Thr Asn Gln Ser Phe His Pro Phe Ile His Asn Ser Lys
260 265 270

Pro His Pro Gly Gln Leu Trp Ala Ala Asp Gln Met Ile Ser Leu Leu
275 280 285

Ala Asn Ser Gln Leu Val Arg Asp Glu Leu Asp Gly Lys His Asp Tyr

```
    290                 295                 300

Arg Asp His Glu Leu Ile Gln Asp Arg Tyr Ser Leu Arg Ser Leu Pro
305                 310                 315                 320

Gln Tyr Leu Gly Pro Ile Val Asp Gly Ile Ser Gln Ile Ala Lys Gln
                325                 330                 335

Ile Glu Ile Glu Ile Asn Ser Val Thr Asp Asn Pro Leu Ile Asp Val
            340                 345                 350

Asp Asn Gln Ala Ser Tyr His Gly Gly Asn Phe Leu Gly Gln Tyr Val
        355                 360                 365

Gly Met Gly Met Asp His Leu Arg Tyr Tyr Ile Gly Leu Leu Ala Lys
    370                 375                 380

His Leu Asp Val Gln Ile Ala Leu Leu Ala Ser Pro Glu Phe Ser Asn
385                 390                 395                 400

Gly Leu Pro Pro Ser Leu Leu Gly Asn Arg Glu Arg Lys Val Asn Met
                405                 410                 415

Gly Leu Lys Gly Leu Gln Ile Cys Gly Asn Ser Ile Met Pro Leu Leu
            420                 425                 430

Thr Phe Tyr Gly Asn Ser Ile Ala Asp Arg Phe Pro Thr His Ala Glu
        435                 440                 445

Gln Phe Asn Gln Asn Ile Asn Ser Gln Gly Tyr Thr Ser Ala Thr Leu
    450                 455                 460

Ala Arg Arg Ser Val Asp Ile Phe Gln Asn Tyr Val Ala Ile Ala Leu
465                 470                 475                 480

Met Phe Gly Val Gln Ala Val Asp Leu Arg Thr Tyr Lys Lys Thr Gly
                485                 490                 495

His Tyr Asp Ala Arg Ala Cys Leu Ser Pro Ala Thr Glu Arg Leu Tyr
            500                 505                 510

Ser Ala Val Arg His Val Val Gly Gln Lys Pro Thr Ser Asp Arg Pro
        515                 520                 525

Tyr Ile Trp Asn Asp Asn Glu Gln Gly Leu Asp Glu His Ile Ala Arg
    530                 535                 540

Ile Ser Ala Asp Ile Ala Ala Gly Gly Val Ile Val Gln Ala Val Gln
545                 550                 555                 560

Asp Ile Leu Pro Cys Leu His
                565
```

<210> 9
<211> 567
<212> PRT
<213> Artificial

<220>
<223> Cysteine to serine substitution at position 503 in Anabaena variabilis PAL

<400> 9

```
Met Lys Thr Leu Ser Gln Ala Gln Ser Lys Thr Ser Ser Gln Gln Phe
1               5                   10                  15

Ser Phe Thr Gly Asn Ser Ser Ala Asn Val Ile Ile Gly Asn Gln Lys
            20                  25                  30

Leu Thr Ile Asn Asp Val Ala Arg Val Ala Arg Asn Gly Thr Leu Val
        35                  40                  45

Ser Leu Thr Asn Asn Thr Asp Ile Leu Gln Gly Ile Gln Ala Ser Cys
    50                  55                  60

Asp Tyr Ile Asn Asn Ala Val Glu Ser Gly Glu Pro Ile Tyr Gly Val
65                  70                  75                  80

Thr Ser Gly Phe Gly Gly Met Ala Asn Val Ala Ile Ser Arg Glu Gln
                85                  90                  95

Ala Ser Glu Leu Gln Thr Asn Leu Val Trp Phe Leu Lys Thr Gly Ala
            100                 105                 110

Gly Asn Lys Leu Pro Leu Ala Asp Val Arg Ala Ala Met Leu Leu Arg
        115                 120                 125

Ala Asn Ser His Met Arg Gly Ala Ser Gly Ile Arg Leu Glu Leu Ile
    130                 135                 140

Lys Arg Met Glu Ile Phe Leu Asn Ala Gly Val Thr Pro Tyr Val Tyr
145                 150                 155                 160

Glu Phe Gly Ser Ile Gly Ala Ser Gly Asp Leu Val Pro Leu Ser Tyr
                165                 170                 175

Ile Thr Gly Ser Leu Ile Gly Leu Asp Pro Ser Phe Lys Val Asp Phe
            180                 185                 190

Asn Gly Lys Glu Met Asp Ala Pro Thr Ala Leu Arg Gln Leu Asn Leu
        195                 200                 205

Ser Pro Leu Thr Leu Leu Pro Lys Glu Gly Leu Ala Met Met Asn Gly
    210                 215                 220
```

