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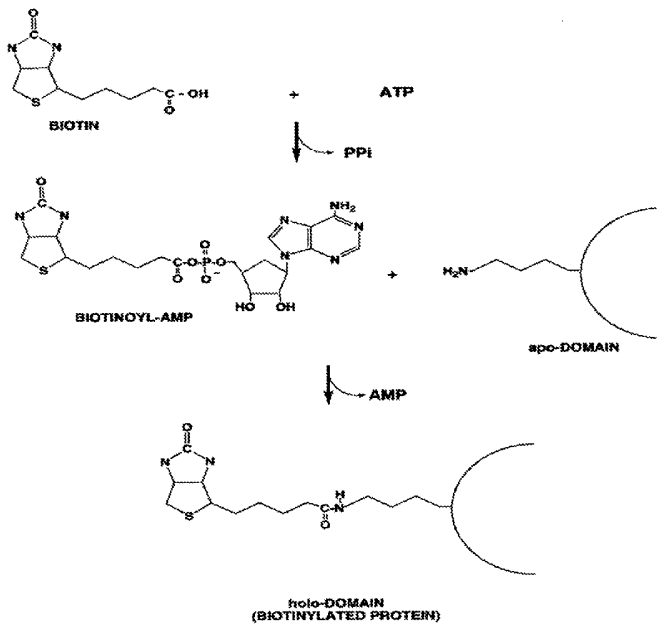
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(54) Title: INHIBITORS OF BIOTIN PROTEIN LIGASE



(57) Abstract: The present invention relates to a method for identifying an inhibitor of a biotin protein ligase. The method include the steps of (a) providing a substrate, wherein the substrate may be biotinylated; (b) contacting the substrate with biotin and a biotin protein ligase in the presence of a test compound; (c) determining the extent of biotinylation of the substrate by the biotin protein ligase in the presence of the test compound; and (d) identifying the test compound as an inhibitor of the biotin protein ligase by a reduction in the biotinylation of the substrate in the presence of the test compound as compared to the extent of biotinylation of the substrate in the absence of the test compound.

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INHIBITORS OF BIOTIN PROTEIN LIGASE

This application claims priority from Australian Provisional Patent Application No. 2004906688 filed on 23 November 2004, the contents of which are to be taken as incorporated herein by this reference.

Field of the Invention

The present invention relates to methods for identifying inhibitors of biotin protein ligases, and in particular, to methods for the identification of inhibitors of biotin protein ligases of pathogenic organisms. The present invention also relates to methods for identifying differential inhibitors of biotin protein ligases.

The present invention also relates to inhibitors of biotin protein ligases identified by the methods of the present invention, biotin protein ligases as targets for identifying inhibitors of biotinylation, and methods and compositions for inhibiting biotinylation using the inhibitors.

Background of the Invention

There is a continuing need for the development of new agents that have the ability to inhibit the growth of pathogenic organisms, and in particular, the development of new agents that have the ability to inhibit the growth of bacterial and fungal pathogens. Infection by bacterial and fungal pathogens represents one of the most significant causes of disease and economic loss.

The treatment of bacterial infections has traditionally relied on the use of antibiotics. However, the treatment of many bacterial infections is becoming increasingly difficult because of resistance to one or more known antibiotics developing in the bacteria. Indeed, in some cases bacteria have become resistant to all known antibiotics, rendering the host organism vulnerable to the effects of infection by the bacteria.

The effective treatment of fungal and parasitic infections also remains problematic. For example, strains of the yeast *Candida* are among the most virulent pathogenic fungal organisms of humans. While there are several topical agents for treatment of candidiases, treatment of systemic infection by *Candida* is much more difficult. Systemic fungal infections are common complications in immune-compromised patients, and as such may be life-threatening. The available drugs for treating systemic fungal infections are often highly toxic to the host.

Plants are also infected by a wide range of pathogens, including bacterial, fungal nematode and insect pathogens. The infection of plants by such pathogens causes extensive losses to agricultural products and also affects the storage and manufacture of agricultural products.

Accordingly, there is a need for the identification of new agents that may prevent or ameliorate infection by pathogenic organisms. In addition, it is desirable that such agents are able not only to kill or significantly inhibit the growth of the pathogenic organism, but they should also not be highly toxic to the host organism.

Biotinylation is a process that is ubiquitous to all organisms. In this process biotin, otherwise known as vitamin H, is covalently attached at the active site of the class of metabolic enzymes known as biotin carboxylases, biotin decarboxylases and biotin transcarboxylases. These are key enzymes involved in gluconeogenesis, lipogenesis, amino acid metabolism and energy transduction. Biotin must be covalently attached to these enzymes for the enzymes to function. For example, biotin must be covalently attached to pyruvate carboxylase so that the enzyme may catalyse the generation of oxaloacetate, a precursor for the synthesis of glucose and fat as well as some amino acids and neurotransmitters.

The enzyme responsible for the covalent attachment of biotin to its cognate proteins is biotin protein ligase (BPL). Biotin is attached post-translationally by BPL in a reaction of stringent specificity via an amide linkage to a specific lysine residue in a two-step reaction as shown in Figure 1.

Despite the fact that biotin-dependent enzymes are present in all organisms, biotinylation is still a rare modification in the cell, with only between one and five distinct protein species actually being biotinylated. The functional interaction between BPL and its protein substrates shows a very high degree of conservation throughout evolution, because biotinylation will occur when the two proteins are from widely-divergent biological sources.

As biotinylation is a process that is ubiquitous in all organisms, it represents a potential target for the development of new compounds that may inhibit the growth of pathogenic organisms. The present invention relates to methods for the identification of inhibitors of biotin protein ligase.

A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

Summary of the Invention

The present invention provides a method for identifying an inhibitor of a biotin protein ligase, the method including the steps of:

(a) providing a substrate, wherein the substrate may be biotinylated;
(b) contacting the substrate with biotin and a biotin protein ligase in the presence of a test compound;
(c) determining the extent of biotinylation of the substrate by the biotin protein ligase in the presence of the test compound; and
(d) identifying the test compound as an inhibitor of the biotin protein ligase by a reduction in the biotinylation of the substrate in the presence of the test compound as compared to the extent of biotinylation of the substrate in the absence of the test compound.

The present invention also provides an anti-pathogenic agent, wherein the agent inhibits biotinylation of a substrate by a biotin protein ligase of a pathogen.

The present invention also provides a method for identifying an agent that inhibits growth and/or survival of a pathogenic organism, the method including the step of identifying an agent that inhibits a biotin protein ligase.

The present invention also provides the use of a biotin protein ligase as a target for identifying an agent that inhibits growth and/or survival of an organism.

The present invention also provides an isolated biotin protein ligase suitable as a target for identifying an inhibitor of biotinylation.

The present invention also provides an isolated biotin protein ligase suitable for use as a target for identifying an agent that inhibits growth and/or survival of an organism.

The present invention also provides a method for identifying an inhibitor of biotinylation in a biological system, the method including the steps of:

(a) identifying a test compound as an inhibitor of a biotin protein ligase;

(b) determining the ability of the test compound to inhibit biotinylation in a biological system; and

(c) identifying the test compound as an inhibitor of biotinylation in the biological system.

The present invention also provides a method for identifying a compound that differentially inhibits a first biotin protein ligase as compared to inhibition of a second biotin protein ligase, the method including the steps of:

(a) identifying a test compound that inhibits a first biotin protein ligase;

(b) determining the ability of the test compound to inhibit a second biotin protein ligase; and

(c) identifying the test compound as a compound that differentially inhibits the first biotin protein ligase as compared to the inhibition of the second biotin protein ligase.

The present invention also provides a method for identifying a compound that differentially inhibits biotinylation in a first biological system as compared to inhibition of biotinylation in a second biological system, the method including the steps of:

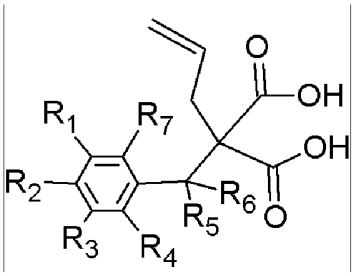
(a) identifying a test compound that inhibits biotinylation in a first biological system;

(b) determining the ability of the test compound to inhibit biotinylation in a second biological system; and

(c) identifying the test compound as a compound that differentially inhibits biotinylation in the first biological system as compared to the inhibition of biotinylation in the second biological system.

The present invention also provides a method for preventing and/or treating an infection by a pathogenic organism of a subject, the method including the step of administering to the subject an effective amount of an agent that inhibits a biotin protein ligase of the pathogenic organism.

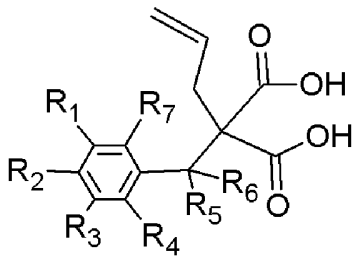
The present invention also provides a composition for preventing and/or treating an infection of a host by a pathogenic organism, the composition including a compound with the formula:



or a salt or ester thereof, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl, arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino,

sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-, $R_9N(R_{10})SO_2R_{11}$-, $R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

The present invention also provides the use of a compound with the formula:



or a salt or ester thereof, in the preparation of a medicament for preventing and/or treating an infection of a host by a pathogenic organism, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl, arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino, sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-,

$R_9N(R_{10})SO_2R_{11}$-, $R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

The present invention arises out of studies into the biotinylation of protein fragments by biotin protein ligases. In particular, it has been found that certain compounds are capable of inhibiting the biotinylation of protein fragments by biotin protein ligases, demonstrating that inhibitors of biotin protein ligases may be identified. Compounds so identified are candidate drugs for inhibiting the growth and/or survival of organisms.

In addition, it has also been found that some compounds are capable of differentially inhibiting biotinylation of a substrate by biotin protein ligases of different species. Given the high degree of conservation between biotin protein ligases of different species, the ability to identify compounds that differentially inhibit biotin protein ligases of different organisms was contrary to expectation.

In the case where the compounds differentially inhibit the biotin protein ligase of a pathogenic organism as compared to the host organism, these compounds are candidate drugs for selectively targeting the pathogen.

Various terms that will be used throughout the specification have meanings that will be well understood by a skilled addressee. However, for ease of reference, some of these terms will now be defined.

The term “biotin protein ligase” as used throughout the specification is to be understood to mean any protein (or a functional fragment thereof) that has the capacity to enzymatically attach a free biotin group covalently to a substrate in a reaction catalysed by the hydrolysis of a nucleoside triphosphate. The biotin protein ligase may be a naturally occurring form of the protein or a fragment thereof, a functional variant of the protein or fragment, a synthetic form of the protein, or an analogue of the protein. For example, the biotin protein ligase may be a protein or fragment thereof isolated from a cell that has the capacity to enzymatically attach a biotin group covalently to a substrate,

or it may be any polypeptide synthesised *in vitro* (for example by chemical synthesis or by *in vitro* translation) that has the capacity to enzymatically attach a biotin group covalently to a substrate.

Methods for identifying biotin protein ligases are known in the art. For example, biotin protein ligases may be identified using the BLAST algorithm, which determines the extent of homology between two nucleotide sequences (blastn) or the extent of homology between two amino acid sequences (blastp). BLAST identifies local alignments between the sequences in the database and predicts the probability of the local alignment occurring by chance. The BLAST algorithm is as described in Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410.

The term "substrate" as used throughout the specification is to be understood to mean any molecule that has the capacity to have a biotin group attached covalently to the molecule by the action of a biotin protein ligase. Examples of suitable substrates include proteins or fragments thereof that have the capacity for a biotin group to be attached to them, polypeptides synthesized *in vitro* that have the capacity for a biotin group to be attached to them, or small molecules that have the capacity for a biotin group to be attached to them, such as hydroxylamine.

The term "biotinylation" as used throughout the specification is to be understood to mean the covalent attachment of a biotin group to one or more molecules. The biotinylation reaction may occur, for example, *in vivo*, in one or more isolated cells, or in a cell free system *in vitro*.

The term "biological system" as used throughout the specification is to be understood to mean any single or multi-cellular system in which biotinylation occurs. For example, the biological system may be one or more isolated cells, an unicellular organism, the part or whole of a tissue or organ, or an entire multi-cellular organism, such as a human, animal or plant.

The term "variant" as used throughout the specification is to be understood to mean an amino acid sequence of a polypeptide or protein that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid

has similar structural or chemical properties to the replaced amino acid (e.g., replacement of leucine with isoleucine). A variant may also have "non-conservative" changes (e.g., replacement of a glycine with a tryptophan) or a deletion and/or insertion of one or more amino acids. The term also includes within its scope any insertions/deletions of amino acids to a particular polypeptide or protein. A “functional variant” will be understood to mean a variant that retains the functional capacity of a reference protein or polypeptide.

The term “nucleic acid” as used throughout the specification is to be understood to mean to any oligonucleotide or polynucleotide. The nucleic acid may be DNA or RNA and may be single stranded or double stranded. The nucleic acid may be any type of nucleic acid, including a nucleic acid of genomic origin, cDNA origin (ie derived from a mRNA), derived from a virus, or of synthetic origin.

In this regard, an oligonucleotide or polynucleotide may be modified at the base moiety, sugar moiety, or phosphate backbone, and may include other appending groups to facilitate the function of the nucleic acid. The oligonucleotide or polynucleotide may be modified at any position on its structure with constituents generally known in the art. For example, an oligonucleotide may include at least one modified base moiety which is selected from the group including 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5- (carboxyliydroxylmethyl) uracil,5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3- (3-amino-3-N-2-carboxypropyl) uracil, (acp3) w, and 2,6-diaminopurine.

The oligonucleotide or polynucleotide may also include at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose. In addition, the oligonucleotide or polynucleotide may include at least one modified phosphate backbone, such as a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or any analogue thereof.

The term "subject" as used throughout the specification is to be understood to mean any multicellular organism, including a human, plant or an animal subject.

For example, in the case where the subject is a human or animal, the subject organism may be a mammal, a primate, a livestock animal (eg. a horse, a cow, a sheep, a pig, or a goat), a companion animal (eg. a dog, a cat), a laboratory test animal (eg. a mouse, a rat, a guinea pig, a bird), an animal of veterinary significance, or an animal of economic significance.

In the case where the subject is a plant, the plant may be for example a commercial crop species (eg barley, oat, millet, alfalfa), a leguminous plant (eg soybean, alfalfa, and pea), a non-leguminous plants (e.g., corn, wheat, and cotton), or an angiosperm or cereal.

The term "isolated" as used throughout the specification is to be understood to mean an agent, for example a protein such a biotin protein ligase, which is purified and/or removed from its natural environment. For example, an isolated biotin protein ligase may be a substantially purified form of the enzyme.

The term "anti-pathogenic agent" as used throughout the specification is to be understood to mean an agent that functions to suppress, destroy, kill, or inhibit the growth, propagation, reproduction or maintenance of an organism. In this regard, the term "survival" of an organism will be understood to encompass the ability of an organism to grow, propagate, reproduce or maintain itself. As will be appreciated, in certain embodiments the term relates to anti-microbial agents, anti-bacterial agents, anti-fungal agents, anti-nematode agents, anti-parasitic agents, and insecticidal agents.

The term "wild type nucleotide sequence" as used throughout the specification is to be understood to mean the nucleotide sequence of a nucleic acid that is present in a naturally occurring organism.

Brief Description of the Figures

Figure 1 shows a schematic of the mechanism for attachment of biotin to a lysine residue in a two step reaction catalysed by a biotin protein ligase.