Thr Ser Val Met Thr Gly Ile Ala Ala Asn Cys Val Tyr Asp Thr Gln
225 230 235 240

Ile Leu Thr Ala Ile Ala Met Gly Val His Ala Leu Asp Ile Gln Ala
245 250 255

Leu Asn Gly Thr Asn Gln Ser Phe His Pro Phe Ile His Asn Ser Lys
260 265 270

Pro His Pro Gly Gln Leu Trp Ala Ala Asp Gln Met Ile Ser Leu Leu
275 280 285

Ala Asn Ser Gln Leu Val Arg Asp Glu Leu Asp Gly Lys His Asp Tyr
290 295 300

Arg Asp His Glu Leu Ile Gln Asp Arg Tyr Ser Leu Arg Cys Leu Pro
305 310 315 320

Gln Tyr Leu Gly Pro Ile Val Asp Gly Ile Ser Gln Ile Ala Lys Gln
325 330 335

Ile Glu Ile Glu Ile Asn Ser Val Thr Asp Asn Pro Leu Ile Asp Val
340 345 350

Asp Asn Gln Ala Ser Tyr His Gly Gly Asn Phe Leu Gly Gln Tyr Val
355 360 365

Gly Met Gly Met Asp His Leu Arg Tyr Tyr Ile Gly Leu Leu Ala Lys
370 375 380

His Leu Asp Val Gln Ile Ala Leu Leu Ala Ser Pro Glu Phe Ser Asn
385 390 395 400

Gly Leu Pro Pro Ser Leu Leu Gly Asn Arg Glu Arg Lys Val Asn Met
405 410 415

Gly Leu Lys Gly Leu Gln Ile Cys Gly Asn Ser Ile Met Pro Leu Leu
420 425 430

Thr Phe Tyr Gly Asn Ser Ile Ala Asp Arg Phe Pro Thr His Ala Glu
435 440 445

Gln Phe Asn Gln Asn Ile Asn Ser Gln Gly Tyr Thr Ser Ala Thr Leu
450 455 460

Ala Arg Arg Ser Val Asp Ile Phe Gln Asn Tyr Val Ala Ile Ala Leu
465 470 475 480

Met Phe Gly Val Gln Ala Val Asp Leu Arg Thr Tyr Lys Lys Thr Gly
485 490 495

His Tyr Asp Ala Arg Ala Ser Leu Ser Pro Ala Thr Glu Arg Leu Tyr

500 505 510

Ser Ala Val Arg His Val Val Gly Gln Lys Pro Thr Ser Asp Arg Pro
515 520 525

Tyr Ile Trp Asn Asp Asn Glu Gln Gly Leu Asp Glu His Ile Ala Arg
530 535 540

Ile Ser Ala Asp Ile Ala Ala Gly Gly Val Ile Val Gln Ala Val Gln
545 550 555 560

Asp Ile Leu Pro Cys Leu His
565

<210> 10
<211> 567
<212> PRT
<213> Artificial

<220>
<223> Cysteine to serine substition at position 565 in Anabaena variabilis PAL