Figure 2 shows the analysis of pyruvate carboxylase fragments as BPL substrates. Peptides that encompassed the predicted biotin domains of *C. albicans* and human pyruvate carboxylase were expressed in *E. coli* as fusions to GST. Expression of each construct was assayed by Western blot using anti-GST antibody and biotinylation determined by Streptavidin blot. The blots used for the analysis of the C-terminal 81 and 108 amino acids of human pyruvate carboxylase are shown in panel A. Quantitation of the blots from three independent experiments is shown in panel B. Similarly, the analysis of three peptides encompassing the predicted biotin domain of *C. albicans* pyruvate carboxylase is shown in panel C. Protein induction was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Uninduced cultures are represented by (-) and induced samples are represented by (+).

Figure 3 shows the purification of the biotin domain of human pyruvate carboxylase and kinetic analysis of the biotinylation of the domain. Panel A shows purification of the biotin domain of human pyruvate carboxylase. Whole cell lysate from BL21 cells expressing hPC-108 as a fusion to GST were separated on a 1 ml GST-Trap column. Material in the unbound fraction is shown in lane 1 and protein in the wash fraction is shown in lane 2. The biotin domain was released from GST by addition of thrombin directly onto the column. Cleaved material (lane 3) was washed off the column and biotin-containing material removed using Streptavidin Sepharose (lane 4). The purification was monitored by SDS-PAGE (top panel) and Streptavidin blot (lower panel). Migration of molecular mass standards (kDa) is shown on the left and the position of hPC-108 shown on the right. Panel B shows kinetic analysis performed using varied concentrations of purified hPC-108 in an *in vitro* assay with human BPL.

The enzyme displays a low K_M for the domain (1 ± 0.2 uM) indicating that it is a good BPL substrate.

Figure 4 shows *in vivo* analysis of exogenous BPLs in *E. coli* BM4062. The *E. coli* strain BM4062 was transformed with vectors derived from pARA13 (A) for constitutive expression of BPL from *S. cerevisiae* (B), *C. albicans* (C) and *H. sapiens* (D). Strains were grown at the restrictive temperature of 42°C (left) or the permissive temperature of 30°C (right).

Figure 5 shows the purification of *C. albicans* and human BPL. Recombinant BPL was expressed and purified as described in Example 8. (A) Purification of *C. albicans* BPL was assessed using SDS-PAGE (top panel) and a Ni-NTA blot to specifically probe for the C-terminal multi-histidine tag (bottom panel). Proteins were fractionated on 12% polyacrylamide gels under reducing conditions. The migration of molecular mass standards is indicated to the left of each panel. Samples for analysis were (1) total protein fraction, (2) soluble protein fraction, (3) unbound fraction, (4) wash fraction, (5) 100 mM imidazole wash fraction and (6) eluted protein. The large arrow indicates the band corresponding to full-length enzyme. The smaller arrow indicates a proteolytic product of the BPL by bacterial proteases. (B) Panel showing the purification of human Met^{1}-Ala^{80}BPL. The arrow represents the expected migration position of this protein.

Figure 6 shows the kinetic determination of optimal pH for *C. albicans* BPL activity. The assay for *C. albicans* BPL activity was performed as described in Example 9. The activity of the enzyme was determined in various buffers in the range of pH 5 – 10.

Figure 7 shows the affect of various salts on *C. albicans* BPL activity. The assay for *C. albicans* BPL activity was performed as described in Example 9. The activity of the enzyme was determined in the presence of various salt concentrations, as indicated under the graph.

Figure 8 shows the activity of *C. albicans* BPL in the presence of various metal ions. The assay for *C. albicans* BPL activity was performed as described in Example 9. The activity of the enzyme was determined in the presence of various mono- and divalent metal ions, as indicated under the graph.

Figure 9 shows the activity of *C. albicans* BPL in the presence of various nucleotide triphosphates. The assay for *C. albicans* BPL activity was performed as described in Example 9. The activity of the enzyme was determined in the presence of various nucleotide triphosphates, as indicated under the graph.

Figure 10 shows the activity of *C. albicans* BPL with varying MgATP concentration. The assay for *C. albicans* BPL activity was performed as described in Example 9. The activity of the enzyme was determined in the presence of varying concentrations of MgATP, as indicated under the graph.

Figure 11 shows the activity of *C. albicans* BPL in the presence of DMSO. The assay for *C. albicans* BPL activity was performed as described in Example 9. The activity of the enzyme was determined in the presence of varying concentrations of DMSO, as indicated under the graph.

Figure 12 shows kinetic analysis of the inhibition of *C. albicans* BPL by pyrophosphate. The assay for *C. albicans* BPL activity was performed as described in Example 9. The activity of the enzyme was determined in the presence of varying concentrations of pyrophosphate.

Figure 13 shows the concentration response curve of *C. albicans* BPL activity inhibition by biotinol-adenylate. The assay for *C. albicans* BPL activity was performed as described in Example 9. The activity of the enzyme was determined in the presence of varying concentrations of biotinol-adenylate.

Figure 14 shows the concentration response curve of human BPL activity inhibition by biotinol-adenylate. The assay for human BPL activity was performed as described in Example 9. The activity of the enzyme was determined in the presence of varying concentrations of biotinol-adenylate.

Figure 15 shows the concentration response curve of bacterial BPL activity inhibition by biotinol-adenylate. The assay for bacterial BPL was performed as described in Example 9. The activity of the enzyme was determined in the presence of varying concentrations of biotinol-adenylate.

Figure 16 shows the results of inhibition of *Candida* BPL activity by each of 329 compounds present in a library of test compounds. The activity is expressed relative to control reactions containing DMSO. The graph shows the activity of each compound assayed at 10 μM (grey bars) and 50 μM (black bars).

Fig 17 shows the differential inhibition of *Candida* and human BPls by selected compounds. Compound 69 (Panel A) and Compound 296 (Panel B) were dissolved in 100% DMSO, added to the reactions to a final concentration of 10 μM or 50 μM and their effect on BPL activity measured. The graph shows the average and standard error from reactions performed in triplicate. The inhibitory activity of the compounds was determined relative to the negative control reactions containing only DMSO ("control"). Biotinol-AMP ("BtnOH") was included in the reactions as a control for inhibition.

Figure 18 shows the concentration response curve of *E. coli*, *S. aureus*, *S. cerevisiae* and human BPLs by compounds 2 to 5. The assay for enzyme activity was performed as described in Example 4 for the two bacterial enzymes and Example 9 for the yeast and human enzymes. The activity of each enzyme was determined in the presence of varying concentrations of each compound

Figure 19 shows the antibiotic activity of Compound 2 against *Staphylococcus aureus* on both solid media (A-C) and in liquid culture (D). The effect on the growth of *S. aureus* was assessed as described in Example 24. A) DMSO (vehicle control), B) 100 μM ampicillin or C) 100 μM. The clear zone surrounding the filter discs in B & C represents sensitivity of the cells to each treatment. D) Quantitation of the half maximal effective dose (ED50) was performed in liquid culture and determined to be 11.6 ± 1.3 μM.

Figure 20 shows the toxicity of mammalian liver cells (Hep3B2-1-17) to compounds 2 – 5. The assay for the compounds was performed as described in Example 25. The toxicity of each compound was determined in the presence of varying concentrations of each compound.

General Description of the Invention

As mentioned above, in one form the present invention provides a method for identifying an inhibitor of a biotin protein ligase, the method including the steps of:

(a) providing a substrate, wherein the substrate may be biotinylated;
(b) contacting the substrate with biotin and a biotin protein ligase in the presence of a test compound;
(c) determining the extent of biotinylation of the substrate by the biotin protein ligase in the presence of the test compound; and
(d) identifying the test compound as an inhibitor of the biotin protein ligase by a reduction in the biotinylation of the substrate in the presence of the test compound as compared to the extent of biotinylation of the substrate in the absence of the test compound.

This form of the present invention is directed to the identification of inhibitors of biotin protein ligase, and in particular, the identification of inhibitors of biotin protein ligases of pathogenic organisms. Inhibitors so identified are candidate compounds for inhibiting bioinylation and as such may be used to inhibit the growth and/or survival of organisms, including the organism from which the biotin protein ligase of interest was derived.

The present invention also provides an inhibitor of a biotin protein ligase identified by the relevant methods for the present invention. As will be appreciated, this form of the present invention may also be used to identify inhibitors of biotinylation of a substrate by a biotin protein ligase.

The biotin protein ligase in the various forms of the present invention may be any biotin protein ligase for which the identification of an inhibitor is desired. Preferably, the biotin protein ligase is a biotin protein ligase of a pathogenic organism of a human, animal or a plant.

Pathogenic organisms of humans or animals for which an inhibitor of a biotin protein ligase may be identified in the various forms of the present invention include bacteria, fungi or parasites. Pathogenic organisms of plants for which an inhibitor of a biotin protein ligase may be identified include bacteria, fungi, insects or nematodes.

Pathogenic bacteria of humans include *Acinetobacter calcoaceticus, Acinetobacter Iwoffi, Actinobacillus* - all species, *Actinomadura madurae, Actinomadura pelletieri, Actinomycetaceae* – all members, *Aeromonas hydrophila, Alcaligenes spp., Arachnia propionica, Arizona spp., Bacillus anthracis, Bacillus cereus, Bacteroides spp., Bartonella* - all species, *Bordetella* - all species, *Borrelia* - all species, *Brucella* - all species, *Campylobacter coli, Campylobacter fetus, Campylobacter jejuni, Cardiobacterium hominis, Chlamydia psittaci, Chlamydia pneumoniae, Chlamydia trachomatis, Chlamydia psittaci, Clostridium botulinum, Clostridium chauvoei, Clostridium difficile, Clostridium haemolyticum, Clostridium histolyticum, Clostridium novyi, Clostridium perfringens, Clostridium septicum, Clostridium sordellii, Clostridium tetani, Corynebacterium diphtheriae, Corynebacterium equi, Corynebacterium haemolyticum, Corynebacterium pseudotuberculosis, Corynebacterium pyogenes, Corynebacterium renale, Coxiella burnetii, Edwardsiella tarda, Eikenella corrodens, Enterobacter spp., Erysipelothrix rusiopathae (insidiosa), Escherichia coli (enterotoxigenic/invasive/haemorrhagic strains), Flavobacterium meningosepticum, Francisella (Pasteurella) tularensis Type A, Francisella tularensis Type B, Francisella novocida, Haemophilus influenzae , Haemophilus ducreyi, Klebsiella - all species and all serotypes, Helicobacter - all species, Legionella - all species, Leptospira interrogans - all serovars, Listeria - all species, Mimae polymorpha, Moraxella - all species, Morganella morganii, Mycobacterium bovis, Mycobacterium chelonae, Mycobacterium fortuitum, Mycobacterium marinum, Mycobacterium paratuberculosis, Mycobacterium africanum, Mycobacterium avium/intracellulare, Mycobacterium bovis, Mycobacterium kansasii, Mycobacterium leprae, Mycobacterium malmoense, Mycobacterium microtic, Mycobacterium scrofulaceum, Mycobacterium simiae, Mycobacterium szulga, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycobacterium xenopi, Mycoplasma - all species, Neisseria elongata, Neisseria gonorrhoeae, Neisseria meningitides, Nocardia spp., Pasteurella multocida, Pasteurella. spp., Peptostreptococcus spp., Plesiomonas*

shigelloides, Porphyromonas spp., Prevotella spp., Proteus - all species, Providencia spp., Pseudomonas aeruginosa, Pseudomonas (Burkholderia) mallei, Pseudomonas (Burkholderia) pseudomallei, Rickettsia - all species, Rhodococcus equi, Salmonella arizonae, Salmonella enteritidis, Salmonella typhimurium, Salmonella paratyphi, Salmonella typhi, Serpulina spp., Serratia liquefaciens, Serratia marcescens, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Shigella dysenteriae, Sphaerophorus necrophorus, Staphylococcus aureus, Stenotrophomonas maltophilia, Streptobacillus moniliformis, Streptococcus spp., Treponema spp., Ureaplasma urealyticum, Vibrio spp., Yersinia (Pasteurella) pestis, Yersinia spp..

Pathogenic fungi of humans include *Asperigillus fumigatus, Blastomyces dermatitidis, Candida spp., Cladophialophora bantiana, Coccidioides immitis, Cryptococcus neoformans var. neoformans, Cryptococcus neoformans var. gattii, Emmonsia parva var. parva, Emmonsia parva var. crescens, Epidermophyton floccosum, Fonsecaea compacta, Fonsecaea pedrosoi, Histoplasma capsulatum var. capsulatum,, Histoplasma capsulatum var. duboisii, Histoplasma capsulatum var. farcinimosum, Madurella grisea, Madurella mycetomatis, Microsporum spp., Neotestudina rosatii, Paracoccidioides brasiliensis, Penicillium marneffei, Scedosporium apiospermum (Pseudallescheria boydii), Scedosporium proliferans (inflatum), Sporothrix schenckii, Trichophyton spp.*

Parasites of humans include *Acanthamoeba spp., Ancylostoma duodenale, Angiostrongylus cantonensis, Angiostrongylus costaricensis, Anisakis simplex, Ascaris lumbricoides, Ascaris suum, Babesia divergens, Babesia microti, Balantidium coli, Blastocystis hominis, Brugia malayi, Brugia pahangi, Brugia timori, Capillaria spp., Contracaecum osculatum, Cryptosporidium spp., Cyclospora spp., Dicrocoelium dendriticum, Dientamoeba fragilis, Diphyllobothrium latum, Dracunculus medinensis, Echinococcus granulosus, Echinococcus multilocularis, Echinococcus vogeli, Entamoeba histolytica, Enterobius vermicularis, Enterocytozoon bieneusi, Fasciola gigantica, Fasciola hepatica, Fasciolopsis buski, Giardia lamblia (Giardia intestinalis), Heterophyes spp., Hymenolepsis diminuta, Hymenolepsis nana (human origin), Isopora belli, Leishmania brasiliensis, Leishmania donovani, Leishmania spp., Loa loa, Mansonella (Dipetalonema) ozzardi, Mansonella (Dipetalonema) perstans, Mansonella (Dipetalonema) streptocerca, Metagonimus spp., Naegleria spp. (especially fowleri),*

Necator americanus, Onchocerca volvulus, Opisthorchis spp. (Clonorchis), Paragonimus spp., Plasmodium falciparum, Plasmodium. spp., Pneumocystis carinii, Pseudoterranova decipiens, Sarcocystis suihominis, Schistosoma spp., Strongyloides spp., Taenia saginata, Taenia solium, Toxocara canis, Toxocara cati, Toxoplasma gondii, Trichinella nativa, Trichinella nelsoni, Trichinella pseudospiralis, Trichinella spiralis, Trichomonas vaginalis, Trichostrongylus spp., Trichuris trichiura, Trypanosoma brucei rhodesiense, Trypanosoma cruzi, Trypanosoma brucei brucei, Trypanosoma brucei gambiense, Trypanosoma rangeli, Wuchereria bancrofti.

Pathogenic bacteria of plants include *Erwinia* species, including *Erwinia amylovora* (Burrill) Winslowet al., *Erwinia carotovora* (Jones) Bergeyet al. subsp.carotovora;, *Pseudomonas* species including *Pseudomonas cichorii* (Swingle) Stapp, *Pseudomonas syringae* pv.glycinea (Coerper) Younget al., *Pseudomonas syringae* pv.pisi (Sackett) Younget al., *Pseudomonas syringae* pv.syringae van Hall x apple, *Pseudomonas syringae* pv.syringae van Hall x apricot, *Pseudomonas syringae* ssp.savastanoi pv.oleae;, *Xanthomonas* species including *Xanthomonas campestris* pv.campestris (Pammel) Dowson, *Xanthomonas campestris* pv.corylina Milleret al., *Xanthomonas campestris* pv.graminis (Egliet al.) Dye, *Xanthomonas campestris* pv.juglandis (Pierce) Dye, *Xanthomonas cynarae* Ridé, Xanthomonas fragariae Kennedy et King, *Agrobacterium* species, and *Corynebacterium* species.