<400> 10

Met Lys Thr Leu Ser Gln Ala Gln Ser Lys Thr Ser Ser Gln Gln Phe
1 5 10 15

Ser Phe Thr Gly Asn Ser Ser Ala Asn Val Ile Ile Gly Asn Gln Lys
20 25 30

Leu Thr Ile Asn Asp Val Ala Arg Val Ala Arg Asn Gly Thr Leu Val
35 40 45

Ser Leu Thr Asn Asn Thr Asp Ile Leu Gln Gly Ile Gln Ala Ser Cys
50 55 60

Asp Tyr Ile Asn Asn Ala Val Glu Ser Gly Glu Pro Ile Tyr Gly Val
65 70 75 80

Thr Ser Gly Phe Gly Gly Met Ala Asn Val Ala Ile Ser Arg Glu Gln
85 90 95

Ala Ser Glu Leu Gln Thr Asn Leu Val Trp Phe Leu Lys Thr Gly Ala
100 105 110

Gly Asn Lys Leu Pro Leu Ala Asp Val Arg Ala Ala Met Leu Leu Arg
115 120 125

Ala Asn Ser His Met Arg Gly Ala Ser Gly Ile Arg Leu Glu Leu Ile
130 135 140

Lys Arg Met Glu Ile Phe Leu Asn Ala Gly Val Thr Pro Tyr Val Tyr
145 150 155 160

```
Glu Phe Gly Ser Ile Gly Ala Ser Gly Asp Leu Val Pro Leu Ser Tyr
                165                 170                 175

Ile Thr Gly Ser Leu Ile Gly Leu Asp Pro Ser Phe Lys Val Asp Phe
            180                 185                 190

Asn Gly Lys Glu Met Asp Ala Pro Thr Ala Leu Arg Gln Leu Asn Leu
        195                 200                 205

Ser Pro Leu Thr Leu Leu Pro Lys Glu Gly Leu Ala Met Met Asn Gly
    210                 215                 220

Thr Ser Val Met Thr Gly Ile Ala Ala Asn Cys Val Tyr Asp Thr Gln
225                 230                 235                 240

Ile Leu Thr Ala Ile Ala Met Gly Val His Ala Leu Asp Ile Gln Ala
                245                 250                 255

Leu Asn Gly Thr Asn Gln Ser Phe His Pro Phe Ile His Asn Ser Lys
            260                 265                 270

Pro His Pro Gly Gln Leu Trp Ala Ala Asp Gln Met Ile Ser Leu Leu
        275                 280                 285

Ala Asn Ser Gln Leu Val Arg Asp Glu Leu Asp Gly Lys His Asp Tyr
    290                 295                 300

Arg Asp His Glu Leu Ile Gln Asp Arg Tyr Ser Leu Arg Cys Leu Pro
305                 310                 315                 320

Gln Tyr Leu Gly Pro Ile Val Asp Gly Ile Ser Gln Ile Ala Lys Gln
                325                 330                 335

Ile Glu Ile Glu Ile Asn Ser Val Thr Asp Asn Pro Leu Ile Asp Val
            340                 345                 350

Asp Asn Gln Ala Ser Tyr His Gly Gly Asn Phe Leu Gly Gln Tyr Val
        355                 360                 365

Gly Met Gly Met Asp His Leu Arg Tyr Tyr Ile Gly Leu Leu Ala Lys
    370                 375                 380

His Leu Asp Val Gln Ile Ala Leu Leu Ala Ser Pro Glu Phe Ser Asn
385                 390                 395                 400

Gly Leu Pro Pro Ser Leu Leu Gly Asn Arg Glu Arg Lys Val Asn Met
                405                 410                 415

Gly Leu Lys Gly Leu Gln Ile Cys Gly Asn Ser Ile Met Pro Leu Leu
            420                 425                 430
```

Thr Phe Tyr Gly Asn Ser Ile Ala Asp Arg Phe Pro Thr His Ala Glu
435 440 445

Gln Phe Asn Gln Asn Ile Asn Ser Gln Gly Tyr Thr Ser Ala Thr Leu
450 455 460

Ala Arg Arg Ser Val Asp Ile Phe Gln Asn Tyr Val Ala Ile Ala Leu
465 470 475 480

Met Phe Gly Val Gln Ala Val Asp Leu Arg Thr Tyr Lys Lys Thr Gly
485 490 495

His Tyr Asp Ala Arg Ala Cys Leu Ser Pro Ala Thr Glu Arg Leu Tyr
500 505 510

Ser Ala Val Arg His Val Val Gly Gln Lys Pro Thr Ser Asp Arg Pro
515 520 525

Tyr Ile Trp Asn Asp Asn Glu Gln Gly Leu Asp Glu His Ile Ala Arg
530 535 540

Ile Ser Ala Asp Ile Ala Ala Gly Gly Val Ile Val Gln Ala Val Gln
545 550 555 560

Asp Ile Leu Pro Ser Leu His
565

<210> 11
<211> 567
<212> PRT
<213> Artificial

<220>
<223> Cysteine to serine substitutions at positions 565 and 503 in Anabaena variabilis PAL