Pathogenic fungi of plants include *Alternaria* species, including *Alternaria helianthi* (Hansf.) Tub. et Nish., *Alternaria brassicae* (Berk.) Sacc. x Cabbage, *Alternaria brassicae* (Berk.) Sacc. x Rape, *Alternaria brassicicola* (Schwein.) Wiltshire, *Alternaria dauci* (Kühn) Gr. et Sk. f. sp.solani (Ell. et Mart.) Neerg., *Alternaria radicina* (Meier) Drechsler et Eddy; *Armilaria* species including *Armilaria mellea* (Vahl) Kummer x grapevine, *Armilaria mellea* (Vahl) P. Kummer x fig; *Ascochyta* species including *Ascochyta fabae* Speg., *Ascochyta hortorum* (Speg.) Smith, *Ascochyta pisi* Lib.; *Athelia* species including *Athelia rolfsii* (Curzi) Tu et Kimbrough; *Botryotinia* species including *Botryotinia fuckeliana* (de Bary) Whetzel x artichoke, *Botryotinia fuckeliana* (de Bary) Whetzel x blackcurrant, *Botryotinia fuckeliana* (de Bary) Whetzel x grapevine, *Botryotinia fuckeliana* (de Bary) Whetzel x hazel, *Botryotinia fuckeliana* (de Bary) Whetzel x raspberry, *Botryotinia fuckeliana* (de Bary) Whetzel x strawberry, *Botryotinia fuckeliana* (de Bary) Whetzel x sunflower, *Botrytis fabae* Sardina; *Bremia*

species including *Bremia lactucae* Regel x artichoke, *Bremia lactucae* Regel x lettuce; *Ceratobasidium cereale* Murray et Burpee; *Cercospora beticola* Sacc.; *Chondrostereum* species including *Chondrostereum purpureum* (Pers.) Pouzar x apple, *Chondrostereum purpureum* (Pers.) Pouzar xPrunus sp.; *Cladosporium cucumerinum* Ell. et Arthur; *Claviceps purpurea* (Fr.) Tul.; *Colletotrichum* species including *Colletotrichum lagenarium* (Pass.) Ellis et Halsted, *Colletotrichum trifolii* Bain et Essary, *Colletotrichum truncatum* (Schwein); *Coniothyrium diplodiella* (Speg.) Sacc.; *Corticium solani* (Prill. et Delacr.) Bourdot et Galzin, *Corticium solani* (Prill. et Delacr.) Bourdot et Galzin; *Coryneum beijerinckii* Oudemans; *Cronartium ribicola* J.C. Fischer; *Cryphonectria parasitica* (Murrill) Barr; *Cryptosporella viticola* (Reddick) Shear; *Diaporthe* species including *Diaporthe cinerescens* (Sacc.), *Diaporthe eres* Nitschke, *Diaporthe helianthi* Muntanola-Cvetkovic Mihaljcevic et Petrov, *Diaporthe phaseolorum* var.sojae (Lehman) Wehmeyer; *Didymella* species including *Didymella applanata* (Niessl.) Sacc., *Didymella lycopersici* Klebahn; *Diplocarpon earliana* (Ell. et Ev.) Wolf; *Drepanopeziza ribis* (Kleb.) Höhnel; *Elsinoe ampelina* Shear; *Erysiphe* species including *Erysiphe betae* (Vanha) Weltzien, *Erysiphe cichoracearum* DC.; *Erysiphe graminis* DC.avenae, *Erysiphe polyfaga* Hammarlund; *Eutypa armeniacae* Hansford et Carter; *Fulvia fulva* (Cooke) Ciferri; *Fusarium* species including *Fusarium oxysporum f. sp.asparagi* Cohen, *Fusarium oxysporum* Schlecht. f. sp.lini (Bolley) Snyd. et Hansen, *Fusarium oxysporum* Schlecht. f. sp.melonis (L. et C.) Snyd. et Hansen x Cucumber, *Fusarium oxysporum* Schlecht. f. sp.melonis (L. et C.) Snyd. et Hansen x melon, *Fusarium oxysporum* Schlecht. f. sp.melonis (L. et C.) Snyd. et Hansen x watermelon, *Fusarium oxysporum* Schlecht. f. sp.pisi (Linford) Snyd. et Hansen, *Fusarium oxysporum* Schlecht. f. sp.radicis-lycopersici Jarvis et Shoem., *Fusarium roseum* (Link) Snyd. et Hansen x asparagus, *Fusarium roseum* (Link) Snyd. et Hansen x gramineae; *Fusicoccum amygdali* Delacr.; *Glomerella* species including *Glomerella cingulata* (Stoneman) Spaulding et Schrenk x almond, *Glomerella cingulata* (Stoneman) Spaulding et Schrenk x apple, *Glomerella cingulata* (Stoneman) Spaulding et Schrenk x olive, *Glomerella glycines* (Hori) Lehmann et Wolf; *Gnomonia leptostyla* (Fr.) Ces. et de Not; *Guignardia baccae* (Cavara) Jacz.; *Helicobasidium brebissonii* (Desm.) Donk x asparagus, *Helicobasidium brebissonii* (Desm.) Donk x beet; *Kabatiella zeae* Narita et Hiratsuka, *Leptosphaeria maculans* (Desm.) Ces. et de Not. x cabbage; *Leptosphaeria* species including *Leptosphaeria maculans* (Desm.) Ces. et de Not. x rape, *Leptosphaeria nodorum* E. Müller; *Leucostoma cinctum* (Fr.) Höhnel,

Leveillula taurica (Lév.) Arn.; *Marssonina panattoniana* (Berlese) Magnus; *Mastigosporium rubricosum* (Dearn. et Barth.) Sprague; *Monilinia* species including *Monilinia fructigena* (Aderhold et Ruhl.) Honeyex Whetzel, *Monilinia laxa* (Aderhold et Ruhland) Honey xPrunus spp.; *Monographella nivalis* (Schaffnit) E. Müller et v. Arx x cereals; *Mycocentrospora cladosporioides* (Sacc.) P. Costaex Deighton; *Mycoplasme* de la Flavescence dorée, *Mycosphaerella pinodes* (Berk. et Blox.) Vestergren; *Mycosphaerella* sp.; *Nectria galligena* Bresad.; *Peronospora* species including *Peronospora destructor* (Berk.) Caspary x onion, *Peronospora farinosa* Fr. f. sp.beta Byford, *Peronospora farinosa* Fr. f. sp.spinaciae Byford, *Peronospora viciae* (Berk.) Caspary f. sp.pisi Sydow; *Pestalotiopsis menezesiana* (Bres. et Torr) Bres. et Torr.; *Phakopsora* species including *Phakopsora euvitis*, *Phakopsora ampelopsidis*, and *Phakopsora vitis*, *Phoma apiicola* Klebhan, *Phoma exigua* Desm., *Phoma medicaginis* Mal. et Roum v.pinodella (L.K. Jones) Boer. x lucerne; *Phoma medicaginis* Mal. et Roum v.pinodella (L.K. Jones) Boer. x peas; *Phragmidium rubi-idaei* (DC.) P. Karsten; *Phytophthora* species including *Phytophthora cactorum* (Lebert et Cohn) Schröter, *Phytophthora capsici Leonian*, *Phytophthora fragariae* Hickman, *Phytophthora infestans* (Mont.) de Bary x potato, *Phytophthora infestans* (Mont.) de Bary x tomato; *Plasmopara* species including *Plasmopara helianthi* Novot., *Plasmopara viticola* (Berk. et Curtisex. de Bary) Berl. et de Toni; *Podosphaera leucotricha* (Ell. et Ev.) Salmon; *Polymyxa betae* Keskin; *Pseudocercosporella herpotrichoides* (Fron) Deighton; *Pseudoperonospora* species including *Pseudoperonospora cubensis* (Berk. et Curtis) Rostovtsev, *Pseudoperonospora humuli* (Miyabe et Takah.) G. Wilson; *Pseudopeziza* species including *Pseudopeziza medicaginis* (Lib.) Sacc. f. sp.medicaginis-lupulinae Schmied., *Pseudopeziza tracheiphila* Müller-Thurgau; *Puccinia* species including *Puccinia allii* (DC.) Rudolph, *Puccinia asparagi* DC., *Puccinia coronata* Corda, *Puccinia coronifera* Kleb., *Puccinia graminis* Persoon f. sp.avenae, *Puccinia hordei* Otth, *Puccinia anomala* Rostrup, *Puccinia recondita* Roberge f. sp.recondita, *Puccinia rubigo-vera* Winter, *Puccinia striiformis* Westendorp f. sp.tritici; *Pyrenochaeta lycopersici* R. Schneider et Gerlach; *Pyrenopeziza brassicae* B. Sutton et Rawlinson; *Pyrenophora* species including *Pyrenophora graminea* Ito et Kuribay, *Pyrenophora teres* Drechsler; *Rhynchosporium secalis* (Oudem.) J. Davis; *Rosellinia necatrix* Prill; *Sclerotinia* species including *Sclerotinia laxa* Aderhold et Ruhland, *Sclerotinia minor* Jagger, *Sclerotinia sclerotiorum* (Lib.) de Bary x artichoke, *Sclerotinia libertiana* Fuckel, *Sclerotinia sclerotiorum* (Lib.) de Bary x carott, *Sclerotinia libertiana* Fuckel,

Sclerotinia sclerotiorum (Lib.) de Bary x rape, *Sclerotinia libertiana* Fuckel, *Sclerotinia sclerotiorum* (Lib.) de Bary x rsunflower, *Sclerotinia libertiana* Fuckel, *Sclerotinia sclerotiorum* (Lib.) de Bary x soybean, *Sclerotinia libertiana* Fuckel, *Sclerotinia trifoliorum* J. Eriksson; *Sclerotium bataticola* Taub., *Sclerotium cepivorum* Berk; *Septoria apiicola* Speg.; *Setosphaeria turcica* (Luttrell) Leonard et Suggs; *Sorosporium reiliana* McAlp; S*phacelotheca* species including *Sphacelotheca reiliana* (Kühn) Clinton, *Sphaerotheca fuliginea* (Schlecht.) Pollacci, *Sphaerotheca mors-uvae* (Schwein.) Berk. et Curtis; *Spilocaea oleagina* (Castagne) Hughes; *Stereum hirsutum* (Willd.) Pers.; *Stigmina carpophila* (Lév.) M.B. Ellis; *Taphrina deformans* (Berk.) Tul. var.persicae; *Thanatephorus cucumeris* (A.B. Frank) Donk x artichoke, *Thanatephorus cucumeris* (A.B. Frank) Donk x chicory, *Uncinula necator* (Schw.) Burr.; *Urocystis cepulae* Frost; *Uromyces betae* Kickx, *Ustilago maydis* (DC.) Corda; *Venturia inaequalis* (Cooke) Winter, *Venturia pirina* Aderhold; *Verticillium* species including *Verticillium albo-atrum* Reinke et Berthold x lucerne, *Verticillium albo-atrum* Reinke et Berthold x sunflower, *Verticillium dahliae* Klebahn x artichoke, *Verticillium dahliae* Klebahn x strawberry, *Verticillium dahliae* Klebahn x sunflower, *Verticillium dahliae* Klebahn x tomato.

Pathogenic insects of plants include insect pests of the orders of *Lepidoptera, Coleoptera, Diptera, Homoptera, Hemiptera, Thysanoptera, and Orthoptera.*

Pathogenic nematodes of plants include nematodes of the genera *Heterodera, Globodera, Umbelliferae, Solanaceae, Pratylinchus, and Meloidogyne.*

In one preferred embodiment, the biotin protein ligase is a biotin protein ligase of a pathogenic organism of a human. Preferably, the biotin protein ligase is a biotin protein ligase from a fungal, bacterial or parasitic pathogen of a human.

In the case of a bacterial pathogen of a human, preferably the biotin protein ligase is a biotin protein ligase from *Eschericia coli* or *Staphyloccus aureus.*

In the case of a fungal pathogen of a human, preferably the biotin protein ligase is a biotin protein ligase from a *Candida* species, such as *Candida albicans,* or a yeast.

In another preferred embodiment, the biotin protein ligase is a biotin protein ligase of a pathogenic organism of a plant. Preferably, the biotin protein ligase is a biotin protein ligase from a fungal, bacterial, insect or nematode pathogen of a plant.

The present invention also provides the use of a biotin protein ligase as a target for identifying agents that inhibit growth of an organism. Preferred biotin protein ligases from various organisms are as herein described.

In another form, the present invention provides a method for identifying an agent that inhibits growth and/or survival of an organism, the method including the step of identifying an agent that inhibits a biotin protein ligase.

In another form, the present invention provides a biotin protein ligase suitable for use as a target for identifying an inhibitor of biotinylation, and/or suitable for use as a target for identifying an inhibitor of growth and/or survival of an organism. Preferably, the biotin protein ligase is an isolated biotin protein ligase. Methods for isolating biotin protein ligases are known in the art.

In a particularly preferred form, the present invention provides an isolated biotin protein ligase when used as a target for identifying an inhibitor of biotinylation, and/or when used as a target for identifying an inhibitor of growth and/or survival of an organism.

Examples of biotin protein ligases particularly suitable for the present invention and their associated sequence data are as follows:

Nematodes:

gi|22532948| *Caenorhabditis elegans*

gi|39597053| *Caenorhabditis briggsae*

Mammals:

gi|1705499| *Homo sapiens*

gi|55655926| *Pan troglodytes*

gi|74001437| *Canis familiaris*

gi|20982837| *Mus musculus*

Birds:

gi|50729961| *Gallus gallus*

Fish:

gi|47223799| *Tetraodon nigrroviridis*

Plants:

gi|50905509| *Oryza sativa*

gi|6623977| *Arabidopsis thaliana*

Fungi:

gi|66852927| *Aspergillus fumigatus*

gi|49097464| *Aspergillus nidulans*

gi|46127903| *Fusarium graminearinearum*

gi|32409781| *Neurospora crassa*

gi|50289965| *Candida glabrata*

gi|1345625| *Saccharomyces cerevesiae*

gi|50310579| *Kluyveromyces lactis*

gi|45187958| *Eremothecium gossypii*

gi|50554375| *Yarrowia lipolytica*

gi|50410019| *Debaryomyces hansenii*

gi|68473197| *Candida albicans*

gi|19113070| *Schizosaccharomyces pombe*

gi|46099450| *Ustilago maydis*

gi|57225614| *Cryptococcus neoformans*

Insects:

gi|23170360| *Drosophila melanogaster*

gi|66565539| *Apis mellifera*

gi|58396066| *Anopheles gambiae*

Microbial:

gi|29377684| *Enterococcus faecalis*

gi|69248979| *Enterococcus faecalis*

gi|77410716| *Streptococcus agalactiae*

gi|71853705| *Streptococcus pyogenes*

gi|55823094| *Streptococcus thermophilus*

gi|50590792| *Streptococcus suis*

gi|15459392| *Streptococcus* pneumoniae

gi|66877104| *Streptococcus pneumoniae*

gi|25528935| *Lactococcus lactis*

gi|62464640| *Lactococcus lactis*

gi|18309655| *Clostridium*

gi|28270529| *Lactobacillus plantarum*

gi|48865543| *Oenococcus oeni*

gi|53689281| *Leuconostoc mesenteroides*

gi|15673762| *Lactococcus*

gi|48870974| *Pediococcus pentosaceus*

gi|27315607| *Staphylococcus epidermidis*

gi|72495112| *Staphylococcus saprophyticus*

gi|49241774| *Staphylococcus aureus*

gi|15924446| *Staphylococcus aureus*

gi|20808369| *Thermoanaerobacter tengcongensis*

gi|76796777| *Thermoanaerobacter ethanolicus*

gi|25500437| *Clostridium acetobutylicum*

gi|28203006| *Clostridium tetani*

gi|67873832| *Clostridium thermocellum*

gi|25329523| *Clostridium acetobutylicum*

gi|20089561| *Methanosarcina acetivorans*

gi|20906352| *Methanosarcina mazei*

gi|12018159| *Methanosarcina barkeri*

gi|68211758| *Methanococcoides burtonii*

gi|15679898| *Methanothermobacter thermautotrophicus*

gi|1168672| *Bacillus subtilis*

gi|52080752| *Bacillus licheniformis*

gi|29895229| *Bacillus cereus*

gi|75760405| *Bacillus thuringiensis*

gi|65318902| *Bacillus anthracis*

gi|47566000| *Bacillus cereus*

gi|52143811| *Bacillus cereus*

gi|49332554| *Bacillus thuringiensis*

gi|56420715| *Geobacillus kaustophilus*

gi|25293806| *Bacillus halodurans*

gi|25517390| *Listeria innocua*

gi|25517191| *Listeria monocytogenes*

gi|22777448| *Oceanobacillus iheyensis*
gi|56963833| *Bacillus clausii*
gi|68009872| *Exiguobacterium*
gi|71547835| *Syntrophobacter fumaroxidans*
gi|73748653| *Dehalococcoides*
gi|57234388| *Dehalococcoides ethenogenes*
gi|76802823| *Natronomonas pharaonis*
gi|55229668| *Haloarcula marismortui*
gi|16554497| *Halobacterium*
gi|66858469| *Anaeromyxobacter dehalogenans*
gi|69260263| *Magnetococcus*
gi|68270618| *Moorella thermoacetica*
gi|71740435| *Pelobacter propionicus*
gi|68178684| *Desulfuromonas acetoxidans*
gi|71545931| *Syntrophobacter fumaroxidans*
gi|68549351| *Pelodictyon phaeoclathratiforme*
gi|67936171| *Chlorobium phaeobacteroides*
gi|67917999| *Chlorobium limicola*
gi|68552171| *Prosthecochloris aestuarii*
gi|67915810| *Chlorobium phaeobacteroides*
gi|71481333| *Prosthecochloris vibrioformis*
gi|14520365| *Pyrococcus abyssi*
gi|14590088| *Pyrococcus horikoshii*
gi|18976449| *Pyrococcus furiosus*
gi|57640922| *Thermococcus kodak*
gi|71541889| *Syntrophomonas wolfei*
gi|55980194| *Thermus thermophilus*
gi|46200063| *Thermus thermophilus*
gi|66798302| *Deinococcus geothermalis*
gi|11497694| *Archaeoglobus fulgidus*
gi|34541252| *Porphyromonas gingivalis*
gi|48856040| *Cytophaga hutchinsonii*
gi|62389598| *Corynebacterium glutamicum*
gi|19551939| *Corynebacterium glutamicum*

gi|25027296| *Corynebacterium efficiens*
gi|38199527| *Corynebacterium diphtheriae*
gi|68264350| *Corynebacterium jeikeium*
gi|15610415| *Mycobacterium tuberculosis*
gi|31794459| *Mycobacterium bovis*
gi|13092858| *Mycobacterium leprae*
gi|15827309| *Mycobacterium leprae*
gi|54022962| *Nocardia farcinica*
gi|69288625| *Kineococcus radiotolerans*
gi|29606981| *Streptomyces avermitilis*
gi|72162952| *Thermobifida fusca*
gi|71369273| *Nocardioides*
gi|66964306| *Arthrobacter*
gi|50954284| *Leifsonia xyli*
gi|68229616| *Frankia*
gi|62423517| *Brevibacterium linens*
gi|23326815| *Bifidobacterium longum*
gi|46190507| *Bifidobacterium longum*
gi|59802309| *Neisseria gonorrhoaea*
gi|11353875| *Neisseria meningitidis*
gi|30043000| *Shigella flexneri*
gi|75176639| *Shigella boydii*
gi|115015| *Escherichia coli*
gi|17865658| *Salmonella typhimurium LT2*
gi|56415970| *Salmonella enterica*
gi|25293809| *Yersinia pestis*
gi|50119174| *Erwinia carotovora*
gi|37528549| *Photorhabdus luminescens*
gi|37681353| *Vibrio vulnificus*
gi|27364604| *Vibrio vulnificus*
gi|75854775| *Vibrio*
gi|75828714| *Vibrio cholerae*
gi|59713032| *Vibrio fischeri*
gi|76793426| *Pseudoalteromonas atlantica*

gi|69163060| *Shewanella frigidimarina*
gi|69942916| *Shewanella denitrificans*
gi|68548555| *Shewanella amazonensis*
gi|68544816| *Shewanella baltica*
gi|56461103| *diomarina loihiensis*
gi|23468178| *Haemophilus somnus*
gi|1168673| *Haemophilus influensae*
gi|75430066| *Actinobacillus succinogenes*
gi|32034277| *Actinobacillus pleuropneumoniae*
gi|33147568| *Haemophilus*
gi|11272589| *Xylella fastidiosa*
gi|21115195| *Xanthomonas campestris*
gi|21110428| *Xanthomonas axonopodis*
gi|77163726| *Nitrosococcus oceani*
gi|30250381| *Nitrosomonas europaea*
gi|71550332| *Nitrosomonas eutropha*
gi|68214112| *Methylobacillus flagellatus*
gi|74316478| *Thiobacillus denitrificans*
gi|34495941| *Chromobacterium violaceum*
gi|56477766| *Azoarcus*
gi|71848990| *Dechloromonas aromatica*
gi|66047791| *Pseudomonas syringae*
gi|70728946| *Pseudomonas fluorescens*
gi|67156990| *Azotobacter vinelandii*
gi|48860573| *Microbulbifer degradans*
gi|67676689| *Chromohalobacter salexigens*
gi|33591356| *Bordetella pertussis*
gi|3800713| *Bordetella pertussis*
gi|33594878| *Bordetella parapertussis*
gi|33575199| *Bordetella bronchiseptica*
gi|74021420| *Rhodoferax ferrireducens*
gi|47573914| *Rubrivivax gelatinosus*
gi|52208452| *Burkholderia pseudomallei*
gi|52428035| *Burkholderia malle*

gi|74014039| *Burkholderia ambifaria*
gi|67668254| *Burkholderia cenocepacia*
gi|67546636| *Burkholderia vietnamiensis*
gi|48786400| *Burkholderia fungorum*
gi|73539808| *Ralstonia*
gi|68554774| *Ralstonia metallidurans*
gi|17545031| *Ralstonia solanacearum*
gi|17987427| *Brucella melitensis*
gi|62289765| *Brucella abortus*
gi|23501703| *Brucella suis*
gi|23347626| *Brucella*
gi|69277800| *Mesorhizobium*
gi|13471392| *Mesorhizobium*
gi|74420934| *Nitrobacter winogradskyi*
gi|69140916| *Nitrobacter hamburgensis*
gi|39649855| *Rhodopseudomonas palustris*
gi|49475644| *Bartonella henselae*
gi|49239695| *Bartonella quintana*
gi|15965033| *Sinorhizobium meliloti*
gi|69298187| *Silicibacter*
gi|56679337| *Silicibacter pomeroyi*
gi|77387506| *Rhodobacter sphaeroides*
gi|69152482| *Paracoccus*
gi|231637| *Paracoccus denitrificans*
gi|68182893| *Jannaschia*
gi|16126179| *Caulobacter cresce*
gi|13423393| *Caulobacter crescentus*
gi|48764633| *Rhodospirillum rubrum*
gi|23014039| *Magnetospirillum magnetotacticum*
gi|61101006| *Erythrobacter litoralis*
gi|68539856| *Sphingopyxis alaskensis*
gi|56544338| *Zymomonas obilis*
gi|58038571| *Gluconobacter oxydans*
gi|70866719| *Trypanosoma cruzi*

gi|70834456| *Trypanosoma brucei*
gi|25534830| *Nostoc*
gi|17232748| *Nostoc*
gi|53764434| *Anabaena variabilis*
gi|23126331| *Nostoc punctiforme*
gi|71675719| *Trichodesmium erythraeum*
gi|67856925| *Crocosphaera watsonii*
gi|7469268| *Synechocystis*
gi|56751177| *Synechococcus elongatus*
gi|37520907| *Gloeobacter violaceus*
gi|62515609| *Lactobacillus delbrueckii*
gi|62513575| *Lactobacillus casei*
gi|7520946| *Syphilis spirochete*
gi|15639348| *Treponema pallidum*
gi|16752173| *Chlamydophila pneumoniae*
gi|29835007| *Chlamydophila caviae*
gi|76167995| *Chlamydia trachomatis*
gi|23509795| *Plasmodium falciparum*
gi|23482140| *Plasmodium yoelii*
gi|56499526| *Plasmodium berghei*
gi|46227802| *Cryptosporidium parvum*
gi|41016764| *Methanocaldococcus jannaschii*
gi|45357682| *Methanococcus maripaludis*
gi|7514332| *Aquifex aeolicus*
gi|51460027| *Rickettsia typhi*
gi|15604390| *Rickettsia prowazekii*
gi|15892696| *Rickettsia conorii*
gi|42453815| *Rickettsia rickettsii*
gi|67004669| *Rickettsia felis*
gi|52698978| *Rickettsia akari*
gi|58417546| *Ehrlichia ruminant*
gi|58616979| *Ehrlichia ruminant*
gi|73666871| *Ehrlichia canis*
gi|58419193| *Wolbachia*

gi|52628131| *Legionella pneumophila*
gi|33517318| *Blochmannia floridanus*
gi|71891971| *Blochmannia pennsylvanicus*
gi|33239881| *Prochlorococcus marinus*
gi|33633186| *Synechococcus*
gi|70607375| *Sulfolobus acidocaldarius*
gi|15897572| *Sulfolobus solfataricus*
gi|57237106| *Campylobacter jejuni*
gi|57504657| *Campylobacter coli*
gi|57505335| *Campylobacter upsaliensis*
gi|57240720| *Campylobacter lari*
gi|71151002| *Thiomicrospira denitrificans*
gi|15645754| *Helicobacter pylori*
gi|46445799| *Parachlamydia*
gi|10580250| *Halobacterium*
gi|34762115| *Fusobacterium nucleatum*
gi|67929504| *Solibacter usitatus*
gi|48764875| *Rhodospirillum rubrum*
gi|71083596| *Pelagibacter ubique*
gi|72009746| *Strongylocentrotus purpuratus*
gi|28492991| *Tropheryma whipple*
gi|76261556| *Chloroflexus*
gi|56707619| *Francisella tularensis*
gi|46580244| *Desulfovibrio vulgaris*
gi|49530030| *Acinetobacter*
gi|29250335| *Giardia lamblia*
gi|67480425| *Entamoeba histolytica*
gi|51246938| *Desulfotalea psychrophila*

Preferably, the use of a biotin protein ligase as a target for identifying agents that inhibit growth of an organism includes the use of one of the above biotin protein ligases.

Under conditions when inhibitors are identified *in vitro*, the biotin protein ligase of interest for use in the methods according to the present invention may be purified by a

suitable method known in the art, including the purification of the protein from cell extracts from the organism of interest, or the expression of the protein in an appropriate expression system from an appropriate expression vector having the cloned gene. In the latter case, the biotin protein ligase may be expressed with an additional moiety that allows the enzyme to be readily purified. Examples of such additional moieties include glutathione S transferase (GST), histidine tags, maltose binding proteins, and calmodulin binding peptides. Such an additional moiety may or may not be removed from the biotin protein ligase before use.

The biotin protein ligase may be purified to a desired extent so as to allow the inhibition of the extent of biotinylation of the substrate by the ligase to be determined.

Under conditions when inhibitors are identified *in vivo*, the biotin protein ligase of interest for use in the methods according to the present invention may be the endogenous biotin protein ligase, or alternatively, may be a cloned biotin protein ligase expressed in a cell.

The substrate according to the various forms of the invention is any molecule that has the capacity to have a biotin group covalently attached to the molecule by the action of a biotin protein ligase. Examples of suitable substrates include biotin-containing proteins such as pyruvate carboxylase, acetyl CoA carboxylase, propionyl CoA carboxylase, B-methylcrotonyl CoA carboxylase, methylmalonyl-CoA carboxyltransferase, oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, glutaconyl-CoA decarboxylase, urea carboxylase, geranoyl-CoA carboxylase and geranoyl-CoA transcarboxylase, or a functional variant of any of the aforementioned proteins; a polypeptide including the biotin domain of any of the aforementioned protein substrates or variants of the proteins; proteins or polypeptides synthesised *in vitro* by chemical synthesis or *in vitro* translation that have the capacity to be biotinylated; or a small molecule that has the ability to have a free biotin group covalently attached to the molecule by a BPL, such as hydroxylamine.

Preferably, the substrate is a polypeptide fragment containing the biotin domain of a biotin-containing protein or hydoxylamine. Most preferably, the substrate is a polypeptide fragment containing the biotin domain of a biotin-containing protein.

Methods for identifying proteins encoding examples of the substrates hereinbefore described may be achieved by a suitable method. For example, pyruvate carboxylases may be identified using the BLAST algorithm, which determines the extent of homology between two nucleotide sequences (blastn) or the extent of homology between two amino acid sequences (blastp). BLAST identifies local alignments between the sequences in the database and predicts the probability of the local alignment occurring by chance. The BLAST algorithm is as described in Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410.

Under conditions when inhibitors are identified *in vitro*, preferably the substrate is a protein substrate derived from the same species or genus as that of the biotin protein ligase of interest. For example, when identifying inhibitors of *Candida* biotin protein ligase, the substrate is preferably a polypeptide from a *Candida* biotin carboxylase, such as *Candida albicans* pyruvate carboxylase. However, it will be appreciated that the substrate may also be derived from an unrelated species or genus as that of the biotin protein ligase of interest.

In the case of the identification of inhibitors of *Candida* biotin protein ligase *in vitro*, the substrate is preferably pyruvate carboxylase or a polypeptide fragment derived from *Candida albicans* pyruvate carboxylase, or a functional variant thereof. Sequence data for *Candida albicans* pyruvate carboxylase may be obtained from the Stanford Genome Technology Centre (orf6.2989 orf6-2189:1646-4594).

Most preferably, the protein substrate includes (or consists of) one of the following polypeptides from *Candida albicans* pyruvate carboxylase (or a functional variant or thereof):

caPC-115:

M^{868}AVGDVSEKTGTREVFFELNGEMRSVSVEDKTVSVELKTRPKASASNEVGA PMAGVVIEIRAHKHQQIAKGDPIAVLSAMKMEMVISAPCSGEIGDILIHEGDSVD ANDLITSIH^{982} (SEQ ID NO.1).

caPC-93:
M[890]RSVSVEDKTVSVELKTRPKASASNEVGAPMAGVVIEIRAHKHQQIAKGDPI AVLSAMKMEMVISAPCSGEIGDILIHEGDSVDANDLITSIH[982] (SEQ ID NO.2).

caPC-74:
K[909]ASASNEVGAPMAGVVIEIRAHKHQQIAKGDPIAVLSAMKMEMVISAPCSGE IGDILIHEGDSVDANDLITSIH[982] (SEQ ID NO.3).