<400> 11

Met Lys Thr Leu Ser Gln Ala Gln Ser Lys Thr Ser Ser Gln Gln Phe
1 5 10 15

Ser Phe Thr Gly Asn Ser Ser Ala Asn Val Ile Ile Gly Asn Gln Lys
20 25 30

Leu Thr Ile Asn Asp Val Ala Arg Val Ala Arg Asn Gly Thr Leu Val
35 40 45

Ser Leu Thr Asn Asn Thr Asp Ile Leu Gln Gly Ile Gln Ala Ser Cys
50 55 60

Asp Tyr Ile Asn Asn Ala Val Glu Ser Gly Glu Pro Ile Tyr Gly Val
65 70 75 80

Thr Ser Gly Phe Gly Gly Met Ala Asn Val Ala Ile Ser Arg Glu Gln
85 90 95

```
Ala Ser Glu Leu Gln Thr Asn Leu Val Trp Phe Leu Lys Thr Gly Ala
            100                 105                 110

Gly Asn Lys Leu Pro Leu Ala Asp Val Arg Ala Ala Met Leu Leu Arg
        115                 120                 125

Ala Asn Ser His Met Arg Gly Ala Ser Gly Ile Arg Leu Glu Leu Ile
    130                 135                 140

Lys Arg Met Glu Ile Phe Leu Asn Ala Gly Val Thr Pro Tyr Val Tyr
145                 150                 155                 160

Glu Phe Gly Ser Ile Gly Ala Ser Gly Asp Leu Val Pro Leu Ser Tyr
                165                 170                 175

Ile Thr Gly Ser Leu Ile Gly Leu Asp Pro Ser Phe Lys Val Asp Phe
            180                 185                 190

Asn Gly Lys Glu Met Asp Ala Pro Thr Ala Leu Arg Gln Leu Asn Leu
        195                 200                 205

Ser Pro Leu Thr Leu Leu Pro Lys Glu Gly Leu Ala Met Met Asn Gly
    210                 215                 220

Thr Ser Val Met Thr Gly Ile Ala Ala Asn Cys Val Tyr Asp Thr Gln
225                 230                 235                 240

Ile Leu Thr Ala Ile Ala Met Gly Val His Ala Leu Asp Ile Gln Ala
                245                 250                 255

Leu Asn Gly Thr Asn Gln Ser Phe His Pro Phe Ile His Asn Ser Lys
            260                 265                 270

Pro His Pro Gly Gln Leu Trp Ala Ala Asp Gln Met Ile Ser Leu Leu
        275                 280                 285

Ala Asn Ser Gln Leu Val Arg Asp Glu Leu Asp Gly Lys His Asp Tyr
    290                 295                 300

Arg Asp His Glu Leu Ile Gln Asp Arg Tyr Ser Leu Arg Cys Leu Pro
305                 310                 315                 320

Gln Tyr Leu Gly Pro Ile Val Asp Gly Ile Ser Gln Ile Ala Lys Gln
                325                 330                 335

Ile Glu Ile Glu Ile Asn Ser Val Thr Asp Asn Pro Leu Ile Asp Val
            340                 345                 350

Asp Asn Gln Ala Ser Tyr His Gly Gly Asn Phe Leu Gly Gln Tyr Val
        355                 360                 365
```

Gly Met Gly Met Asp His Leu Arg Tyr Tyr Ile Gly Leu Leu Ala Lys
370 375 380

His Leu Asp Val Gln Ile Ala Leu Leu Ala Ser Pro Glu Phe Ser Asn
385 390 395 400

Gly Leu Pro Pro Ser Leu Leu Gly Asn Arg Glu Arg Lys Val Asn Met
405 410 415

Gly Leu Lys Gly Leu Gln Ile Cys Gly Asn Ser Ile Met Pro Leu Leu
420 425 430

Thr Phe Tyr Gly Asn Ser Ile Ala Asp Arg Phe Pro Thr His Ala Glu
435 440 445

Gln Phe Asn Gln Asn Ile Asn Ser Gln Gly Tyr Thr Ser Ala Thr Leu
450 455 460

Ala Arg Arg Ser Val Asp Ile Phe Gln Asn Tyr Val Ala Ile Ala Leu
465 470 475 480

Met Phe Gly Val Gln Ala Val Asp Leu Arg Thr Tyr Lys Lys Thr Gly
485 490 495

His Tyr Asp Ala Arg Ala Ser Leu Ser Pro Ala Thr Glu Arg Leu Tyr
500 505 510

Ser Ala Val Arg His Val Val Gly Gln Lys Pro Thr Ser Asp Arg Pro
515 520 525

Tyr Ile Trp Asn Asp Asn Glu Gln Gly Leu Asp Glu His Ile Ala Arg
530 535 540

Ile Ser Ala Asp Ile Ala Ala Gly Gly Val Ile Val Gln Ala Val Gln
545 550 555 560

Asp Ile Leu Pro Ser Leu His
565

<210> 12
<211> 38
<212> DNA
<213> Artificial

<220>
<223> Nostoc punctiforme PAL primer 1 (forward)