In the case of the identification of inhibitors of an insect biotin protein ligase *in vitro*, the substrate is preferably pyruvate carboxylase or a polypeptide fragment derived from a *Drosophila* pyruvate carboxylase (gi|19921944|ref|NP_610527.1| BcDNA:GH06348 gene product), or a functional variant thereof. Most preferably, the protein substrate includes the following polypeptide from *Drosophila melanogaster* pyruvate carboxylase (or a functional variant thereof):

dPC-125:
G[1057]KTLSVKALAVSADLKPNGIREVFFELNGQLRAVHILDKEAVKEIHVHPKAN KSNKSEVGAPMPGTVIDIRVKVGDKVEKGQPLVVLSAMKMEMVVQSPLAGVV KKLEIANGTKLEGEDLIMIIE[1181] (SEQ ID NO.4).

In the case of the identification of an inhibitor of a bacterial biotin protein ligase *in vitro*, the substrate is preferably pyruvate carboxylase or a polypeptide derived from *E.coli* acetyl CoA carboxylase, or a functional variant thereof (gi|1789653|gb|AAC76287.1|[1789653]). Most preferably, the protein substrate includes the following polypeptide from *E. coli* acetyl CoA carboxylase (or a functional variant thereof):

BCCP-87:
M[70]EAPAAAEISGHIVRSPMVGTFYRTPSPDAKAFIEVGQKVNVGDTLCIVEAMK MMNQIEADKSGTVKAILVESGQPVEFDEPLVVIE[156] (SEQ ID NO.5).

Additional polypeptides that may be used as protein substrates *in vitro* for *E. coli* BPL include the following polypeptides:
LGGIFEAMKMELRD (SEQ ID NO.6);
LFLHDFLNAQKVELYPVTSSG (SEQ ID NO.7);
MAGGLNDIFEAQKIEWHEDTGGS (SEQ ID NO.8);
or a functional variant of these polypeptides.

As will be appreciated, under conditions when inhibitors are identified *in vitro*, the substrate of interest for use in the methods according to the present invention will be present at such a concentration and such a level of purity to allow biotinylation of the substrate by the biotin protein ligase to be measured.

In the case of the substrate being a protein substrate, the protein substrate may be purified by a suitable method known in the art, including the purification of the protein from cell extracts from the organism of interest, or the expression of the protein in an appropriate expression system from an appropriate expression vector having the cloned nucleotide sequence. The protein substrate may be expressed with an additional moiety that allows the protein substrate to be readily purified. Examples of such additional moieties include glutathione S transferase (GST), histidine tags, maltose binding proteins, and calmodulin binding peptides. Such an additional moiety may or may not be removed from the biotin protein ligase before use. As discussed, the protein substrate will be purified to an extent to allow the inhibition of the extent of biotinylation of the protein substrate by the ligase to be determined.

Contacting the substrate with biotin and the biotin protein ligase in the various forms of the present invention may be performed under any suitable conditions that allow the substrate to be biotinylated by the biotin protein ligase. Under conditions when inhibitors are identified *in vitro*, preferably the contacting of the substrate with biotin and biotin protein ligase occurs when the substrate is directly or indirectly coupled to a solid phase. In this regard, it has been determined that the ability to identify inhibitors of biotin protein ligases is improved by contacting the substrate with biotin and a biotin protein ligase when the substrate is coupled to a solid phase.

Examples of direct coupling include direct adsorption of the substrate to a solid phase, or direct covalent attachment of the substrate to the solid phase utilising the reaction of a reactive chemical moiety on the column with a reactive moiety on the substrate, with or without a linker or spacer. Preferably, the direct coupling is by way of direct adsorption to a solid phase.

Indirect coupling involves the coupling of the substrate to the solid phase using a molecule or moiety attached to the solid phase that has the ability to capture the substrate non-covalently. Examples of indirect coupling include the coupling of an antibody to the substrate to the solid phase and capturing the substrate (ie antigen) with the antibody, or the capturing of a six histidine sequence by a Nickel column.

In the case where the contacting occurs *in vitro*, the concentration of each of the substrate, biotin and biotin protein ligase in the reaction will be selected so as to allow sufficient biotinylation of the substrate to occur to allow for the determination of whether a test compound at a particular concentration has the capacity to inhibit biotinylation of the substrate.

In the case where the contacting occurs *in vivo*, the contacting may occur in a cell type that allows the extent of biotinylation of the substrate by the biotin protein ligase to be determined. The biotin protein ligase may be present endogenously in the cell, or alternatively, may be expressed for example from an introduced nucleic acid. Similarly, the substrate may either be expressed endogenously by the cell or be expressed from an introduced nucleic acid. In some cases, the biotin protein ligase and/or the substrate may also be contacted with the cell so as to enter the cell.

In this regard, methods for the isolation of nucleic acid sequences and their cloning into a suitable expression vector are essentially as described in Sambrook, J, Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual 2nd. ed. Cold Spring Harbor Laboratroy Press, New York. (1989). The recombinant molecule may then be introduced into the cell by a method known in the art and the cloned nucleic acid expressed.

Methods for introducing nucleic acids into cells and expressing proteins in the various forms of the present invention are known in the art. For example, a nucleic acid may be introduced into a cell by various methods, including transformation using calcium phosphate, viral infection, electroporation, lipofection, and particle bombardment. Methods for introducing DNAs into cells are essentially as described in Sambrook, J, Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual 2nd. ed. Cold Spring Harbor Laboratory Press, New York. (1989).

Under conditions when inhibitors are identified *in vitro*, the biotinylation reaction is dependent upon the presence of a suitable divalent metal ion. Preferably, the divalent metal ion is a magnesium ion or calcium ion. Most preferably, the divalent metal ion is a magnesium ion. The concentration of the divalent metal ion is preferably in the range of 0.5 to 10 mM.

The biotinylation reaction is also dependent upon the presence of ATP. Preferably, the concentration of ATP is in the range of 0.5 uM to 5 mM. Most preferably, the concentration of ATP is 100 uM.

Under conditions when inhibitors are identified *in vitro*, the biotinylation reaction may be buffered by any suitable buffer, including acetate, carbonate or Tris buffers, and the pH of the reaction may be within a suitable range to allow biotinylation of the substrate by the biotin protein ligase. Preferably, the pH of the reaction is in the range of 7.0 to 8.5. Most preferably, the pH of the reaction is in the range from 7.5 to 8.0.

Under conditions when inhibitors are identified *in vitro*, the biotinylation reaction may be performed at a suitable temperature and for a suitable time, and may also include the presence of a salt and/or a reducing agent. Similarly, under conditions when inhibitors are identified *in vivo*, the test compound will be contacted with cells for an appropriate time to allow inhibition of biotinylation to be determined.

The test compound may be any compound that may act as a potential inhibitor of the biotinylation of the protein substrate. For example, the test compound may be a small molecule, drug, protein, peptide, polypeptide, antibody or antigen binging portion thereof, polysaccharide, glycoprotein, lipid, metabolite, cofactor, transition state

analogue, nucleotide, nucleotide analogue, or nucleic acid. Under conditions when inhibitors are identified *in vivo*, the test compound will need to be able to enter the cell and have the capacity to exert an inhibitory action on the biotin protein ligase. Alternatively, the test compound may be expressed in the cells, such as for the case of a potentially inhibitory polypeptide.

As will be appreciated, the ability of the test compound to inhibit biotinylation of the protein substrate will depend on the concentration of the test molecule. Accordingly, the concentration of the test molecule will be selected so as to determine the ability of the test molecule to inhibit biotinylation of the protein substrate at that concentration.

For *in vitro* purposes, to separate biotinylated substrate from free biotin, the substrate may be partitioned from free biotin by washing the substrate bound to a solid phase under conditions to remove free biotin. Alternatively, free biotin may be removed from the substrate by the preferential binding of the free biotin to a solid phase, such as the binding of biotin to anion exchange resin. Preferably, biotinylated substrate is partitioned from free biotin by the coupling of the substrate to a solid phase and removing free biotin by washing of the solid phase.

The determination of the extent of biotinylation of the substrate in the various forms of the present invention may be performed by a suitable method known in the art.

For example, under conditions when inhibitors are identified *in vitro*, biotinylation of the substrate may be detected by use of detectably labelled streptavidin or avidin. Alternatively, detectably labelled biotin may be employed. The extent of biotinylation of the substrate in the presence and absence of the test compound may then be determined, and the test compound identified as an inhibitor of the biotin protein ligase by a reduction in the extent of biotinylation of the substrate in the presence of the test compound as compared to the extent of biotinylation in the absence of the test compound.

For detection of the extent of biotinylation of the protein substrate, the presence of biotin may be detected by any suitable method that allows the determination of the extent of biotinylation of the protein substrate. For example, detectably labelled streptavidin may be utilised.

Under conditions when inhibitors are identified *in vivo,* a protein substrate may be detected by preparing a cell lysate, isolating one or more protein substrates and determining the extent of biotinylation of the protein substrates. For example, the cell lysate may be fractioned by SDS-PAGE, the protein detected with a suitable antibody and the extent of biotinylation determined by a streptavidin blot. Alternatively, one or more protein substrates may be immunoprecipitated and the extent of biotinylation determined by detectably labeled streptavidin or avidin.

Identification of the test compound as an inhibitor of a biotin protein ligase will be made by a comparison of the extent of biotinylation of the substrate in the presence and absence of the test compound. Preferably, the maximum extent of inhibition of activity by the test compound is greater than 70%. More preferably, the maximum extent of inhibition of activity is greater than 90%.

Preferably, the selection of a suitable compound as a test compound is first made by the use of an *in silico* selection method.

Preferably, the *in silico* method utilises the structure of a biotin protein ligase to identify a potential test compound. More preferably, the *in silico* method utilises the structure of the biotin protein ligase in the absence and presence of biotin to identify potential test compounds.

For example, the X-ray structures of BPL from *Escherichia coli*, in the absence (Wilson *et al* (1992) "Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains" *Proc. Natl. Acad. Sci. U S A.* 89; 9257-9261) and presence of biotin (Weaver *et al* (2001) "Corepressor-induced organization and assembly of the biotin repressor: A model for the allosteric activation of a transcriptional regulator" *Proc Natl. Acad. Sci. USA.* 98; 6045-6050) may be used to identify potential inhibitory compounds using *in silico* technology. Missing amino

acids and hydrogen atoms in the structure (PDB: 1BIB) may be modelled using the Biopolymer module of SYBYL v7.0 (Tripos, Inc. SYBYL, Version 7.0, St. Louis, MO 2004). Modelled sections of the protein may be refined by conjugate gradient energy minimisation in explicit water using the program NAMD (Linge *et al* (2003) "Refinement of protein structures in explicit solvent" *Proteins* 50; 496-506; Kale *et al.* (1999) "NAMD2: greater scalability for parallel molecular dynamics" *J. Comput. Phys.* 151: 283-312). Partial charges for BPL and the 24/07/2004 compounds of interest, for example as present in a compound library (eg ZINC) may be calculated using Biopolymer and the SYBYL Programming Language (SPL) respectively. A negative image of the active sites for the prepared BirA may be constructed with ATPTS 2001 (Moreno *et al.* (2002) "Geometric and chemical patterns of interaction in protein--ligand complexes and their application in docking" *Proteins* 47: 1-13). The compound library may then be docked with DOCK v5.1.0. (Ewing *et al.* (2001) "DOCK 4.0: search strategies for automated molecular docking of flexible molecule databases" *J. Comput. Aided Mol. Des.* 15: 411-428). Poses may be scored using Scorer v1.3 and ranked with a threshold of 10% in an in-house consensus scoring program based on the CScore module of SYBYL. Compounds with a score of 6 and the top compounds based on the internal Dock Energy Score may be visually inspected and non-viable candidates filtered out.

Potential test compounds selected by the *in silico* methodology may then be screened for their ability to inhibit biotinylation as previously herein described.

As described previously, the present invention also provides an inhibitor of a biotin protein ligase identified according to the relevant methods for the present invention.

For example, the present invention may be used to identify an inhibitor of a biotin protein ligase of a pathogenic organism.

Accordingly, in another form the present invention provides an anti-pathogenic agent, wherein the agent inhibits biotinylation of a substrate by a biotin protein ligase of a pathogen.

In another form, the present invention provides an inhibitor of a biotin protein ligase of a pathogenic organism. Examples of pathogenic organisms and their biotin protein ligases are as previously hereinbefore described.

For example, the present invention may be used to identify an inhibitor of a bacterial biotin protein ligase or an inhibitor of a microbial protein ligase.

Accordingly, in another form the present invention provides an inhibitor of a bacterial biotin protein ligase.

In another form, the present invention provides an anti-bacterial agent, wherein the agent inhibits biotinylation of a substrate by a bacterial biotin protein ligase.

Preferably, the anti-bacterial agent is an inhibitor of (and inhibits biotinylation of a substrate by) a biotin protein ligase from a bacterium selected from the group consisting the genus or species of *Enterococcus faecalis, Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus thermophilus, Streptococcus suis, Streptococcus pneumoniae, Lactococcus lactis, Clostridium, Lactobacillus plantarum, Oenococcus oeni, Leuconostoc mesenteroides, Lactococcus pentosaceus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus aureus, Thermoanaerobacter tengcongensis, Thermoanaerobacter ethanolicus, Clostridium acetobutylicum, Clostridium tetani, Clostridium thermocellum, Clostridium acetobutylicum, Methanosarcina acetivorans, Methanosarcina mazei, Methanosarcina barkeri, Methanococcoides burtonii, Methanothermobacter thermautotrophicus, Bacillus subtilis, Bacillus licheniformis, Bacillus cereus, Bacillus thuringiensis, Bacillus anthracis, Bacillus cereus, Geobacillus kaustophilus, Bacillus halodurans, Listeria innocua, Listeria monocytogenes, Oceanobacillus iheyensis, Bacillus clausii, Exiguobacterium, Syntrophobacter fumaroxidans, Dehalococcoides including Dehalococcoides ethenogenes, Natronomonas pharaonis, Haloarcula marismortui, Halobacterium, Anaeromyxobacter dehalogenans, Magnetococcus, Moorella thermoacetica, Pelobacter propionicus, Desulfuromonas acetoxidans, Syntrophobacter fumaroxidans, Pelodictyon phaeoclathratiforme, Chlorobium phaeobacteroides, Chlorobium limicola, Prosthecochloris aestuarii, Chlorobium phaeobacteroides, Prosthecochloris vibrioformis, Pyrococcus abyssi, Pyrococcus horikoshii, sPyrococcus*

furiosus, Thermococcus Kodak, Syntrophomonas wolfei, Thermus thermophilus, Deinococcus geothermalis, Archaeoglobus fulgidus, Porphyromonas gingivalis, Cytophaga hutchinsonii, Corynebacterium glutamicum, Corynebacterium glutamicum, Corynebacterium efficiens, Corynebacterium diphtheriae, Corynebacterium jeikeium, Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium leprae, Nocardia farcinica, Kineococcus radiotolerans, Streptomyces avermitilis, Thermobifida fusca, Nocardioides, Arthrobacter, Leifsonia xyli, Frankia, Brevibacterium linens, longum, Bifidobacterium longum, Neisseria gonorrhoaea, Neisseria meningitides, Shigella flexneri, Shigella boydii, Escherichia coli, Salmonella typhimurium LT2, Salmonella enterica, Yersinia pestis, Erwinia carotovora, Photorhabdus luminescens, Vibrio vulnificus, Vibrio vulnificus, Vibrio, Vibrio cholerae, Vibrio fischeri, Pseudoalteromonas atlantica, Shewanella frigidimarina, Shewanella denitrificans, Shewanella amazonensis, Shewanella baltica, diomarina loihiensis, Haemophilus somnus, Haemophilus influensae, Actinobacillus succinogenes, Actinobacillus pleuropneumoniae, Haemophilus, Xylella fastidiosa, Xanthomonas campestris, Xanthomonas axonopodis, Nitrosococcus oceani, Nitrosomonas europaea, Nitrosomonas eutropha, Methylobacillus flagellatus, Thiobacillus denitrificans, Chromobacterium violaceum, Azoarcus, Dechloromonas aromatica, Pseudomonas syringae, Pseudomonas fluorescens, Azotobacter vinelandii, Microbulbifer degradans, Chromohalobacter salexigens, Bordetella pertussis, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Rhodoferax ferrireducens, Rubrivivax gelatinosus, Burkholderia pseudomallei, Burkholderia malle, Burkholderia ambifaria, Burkholderia cenocepacia, Burkholderia vietnamiensis, Burkholderia fungorum, Ralstonia, Ralstonia metallidurans, Ralstonia solanacearum, Brucella melitensis, Brucella abortus, Brucella suis, Brucella, Mesorhizobium, Nitrobacter winogradskyi, Nitrobacter hamburgensis, Rhodopseudomonas palustris, Bartonella henselae, Bartonella Quintana, Sinorhizobium meliloti, Silicibacter, Silicibacter pomeroyi, Rhodobacter sphaeroides, Paracoccus, Paracoccus denitrificans, Jannaschia, Caulobacter cresce, Caulobacter crescentus, Rhodospirillum rubrum, Magnetospirillum magnetotacticum, Erythrobacter litoralis, Sphingopyxis alaskensis, Zymomonas obilis, Gluconobacter oxydans, Trypanosoma cruzi, Trypanosoma brucei, , Anabaena variabilis, Nostoc punctiforme, Trichodesmium erythraeum, Crocosphaera watsonii, Synechocystis, Synechococcus elongates, Gloeobacter violaceus, Lactobacillus delbrueckii, Lactobacillus casei, Syphilis spirochete, Treponema pallidum,

Chlamydophila pneumoniae, Chlamydophila caviae, Chlamydia trachomatis, Plasmodium falciparum, Plasmodium yoelii, Plasmodium berghei, Cryptosporidium parvum, Methanocaldococcus jannaschii, Methanococcus maripaludis, Aquifex aeolicus, Rickettsia typhi, Rickettsia prowazekii, Rickettsia conorii, Rickettsia rickettsii, Rickettsia felis, Rickettsia akari, Ehrlichia ruminant, Ehrlichia ruminant, Ehrlichia canis, Wolbachia, Legionella pneumophila, Blochmannia floridanus, Blochmannia pennsylvanicus, Prochlorococcus marinus, Synechococcus, Sulfolobus acidocaldarius, Sulfolobus solfataricus, Campylobacter jejuni, Campylobacter coli, Campylobacter upsaliensis, Campylobacter lari, Thiomicrospira denitrificans, Helicobacter pylori, Parachlamydia, Halobacterium, Fusobacterium nucleatum, Solibacter usitatus, Rhodospirillum rubrum, Pelagibacter ubique, Strongylocentrotus purpuratus, Tropheryma whipple, Chloroflexus, Francisella tularensis, Desulfovibrio vulgaris, Acinetobacter, Giardia lamblia, Entamoeba histolytica, and *Desulfotalea psychrophila*

Preferably, the anti-bacterial agent has an IC_{50} for the biotin protein ligase of less than 1 μM. More preferably, the anti-bacterial agent has an IC_{50} for the biotin protein ligase of less than 1 nM.

Such bacterial inhibitors may have also anti-bacterial activity generally, and as such may be used to inhibit the growth of one or more bacteria. The inhibitors may be bacteriocidal or bacteriostatic.

In another form the present invention provides an anti-bacterial agent, the agent inhibiting biotinylation by a bacterial biotin protein ligase.

Preferably, the screening methods for the present invention may be used to identify an inhibitor of a bacterial biotin protein ligase with an IC_{50} (inhibitory concentration) of less than 3 μM, more preferably with an IC_{50} of less than 1 μM, more preferably with an IC_{50} of less than 15 nM, more preferably with an with an IC_{50} of less than 2 nM, and most preferably with an IC_{50} of 1 nM or less. In this regard, the screening method for the present invention has been used to identify inhibitors of *E.coli* biotin protein ligase with an IC_{50} of less than 1 nM.

The present invention may also be used to identify inhibitors of biotin protein ligases of other pathogenic organisms.

For example, the present invention may be used to identify an inhibitor of a fungal biotin protein ligase.

Accordingly, in another form the present invention provides an inhibitor of a fungal biotin protein ligase.

In another form, the present invention provides an anti-fungal agent, wherein the agent inhibits biotinylation of a substrate by a fungal biotin protein ligase.

Preferably, the anti-fungal agent is an inhibitor of (and inhibits biotinylation of a substrate by) a biotin protein ligase from a fungus selected from the group consisting the genus or species of *Aspergillus fumigatus, Aspergillus nidulans, Fusarium graminearinearum, Neurospora crassa, Candida glabrata, Saccharomyces cerevesiae, Kluyveromyces lactis, Eremothecium gossypii, Yarrowia lipolytica, Debaryomyces hansenii, Candida albicans, Schizosaccharomyces pombe, Ustilago maydis, Cryptococcus neoformans.*

The present invention may be used to identify an inhibitor of a nematode biotin protein ligase.

Accordingly, in another form the present invention provides an inhibitor of a nematode biotin protein ligase.

In another form, the present invention provides an anti-nematode agent, wherein the agent inhibits biotinylation of a substrate by a nematode biotin protein ligase.

The present invention may be used to identify an inhibitor of a parasite biotin protein ligase.

Accordingly, in another form the present invention provides an inhibitor of a parasite biotin protein ligase.

In another form, the present invention provides an anti-parasitic agent, wherein the agent inhibits biotinylation of a substrate by a nematode biotin protein ligase.

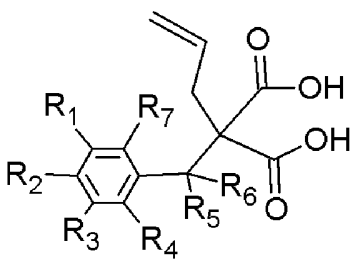
The present invention may be used to identify an inhibitor of an insect biotin protein ligase.

Accordingly, in another form the present invention provides an inhibitor of a insect biotin protein ligase.

In another form, the present invention provides an insecticidal agent, wherein the agent inhibits biotinylation of a substrate by a nematode biotin protein ligase.

The inhibitors may inhibit growth and/or survival of an organism. Accordingly, in another form the present invention provides a method for identifying an inhibitor of growth and/or survival of an organism, the method including the step of identifying an inhibitor of a biotin protein ligase.

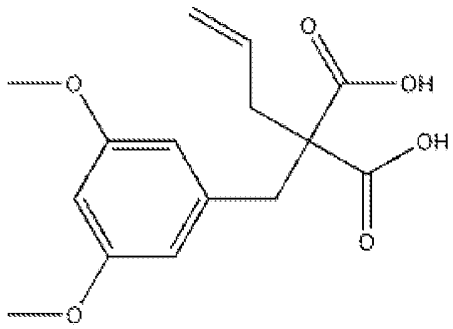
In this regard, the screening method for the present invention has been used to identify compounds that inhibit *E.coli* (IC_{50}= 0.9 nM) and *S. aureus* (IC_{50}= 2.7 μM) biotin protein ligases, with the following formula:



or a salt or ester thereof, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl,

arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino, sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-, $R_9N(R_{10})SO_2R_{11}$-, $R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

In a preferred embodiment, the compound is 2-allyl-2-(3,5-dimethoxybenzyl)malonic acid, or a salt or ester thereof , as follows:



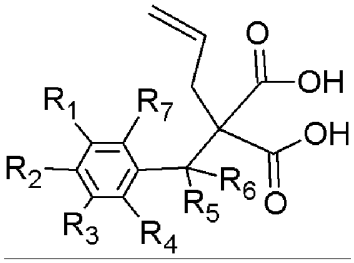
The present invention also provides compositions including an inhibitor of a biotin protein ligase.

The compositions may be used as anti-pathogenic compositions, such as anti-microbial compositions, anti-bacterial compositions, anti-fungal compositions, anti-parasitic compositions, anti-nematode composoitions or insecticidal compositions.

In one embodiment, the compositions may be used to inhibit growth and/or survival of an organism, and in particular, for inhibiting the growth and/or survival of pathogenic organisms, such as bacteria and fungi.

Accordingly, in another form the present invention provides a composition for inhibiting growth of a pathogen, the composition including an effective amount of an agent that inhibits a biotin protein ligase.

In a particular preferred form, the compositions include a compound with the following formula:



or a salt or ester thereof, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl, arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino, sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-, $R_9N(R_{10})SO_2R_{11}$-, $R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

In a preferred embodiment, the compound is 2-allyl-2-(3,5-dimethoxybenzyl)malonic acid, or a salt or ester thereof .

As discussed, the inhibitors of the present invention may also used to inhibit growth and/or survival of an organism, and in particular, the growth and survival of pathogenic organisms of humans, animal or plants.

Accordingly, in another form the present invention provides a method of inhibing the growth and/or survival of a pathogen organism, the method including the step of contacting a biotin protein ligase of the pathogenic organism with an agent that inhibits biotinylation by the biotin protein ligase.

On one embodiment, the pathogen may be exposed to the agent to inhibit growth and/or survival.

In another form, the present invention provides a method of inhibiting the growth and/or survival of a pathogenic organism, the method including the step of exposing the patheogen to an agent that inhibits biotinylation in the pathogenic organism.

The present invention also provides a composition for inhibiting the growth and/or survival of a pathogenic organism.

In one form, the present invention provides a composition for inhibiting the growth and/or survival of a pathogenic organism, the composition including an effective amount of an agent that inhibits biotinylation in the pathogenic organism.

As discussed previously, the present invention may be used to identify an inhibitor of a biotin protein ligase in one or more cells.

In one embodiment, the present invention may be used to identify an inhibitor of biotinylation in a biological system.

Accordingly, the present invention provides a method for identifying an inhibitor of biotinylation in a biological system, the method including the steps of:

(a) identifying a test compound as an inhibitor of a biotin protein ligase;

(b) determining the ability of the test compound to inhibit biotinylation in a biological system; and

(c) identifying the test compound as an inhibitor of biotinylation in the biological system.

This form of the present invention is directed to the identification of inhibitors of biotinylation by first identifying an inhibitor of a biotin protein ligase. Inhibitors so identified are candidate compounds for inhibiting the growth of pathogenic organisms, and in particular, pathogenic organisms from which the biotin protein ligase was derived.

The biological system in the various forms of the present invention may be any cellular or multi-cellular system for which an inhibition of biotinylation is desired. For example, the biological system may be a cell cultured *in vitro*, a bacterial cell, a fungal cell, or an entire organism.

Preferably, the biological system includes a cell derived from a pathogenic organism of a human, animal or plant, such as a bacteria, fungus, parasite, nematode or insect. Examples of such pathogenic organisms are as previously hereinbefore described.

Accordingly, in a preferred form the present invention provides a method for identifying an inhibitor of biotinylation in a pathogenic organism of a human, animal or plant, the method including the steps of:

(a) identifying a test compound as an inhibitor of a biotin protein ligase;

(b) determining the ability of the test compound to inhibit biotinylation in a pathogenic organism of a human, animal or plant; and

(c) identifying the test compound as an inhibitor of biotinylation in the pathogenic organism.

Examples of pathogens are as previously herein described. Preferably, the pathogenic organism is a bacterial or fungal pathogen of a human, animal or plant.

The biotin protein ligase may be any suitable biotin protein ligase for which the identification of an inhibitor if biotinylation is required. The biotin protein ligase may be a biotin protein ligase derived from the biological system for which an inhibitor is required, or alternatively, may be a biotin protein ligase from an unrelated biological system. Preferably, the biotin protein ligase is a biotin protein ligase derived from the biological system for which an inhibitor of biotinylation is to be identified. For example, for the identification of an inhibitor of biotinylation in fungi, preferably the biotin ligase is derived from a fungus such as *Candida* or a yeast.

The identification of a test compound as an inhibitor of a biotin protein ligase may be achieved as previously herein discussed. To determine the ability of the test compound to inhibit biotinylation in the biological system, the test compound will be contacted with the biological system and the extent of biotinylation determined by a suitable method known in the art, including determination by streptavidin blots on enzyme preparations to quantitate the amount of protein-bound biotin and/or enzyme kinetics to determine the specific activity of biotin enzymes.

As will be appreciated, the test compound will be contacted with the biological system in a form and for a time that allows the test compound to be taken up by the biological system. The test compound may be contacted with the biological system in any form or state that allows the test compound to inhibit biotinylation in the biological system. Alternatively, and if feasible, the test compound may be expressed within a cell. An appropriate concentration of the test compound will be selected for determination of the test compound to inhibit biotinylation at that particular concentration.

The identification of the test compound as an inhibitor of a biotinylation in the biological system will be made by a comparison of the extent of biotinylation in the biological system in the presence and absence of the test compound. Preferably, the maximum extent of inhibition of activity by the test compound is greater than 70%. More preferably, the maximum extent of inhibition of activity is greater than 90%.

It has been additionally determined that the present invention may be used to identify compounds that are capable of differentially inhibiting biotin protein ligases from different species. In particular, it has been determined that it is possible to identify compounds that inhibit a biotin protein ligase from a pathogen of a human more effectively than the inhibition of human biotin protein ligase.

As such, these compounds are candidate compounds that may be used to treat an organism for infection by a pathogenic organism.

Accordingly, in another form the present invention provides a method for identifying a compound that differentially inhibits a first biotin protein ligase as compared to inhibition of a second biotin protein ligase, the method including the steps of:

(a) identifying a test compound that inhibits a first biotin protein ligase;

(b) determining the ability of the test compound to inhibit a second biotin protein ligase; and

(c) identifying the test compound as a compound that differentially inhibits the first biotin protein ligase as compared to the inhibition of the second biotin protein ligase.

As will be appreciated, this form of the present invention is directed to the identification of inhibitors that differentially inhibit biotin protein ligases derived from different organisms. Inhibitors so identified are candidate compounds for differentially inhibiting the growth and/or survival of one organism compared to another organism.

In another embodiment, the present invention provides the use of a biotin protein ligase as a target for identifying an agent that differentially inhibits growth of a first organism as compared to growth of a second organism.

In another form, the present invention provides a method for identifying an agent that differentially inhibits growth of a first organism as compared to a second organism, the method including the step of identifying an agent that differentially inhibits a biotin protein ligase from the first organism as compared to a biotin protein ligase from the second organism.

As discussed previously herein, given the high degree of conservation between biotin protein ligases of different species, the ability to identify compounds that differentially inhibit biotin protein ligases of different organisms was contrary to expectation.

Preferably, the differential inhibition is such that the first biotin protein ligase is inhibited and the second biotin protein ligase is not substantially inhibited.

Preferably, the first biotin protein ligase is a biotin protein ligase from an organism that is a pathogen of the organism from which the second biotin protein ligase is derived. More preferably, the first biotin protein ligase is derived from a pathogen of a human, animal or plant, and the second biotin protein ligase is isolated from a host of the pathogen. Examples of pathogenic organisms are as previously hereinbefore described.