<400> 12
cactgtcata tgaatataac atctctacaa cagaacat 38

<210> 13
<211> 45
<212> DNA
<213> Artificial

<220>
<223> Nostoc punctiforme PAL primer 2 (reverse)

<400> 13
gacagtggcg gccgctcacg ttgactttaa gctcgaaaaa atatg 45

<210> 14
<211> 38
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer 1 (forward, N-terminal fragment)

<400> 14
cactgtgcta gcatgaagac actatctcaa gcacaaag 38

<210> 15
<211> 49
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer 2 (reverse, N-terminal fragment)

<400> 15
ggaaatttcc tccatgatag ctggcttggt tatcaacatc aattagtgg 49

<210> 16
<211> 49
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer 3 (forward, C-terminal fragment)

<400> 16
ccactaattg atgttgataa ccaagccagc tatcatggag gaaatttcc 49

<210> 17
<211> 41
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer 4 (reverse, C-terminal fragment)

<400> 17
cactgtgcgg ccgcttaatg caagcagggt aagatatctt g 41

<210> 18
<211> 35
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL forward primer

<400> 18
cactgtcata tgaagacact atctcaagca caaag 35

<210> 19

<211> 36
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL reverse primer

<400> 19
cactgtctcg agatgcaagc agggtaagat atcttg 36

<210> 20
<211> 38
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create 5' NheI site and delete internal NheI site (forward, N-terminal)

<400> 20
cactgtgcta gcatgaagac actatctcaa gcacaaag 38

<210> 21
<211> 49
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create 5' NheI site and delete internal NheI site (reverse, N-terminal)

<400> 21
ggaaatttcc tccatgatag ctggcttggt tatcaacatc aattagtgg 49

<210> 22
<211> 49
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create 5' NheI site and delete internal NheI site (forward, C-terminal fragment)

<400> 22
ccactaattg atgttgataa ccaagccagc tatcatggag gaaatttcc 49

<210> 23
<211> 41
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create 5' NheI site and delete internal NheI site (reverse, C-terminal fragment)

<400> 23
acagtggcgg ccgcttaatg caagcagggt aagatatctt g 41

<210> 24
<211> 38
<212> DNA
<213> Artificial

<220>

<223> Anabaena variabilis PAL primer to create 5' NheI site and 3' SmaI site (forward)

<400> 24
cactgtgaat tcatgaagac actatctcaa gcacaaag 38

<210> 25
<211> 37
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create 5' NheI site and 3' SmaI site (reverse)

<400> 25
cactgtcccg ggttaatgca agcagggtaa gatatct 37

<210> 26
<211> 41
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create cysteine to serine substitution at position 503 (forward)

<400> 26
gtcattacga tgcacgcgcc tctctatcac ctgcaactga g 41

<210> 27
<211> 41
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create cysteine to serine substitution at position 503 (reverse)

<400> 27
ctcagttgca ggtgatagag aggcgcgtgc atcgtaatga c 41

<210> 28
<211> 42
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create cysteine to serine substitution at position 565 (forward)

<400> 28
cagttcaaga tatcttaccc tccttgcatt aacccgggct gc 42

<210> 29
<211> 42
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create cysteine to serine substitution at position 565 (reverse)

<400> 29

gcagcccggg ttaatgcaag gagggtaaga tatcttgaac tg 42

<210> 30
<211> 44
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create cysteine to serine substitution at position 64 (forward)

<400> 30
gcagggtatt caggcatctt ctgattacat taataatgct gttg 44

<210> 31
<211> 44
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create cysteine to serine substitution at position 64 (reverse)

<400> 31
caacagcatt attaatgtaa tcagaagatg cctgaatacc ctgc 44

<210> 32
<211> 43
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create cysteine to serine substitution at position 318 (forward)

<400> 32
caagatcgtt actcactccg atcccttccc cagtatttgg ggc 43

<210> 33
<211> 43
<212> DNA
<213> Artificial

<220>
<223> Anabaena variabilis PAL primer to create cysteine to serine substitution at position 318 (reverse)

<400> 33
gccccaaata ctggggaagg gatcggagtg agtaacgatc ttg 43