Accordingly, in another form the present invention provides a method for identifying a compound that differentially inhibits a biotin protein ligase of a pathogenic organism of a host human, animal or plant, as compared to inhibition of a biotin protein ligase of the host human, animal, or plant, the method including the steps of:

(a) identifying a test compound that inhibits a biotin protein ligase of a pathogenic organism of a host human, animal or plant;

(b) determining the ability of the test compound to inhibit a biotin protein ligase of the host human, animal or plant; and

(c) identifying the test compound as a compound that differentially inhibits the biotin protein ligase of the pathogenic organism as compared to the inhibition of the biotin protein ligase of the host human, animal or plant.

For example, in the case of the second biotin protein ligase being derived from a human or animal, the first biotin protein ligase may be derived from a bacterium, a fungus or a parasite. In the case of the second biotin protein ligase being derived from a plant, the first biotin protein ligase may be derived from a bacterium, a fungus, an insect or a nematode.

Preferably, the first biotin protein ligase is derived from a bacteria or fungus, and the second biotin protein ligase is derived from a human or animal. Most preferably, the first biotin protein ligase is derived from a fungus such a *Candida* or yeast species, or a

bacterium such as *E.coli* or *S. aureus*, and the second biotin protein ligase is derived from a human or animal.

The test compound may be identified as a compound capable of differentially inhibiting the first biotin protein ligase as compared to the second biotin protein ligase, by comparing the inhibition of each of the biotin protein ligases in the presence and absence of the test compound. An inhibitor that inhibits the first biotin protein ligase in the presence of the test compound as compared to the absence of the test compound, but which does not inhibit the second biotin protein ligase to the same extent, will be identified as a suitable compound.

Preferably, the test compound inhibits the activity of the first biotin protein ligase by a maximum of 70% or greater. More preferably, the test compound inhibits the activity of the first biotin protein ligase by a maximum of 90% or greater.

Preferably, the inhibitor does not inhibit the activity of a second biotin protein ligase by more than 10%. More preferably, the inhibitor does not inhibit the activity of the second biotin protein ligase by more than 5%.

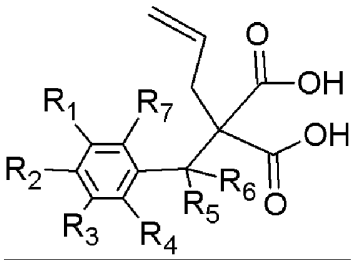
The present invention also provides a differential inhibitor identified by the relevant methods for the present invention. Accordingly, the present invention also provides an agent that differentially inhibits a first biotin protein as compared to a second biotin protein ligase.

The differential inhibitor may inhibit a biotin protein ligase of a pathogen of a human, animal or plant, and not substantively inhibit a human, animal or plant biotin protein ligase. Accordingly, in another form the present invention provides an inhibitor of a biotin protein ligase of a pathogen of a human, animal or plant, wherein the inhibitor does not substantively inhibit a human, plant or animal biotin protein ligase.

Examples of pathogenic organisms are as previously hereinbefore described.

For example, the present invention may be used to identify a compound that inhibits a bacterial biotin protein ligase and which does not substantively inhibit a human, plant or animal biotin protein ligase. Accordingly, in another form the present invention provides an inhibitor of a bacterial biotin protein ligase, wherein the inhibitor does not substantively inhibit a human, plant or animal biotin protein ligase.

In this regard, the screening method for the present invention has been used to identify a compound that inhibits *E.coli* (IC_{50}= 0.9 nM) and *S. aureus* (IC_{50}= 2.7 μM) biotin protein ligases, but which does not substantively inhibit human biotin protein ligase (IC_{50}>50 μM), with the following formula:



or a salt or ester thereof, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl, arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino, sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-, $R_9N(R_{10})SO_2R_{11}$-, $R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl,

heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

In a preferred embodiment, the compound is 2-allyl-2-(3,5-dimethoxybenzyl)malonic acid, or a salt thereof .

The present invention may be used to identify a compound that inhibits a fungal biotin protein ligase and which does not substantively inhibit a human, plant or animal biotin protein ligase. Accordingly, in another form the present invention provides an inhibitor of a fungal biotin protein ligase, wherein the inhibitor does not substantively inhibit a human, plant or animal biotin protein ligase.

The present invention also provides a method for identifying a compound that differentially inhibits biotinylation in a first biological system as compared to inhibition of biotinylation in a second biological system, the method including the steps of:

(a) identifying a test compound that inhibits biotinylation in a first biological system;

(b) determining the ability of the test compound to inhibit biotinylation in a second biological system; and

(c) identifying the test compound as a compound that differentially inhibits biotinylation in the first biological system as compared to the inhibition of biotinylation in the second biological system.

As will be appreciated, this form of the present invention is directed to the identification of inhibitors that differentially inhibit biotinylation in a first biological system as compared to a second biological system. Inhibitors so identified are candidate compounds for differentially inhibiting the growth of one organism compared to another organism.

Preferably, the differential inhibition is such that the biotinylation in the first biological system is inhibited and biotinylation in the second biological system is not substantially inhibited.

Preferably, the first biological system is a biological system derived from an organism that is a pathogen of the organism from which the second biological system is derived. More preferably, the first biological system is derived from a pathogen of a human, animal or plant, and the second biological system is isolated from the host of the pathogen of the first biological system.

Accordingly, in another form the present invention provides a method for identifying a compound that differentially inhibits biotinylation in a pathogenic organism of a host human, animal or plant, as compared to inhibition of biotinylation in the host human, animal or plant, the method including the steps of:

(a) identifying a test compound that inhibits biotinylation in a pathogenic organism of a host human, animal or plant;

(b) determining the ability of the test compound to inhibit biotinylation in the host human, animal or plant; and

(c) identifying the test compound as a compound that differentially inhibits biotinylation in the pathogenic organism as compared to the inhibition of biotinylation in the host human, animal or plant.

For example, in the case of the second biological system being derived from a human or animal, the first biological system may be derived from a bacteria, fungus or parasite. In the case of the second biological system being derived from a plant, the first biological system may be derived from a bacteria, fungus, insect or nematode.

More preferably, the first biological system is derived from a bacteria or a fungus, and the second biological system is derived from a human or animal.

The test compound may be identified as a compound capable of differentially inhibiting biotinylation in the first biological system as compared to the second biological system, by methods as previously described.

The test compound may be identified as a compound capable of differentially inhibiting biotinylation in a first biological system as compared to a second biological system, by comparing the inhibition of biotinylation for each of the biological systems in the presence and absence of the test compound. A compound that inhibits the biotinylation

in the first biological system in the presence of the test compound as compared to the absence of the test compound, and which does not inhibit biotinylation in a second biological system to the same extent, will be identified as a suitable compound.

Preferably, the test compound inhibits biotinylation in the first biological system by 70% or greater. More preferably, the test compound inhibits biotinylation in the first biological system by 90% or greater.

Preferably, the test compound does not inhibit biotinylation in the second biological system by more than 10%. More preferably, the test compound does not inhibit biotinylation in the second biological system by more than 5%.

Differential inhibitors identified by the various methods for the present invention may be used to prevent and/or treat the growth of a pathogenic organism in a subject.

For example, the differential inhibitors may be used to prevent and/or treat a human, animal or plant subject having a local and/or systemic infection by a pathogenic organism, by inhibiting the activity of a biotin protein ligase of the pathogenic organism.

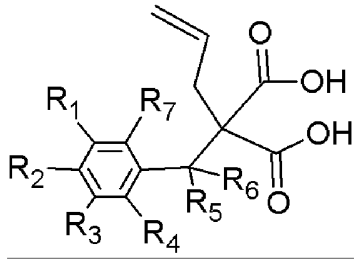
Accordingly, in another form the present invention provides a method for preventing and/or treating an infection by a pathogenic organism of a subject, the method including the step of administering to the subject an effective amount of an agent that inhibits a biotin protein ligase of the pathogenic organism.

This form of the present invention results in biotinylation in the pathogenic organism being inhibited, thus resulting in an inhibition of growth and/or survival of the pathogenic organism in the subject.

Accordingly, in another the present invention provides a method for preventing and/or treating an infection by a pathogenic organism of a subject, the method including the step of administering to the subject an effective amount of an agent that inhibits biotinylation in the pathogenic organism.

Examples of agents include drugs, small molecules, nucleic acids, oligonucleotides, peptides, polypeptides, proteins, enzymes, polysaccharides, glycoproteins, lipids, antibodies or a part thereof, and aptamers.

In particular preferred form, the agent administered is a compound with the following chemical formula:



or an acceptable salt or ester thereof, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl, arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino, sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-, $R_9N(R_{10})SO_2R_{11}$-, $R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

In a preferred embodiment, the compound is 2-allyl-2-(3,5-dimethoxybenzyl)malonic acid, or an acceptable salt thereof .

This compound may be synthesized as described in Zhao, Ya Jun; Tang, Hui Tong; Zhang, Pang (1997) "A study on cycloacylation related to synthesis of perylenequinones." *Chinese Chemical Letters* 8(9): 769-772.

In another form, the present invention provides a composition including the above agents for the prevention and/or treatment of an infection of a host by a pathogenic organism, and the use of such agents for the preparation of a medicament for preventing and/or treating an infection of a host by a pathogenic organism.

In one form the present invention provides a composition for preventing and/or treating method an infection of a host by a pathogenic organism of a subject, the composition including an effective amount of an agent that inhibits a biotin protein ligase of the pathogenic organism.

In another form the present invention provides a composition for preventing and/or treating an infection of a host by a pathogenic organism, the composition including an effective amount of an agent that inhibits biotinylation in the pathogenic organism.

In the case of administration of an agent to a human or animal subject (ie host), the agent may be administered to the subject in a suitable form to inhibit the activity of the biotin protein ligase in the pathogenic organism. The effective amount of agent to be administered is not particularly limited, so long as it is within such an amount and in such a form that generally exhibits a useful or therapeutic effect.

In this regard, an effective amount of the agent may be appropriately chosen, depending upon, for example, the type and extent of infection by the pathogenic organism, the age and body weight of the subject, the frequency of administration, and the presence of other active agents.

The administration of the agent may be within any time suitable to produce the desired effect of preventing and/or treating infection by the pathogenic organism.

The agent may be administered orally, parenterally, topically or by any other suitable means, and therefore transit time of the agent must be taken into account.

The administration of the agent may also include the use of one or more pharmaceutically acceptable additives, including pharmaceutically acceptable salts, amino acids, polypeptides, polymers, solvents, buffers, excipients and bulking agents, taking into consideration the particular physical and chemical characteristics of the agent to be administered.

For example, the agent can be prepared into a variety of pharmaceutical compositions in the form of, e.g., an aqueous solution, an oily preparation, a fatty emulsion, an emulsion, a gel, etc., and these preparations can be administered as intramuscular or subcutaneous injection or as injection to an organ, or as an embedded preparation or as a transmucosal preparation through nasal cavity, rectum, uterus, vagina, lung, etc. The composition may be administered in the form of oral preparations (for example solid preparations such as tablets, capsules, granules or powders; liquid preparations such as syrup, emulsions or suspensions). Compositions containing the agent may also contain a preservative, stabiliser, dispersing agent, pH controller or isotonic agent. Examples of suitable preservatives are glycerin, propylene glycol, phenol or benzyl alcohol. Examples of suitable stabilisers are dextran, gelatin, a-tocopherol acetate or alpha-thioglycerin. Examples of suitable dispersing agents include polyoxyethylene (20), sorbitan mono-oleate (Tween 80), sorbitan sesquioleate (Span 30), polyoxyethylene (160) polyoxypropylene (30) glycol (Pluronic F68) or polyoxyethylene hydrogenated castor oil 60. Examples of suitable pH controllers include hydrochloric acid, sodium hydroxide and the like. Examples of suitable isotonic agents are glucose, D-sorbitol or D-mannitol.

The administration of the agent in the various forms of the present invention may also be in the form of a composition containing a pharmaceutically acceptable carrier, diluent, excipient, suspending agent, lubricating agent, adjuvant, vehicle, delivery system, emulsifier, disintegrant, absorbent, preservative, surfactant, colorant, flavorant or sweetener, taking into account the physical and chemical properties of the agent being administered.

For these purposes, the composition may be administered orally, parenterally, by inhalation spray, adsorption, absorption, topically, rectally, nasally, bucally, vaginally, intraventricularly, via an implanted reservoir in dosage formulations containing conventional non-toxic pharmaceutically-acceptable carriers, or by any other convenient dosage form. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intraperitoneal, intrathecal, intraventricular, intrasternal, and intracranial injection or infusion techniques.

When administered parenterally, the composition will normally be in a unit dosage, sterile injectable form (solution, suspension or emulsion) which is preferably isotonic with the blood of the recipient with a pharmaceutically acceptable carrier. Examples of such sterile injectable forms are sterile injectable aqueous or oleaginous suspensions. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable forms may also be sterile injectable solutions or suspensions in non-toxic parenterally-acceptable diluents or solvents, for example, as solutions in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, saline, Ringer's solution, dextrose solution, isotonic sodium chloride solution, and Hanks' solution. In addition, sterile, fixed oils are conventionally employed as solvents or suspending mediums. For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides, corn, cottonseed, peanut, and sesame oil. Fatty acids such as ethyl oleate, isopropyl myristate, and oleic acid and its glyceride derivatives, including olive oil and castor oil, especially in their polyoxyethylated versions, are useful in the preparation of injectables. These oil solutions or suspensions may also contain long-chain alcohol diluents or dispersants.

The carrier may contain minor amounts of additives, such as substances that enhance solubility, isotonicity, and chemical stability, for example anti-oxidants, buffers and preservatives.

When administered orally, the agent will usually be formulated into unit dosage forms such as tablets, cachets, powder, granules, beads, chewable lozenges, capsules, liquids, aqueous suspensions or solutions, or similar dosage forms, using conventional equipment and techniques known in the art. Such formulations typically include a solid,

semisolid, or liquid carrier. Exemplary carriers include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, mineral oil, cocoa butter, oil of theobroma, alginates, tragacanth, gelatin, syrup, methyl cellulose, polyoxyethylene sorbitan monolaurate, methyl hydroxybenzoate, propyl hydroxybenzoate, talc, magnesium stearate, and the like.

A tablet may be made by compressing or molding the agent optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, surface active, or dispersing agent. Moulded tablets may be made by moulding in a suitable machine, a mixture of the powdered active ingredient and a suitable carrier moistened with an inert liquid diluent.

The administration of the agent may also utilize controlled release technology. The agent may also be administered as a sustained-release pharmaceutical. To further increase the sustained release effect, the agent may be formulated with additional components such as vegetable oil (for example soybean oil, sesame oil, camellia oil, castor oil, peanut oil, rape seed oil); middle fatty acid triglycerides; fatty acid esters such as ethyl oleate; polysiloxane derivatives; alternatively, water-soluble high molecular weight compounds such as hyaluronic acid or salts thereof (weight average molecular weight: ca. 80,000 to 2,000,000), carboxymethylcellulose sodium (weight average molecular weight: ca. 20,000 to 400,000), hydroxypropylcellulose (viscosity in 2% aqueous solution: 3 to 4,000 cps), atherocollagen (weight average molecular weight: ca. 300,000), polyethylene glycol (weight average molecular weight: ca. 400 to 20,000), polyethylene oxide (weight average molecular weight: ca. 100,000 to 9,000,000), hydroxypropylmethylcellulose (viscosity in 1% aqueous solution: 4 to 100,000 cSt), methylcellulose (viscosity in 2% aqueous solution: 15 to 8,000 cSt), polyvinyl alcohol (viscosity: 2 to 100 cSt), polyvinylpyrrolidone (weight average molecular weight: 25,000 to 1,200,000).

Alternatively, the agent may be incorporated into a hydrophobic polymer matrix for controlled release over a period of days. The agent may then be moulded into a solid implant, or externally applied patch, suitable for providing efficacious concentrations of

the agent over a prolonged period of time without the need for frequent re-dosing. Such controlled release films are well known to the art. Other examples of polymers commonly employed for this purpose that may be used include nondegradable ethylene-vinyl acetate copolymer a degradable lactic acid-glycolic acid copolymers which may be used externally or internally. Certain hydrogels such as poly(hydroxyethylmethacrylate) or poly(vinylalcohol) also may be useful, but for shorter release cycles than the other polymer release systems, such as those mentioned above.

The carrier may also be a solid biodegradable polymer or mixture of biodegradable polymers with appropriate time release characteristics and release kinetics. The agent may then be moulded into a solid implant suitable for providing efficacious concentrations of the agent over a prolonged period of time without the need for frequent re-dosing. The agent can be incorporated into the biodegradable polymer or polymer mixture in any suitable manner known to one of ordinary skill in the art and may form a homogeneous matrix with the biodegradable polymer, or may be encapsulated in some way within the polymer, or may be moulded into a solid implant.

For topical administration, the composition of the present invention may be in the form of a solution, spray, lotion, cream (for example a non-ionic cream), gel, paste or ointment. Alternatively, the composition may be delivered via a liposome, nanosome, rivosome, or nutri-diffuser vehicle. Topical administration is particularly suitable for local infections by a pathogenic organism.

A cream is a formulation that contains water and oil and is stabilized with an emulsifier. Lipophilic creams are called water-in-oil emulsions, and hydrophilic creams oil-in-water emulsions. The cream base for water-in-oil emulsions are normally absorption bases such as vaseline, ceresin or lanolin. The bases for oil-in-water emulsions are mono-, di- and triglycerides of fatty acids or fatty alcohols with soaps, alkyl sulphates or alkyl polyglycol ethers as emulsifiers.

A lotion is an opaque, thin, non-greasy emulsion liquid dosage form for external application to the skin, which generally contains a water-based vehicle with greater than 50% of volatiles and sufficiently low viscosity that it may be delivered by pouring.

Lotions are usually hydrophilic, and contain greater than 50% of volatiles as measured by LOD (loss on drying). A lotion tends to evaporate rapidly with a cooling sensation when rubbed onto the skin.

A paste is an opaque or translucent, viscous, greasy emulsion or suspension semisolid dosage form for external application to the skin, which generally contains greater than 50% of hydrocarbon-based or a polyethylene glycol-based vehicle and less than 20% of volatiles. A paste contains a large proportion (20-50%) of dispersed solids in a fatty or aqueous vehicle. An ointment tends not to evaporate or be absorbed when rubbed onto the skin.

An ointment is an opaque or translucent, viscous, greasy emulsion or suspension semisolid dosage form for external application to the skin, which generally contains greater than 50% of hydrocarbon-based or a polyethylene glycol-based vehicle and less than 20% of volatiles. An ointment is usually lipophilic, and contains > 50% of hydrocarbons or polyethylene glycols as the vehicle and < 20% of volatiles as measured by LOD. An ointment tends not to evaporate or be absorbed when rubbed onto the skin.

A gel is usually a translucent, non-greasy emulsion or suspension semisolid dosage form for external application to the skin, which contains a gelling agent in quantities sufficient to impart a three-dimensional, cross-linked matrix. A gel is usually hydrophilic, and contains sufficient quantities of a gelling agent such as starch, cellulose derivatives, carbomers, magnesium-aluminium silicates, xanthan gum, colloidal silica, aluminium or zinc soaps.

The composition for topical administration may further include drying agents, anti-foaming agents; buffers, neutralizing agents, agents to adjust pH; colouring agents and decolouring agents; emollients; emulsifying agents, emulsion stabilizers and viscosity builders; humectants; odorants; preservatives, antioxidants, and chemical stabilizers; solvents; and thickening, stiffening, and suspending agents, and a balance of water or solvent.

It should also be appreciated that other methods for delivery of an agent are contemplated. For example, the agent may be delivered by way of a nucleic acid or vector that allows for expression of the agent in the appropriate target cells. For example, the agent may be delivered by way of a viral vector that causes expression of the agent in target cells.

Methods are also known in the art for administering an agent to a plant to prevent and/or treat an infection by a pathogenic organism. For example, the plant may be treated for a systemic infection by a pathogen, a local infection by a pathogen, or seeds may be treated to remove surface pathogens. As will be appreciated by a person skilled in the art, the formulation of the agent will vary depending upon the particular treatment or prevention regime adopted.

The present invention also provides an isolated nucleic acid sequence encoding a biotin protein ligase or a fragment thereof, wherein the nucleic acid includes at least one codon that is modified from the wild type nucleic acid sequence encoding the biotin protein ligase or fragment thereof.

Preferably, the isolated nucleic acid encodes a biotin protein ligase (or a part thereof) from a biotin protein ligase of a pathogenic organism of a human, plant or animal, such as a bacteria, fungi, parasite, insect or nematode. More preferably, the isolated nucleic acid sequence encodes a biotin protein ligase from a fungus. More preferably, the nucleic acid encodes a biotin protein ligase from a *Candida* species. Most preferably, the nucleic acid encodes a biotin protein ligase from *Candida albicans*.

Modifications to the at least one codon include modifications to change the identity of the amino acid encoded by the codon, or modifications to change the sequence of a codon encoding the same amino acid.

In this regard, it has been found that the expression and activity of biotin protein ligase expressed in bacteria from a nucleic acid sequence encoding a biotin protein ligase from *Candida* may be enhanced by modifying a CUG codon (which normally encodes a leucine amino acid) to a codon specifying a serine amino acid (ie UCU, UCC, UCG, UCA, AGU, AGC).

Accordingly, in a preferred form the present invention provides an isolated nucleic acid encoding a *Candida* biotin protein ligase or a fragment thereof, wherein the nucleic acid includes at least one codon specifying a serine amino acid serine as a replacement for a CUG codon in the corresponding position of the wildtype sequence of the biotin protein ligase.

Preferably the codon specifying the amino acid serine is an AGU codon.

The modifications to the at least one codon may be made by a suitable method known in the art, including the use of site-directed mutagenesis, PCR using primers having the desired mutation or mutations incorporated therein, or by random mutagenesis, such methods as essentially described in Sambrook, J., Fritsch, E.F., and Maniatis, T., in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989). The nucleic acid sequence may be isolated by a suitable method known in the art. The isolated nucleic acid may encode an entire biotin protein ligase containing at least one codon that is modified from the wild type nucleic acid sequence, or part of a biotin protein ligase containing at least one codon that is modified from the wild type nucleic acid sequence.

The isolated nucleic acid may be further cloned into a plasmid for expression of the cloned biotin protein ligase. The plasmid encoding the cloned biotin protein ligase may then be transformed or transfected into a prokaryotic or eukaryotic cell for expression of the cloned biotin protein ligase.

The present invention also provides an isolated polypeptide fragment of a human or *Candida* pyruvate carboxylase, wherein the fragment is capable of biotinylation by a biotin protein ligase.

In this regard, it has been found that isolated polypeptide fragments of a human or *Candida* pyruvate carboxylase may be produced that still retain the capacity to be biotinylated by a biotin protein ligase.

In the case of an isolated polypeptide fragment of human pyruvate carboxylase, preferably the isolated polypeptide fragment of human pyruvate carboxylase includes (or consists of) the following amino acid sequence (or a functional variant thereof):

M^{1098}HFHPKALKDVKGQIGAPMPGKVIDIKVVAGAKVAKGQPLCVLSAMKMET VVTSPMEGTVRKVHVTKDMTLEGDDLILEIE^{1178} (SEQ ID No. 9)

Most preferably, the isolated polypeptide fragment of human pyruvate carboxylase includes (or consists of) one of the following amino acid sequences (or a functional variant thereof):

hPC-108:
A^{1071}GQRQVFFELNGQLRSILVKDTQAMKEMHFHPKALKDVKGQIGAPMPGKV IDIKVVAGAKVAKGQPLCVLSAMKMETVVTSPMEGTVRKVHVTKDMTLEGD DLILEIE^{1178} (SEQ ID No. 10)

hPC-81:
M^{1098}HFHPKALKDVKGQIGAPMPGKVIDIKVVAGAKVAKGQPLCVLSAMKMET VVTSPMEGTVRKVHVTKDMTLEGDDLILEIE^{1178} (SEQ ID No.11)

In the case of an isolated polypeptide fragment of a *Candida* pyruvate carboxylase, preferably the polypeptide fragment is from *Candida albicans*. Most preferably, the isolated polypeptide fragment includes (or consists of) the following amino acid sequence (or a functional variant thereof):

K^{909}ASASNEVGAPMAGVVIEIRAHKHQQIAKGDPIAVLSAMKMEMVISAPCSGE IGDILIHEGDSVDANDLITSIH^{982} (SEQ ID No.12)

More preferably, the isolated polypeptide fragment of *Candida* pyruvate carboxylase includes (or consists of) of one of the following amino acid sequences (or a functionally variant thereof):

caPC-115:
M[868]AVGDVSEKTGTREVFFELNGEMRSVSVEDKTVSVELKTRPKASASNEVGA
PMAGVVIEIRAHKHQQIAKGDPIAVLSAMKMEMVISAPCSGEIGDILIHEGDSVD
ANDLITSIH[982] (SEQ ID No. 13)

caPC-93:
M[890]RSVSVEDKTVSVELKTRPKASASNEVGAPMAGVVIEIRAHKHQQIAKGDPI
AVLSAMKMEMVISAPCSGEIGDILIHEGDSVDANDLITSIH[982] (SEQ ID No. 14)

caPC-74:
K[909]ASASNEVGAPMAGVVIEIRAHKHQQIAKGDPIAVLSAMKMEMVISAPCSGE
IGDILIHEGDSVDANDLITSIH[982] (SEQ ID No.15)

The polypeptides may be produced by methods known in the art using chemical synthesis or by expression and purification of the appropriate cloned nucleotide sequence in an appropriate expression system. In the case of expression and purification of the polypeptides, preferably the polypeptides are fused to a moiety to facilitate purification of the polypeptides, such as the fusion of the polypeptides with GST or the addition of a six histidine tag.

Confirmation of the ability of an isolated polypeptide to be biotinylated by a biotin protein ligase may be determined by a suitable method. For example, the isolated polypeptide may be used as a substrate in an *in vitro* reaction with biotin and biotin protein ligase, and the extent of biotinylation of the isolated polypeptide determined. Alternatively, the polypeptide may be expressed in a cell and the extent of biotinylation by the endogenous biotin protein ligase (or a cloned biotin protein ligase expressed in the cell) may be determined by a suitable method known in the art.

Substrates that are efficiently biotinlyated by biotin protein ligase, as compared to the biotinylation of a known substrate of a biotin protein ligase, may then be identified.

An example of a suitable reaction mix for determination of the ability of an isolated polypeptide to be biotinylated *in vitro* is as follows:

The isolated polypeptide may be adsorbed onto wells (eg Griener Lumitrac 600 White 96-well plates; Stennick Scientific) by coating overnight at 4°C in 100 uL of Tris Buffered saline (TBS, pH 7.5). Wells may then be blocked in 1% BSA solution in TBS for 1 hr at 37°C, the blocking buffer removed and wells washed in TBS buffer containing 0.1% Tween-20. BPL reaction mix may then be added to each well. The BPL reaction mix is 50 mM Tris-HCl pH 8.0, 100 uM ATP, 5.5 mM $MgCl_2$, 0.1 mg/ml BSA, 10 uM biotin, 0.1 uM dithiothreitol. BPL reactions are initiated by the addition of enzyme to a final concentration of 1-5 nM. The reaction is then allowed to proceed at 37°C for 15 minutes at which point it is terminated by the addition of 5 ul 0.5 M EDTA (final concentration 25 mM). The reaction mix may then be discarded and wells washed in TBS-TD (TBS, 0.1% Tween-20 and 100 uM diethylenetriaminepentaacetic acid (DPTA)). The quantitative analysis of biotinylated protein formed in the reaction and coupled onto the well surface may then be determined using time resolved fluorescence. Europium labelled streptavidin (Perkin-Elmer) solution, diluted to 0.1 ug/ml in TBS-TD, is added to each well. The plates may then be incubated at 37°C for 1 hr before being washed in TBS-TD and in water. DELFIA Enhancement solution (Perkin Elmer) may then be added and incubated for ten minutes at room temperature before quantitation.

Description of the Preferred Embodiments

Reference will now be made to experiments that embody the above general principles of the present invention. However, it is to be understood that the following description is not to limit the generality of the above description.

Example 1

Cloning the biotin domains of Candida albicans and human pyruvate carboxylase

Biotin protein ligases recognise a structured biotin domain and covalently modify a specific target lysine in the folded substrate. The ability of a number of peptides to function as a substrate for the endogenous biotin protein ligase in *E. coli* was determined using an *in vivo* biotinylation assay.

Two polypeptides (hPC-108 and hPC-81) containing the biotin domain from human pyruvate carboxylase were used. Three polypeptides (caPC-115, caPC-93 and caPC-74) from *Candida albicans* pyruvate carboxylase were also used.

Peptides encompassing predicted biotin domains of human and *C. albicans* pyruvate carboxylase were expressed as fusions to GST to facilitate high-level expression of each sequence and the quantitation of protein yields by Western blot. Biotinylation of the substrates was determined by Western-blot using the biotin-binding protein streptavidin as a probe.

The amino acid sequence for each of the human and *Candida* peptides are as follows (amino acid positions indicated are the relative position of the corresponding amino acid in the sequence of the full-length protein):

hPC-108:
A^{1071}GQRQVFFELNGQLRSILVKDTQAMKEMHFHPKALKDVKGQIGAPMPGKV IDIKVVAGAKVAKGQPLCVLSAMKMETVVTSPMEGTVRKVHVTKDMTLEGD DLILEIE^{1178} (SEQ ID No. 16)

hPC-81:
M^{1098}HFHPKALKDVKGQIGAPMPGKVIDIKVVAGAKVAKGQPLCVLSAMKMET VVTSPMEGTVRKVHVTKDMTLEGDDLILEIE^{1178} (SEQ ID No. 17)

caPC-115:
M^{868}AVGDVSEKTGTREVFFELNGEMRSVSVEDKTVSVELKTRPKASASNEVGA PMAGVVIEIRAHKHQQIAKGDPIAVLSAMKMEMVISAPCSGEIGDILIHEGDSVD ANDLITSIH^{982} (SEQ ID No. 18)

caPC-93:
M^{890}RSVSVEDKTVSVELKTRPKASASNEVGAPMAGVVIEIRAHKHQQIAKGDPI AVLSAMKMEMVISAPCSGEIGDILIHEGDSVDANDLITSIH^{982} (SEQ ID No. 19)

caPC-74:
K^{909}ASASNEVGAPMAGVVIEIRAHKHQQIAKGDPIAVLSAMKMEMVISAPCSGE IGDILIHEGDSVDANDLITSIH^{982} (SEQ ID No. 20)

To construct plasmids suitable for expressing the C-terminal 74, 93 and 115 amino acids of *C. albicans* pyruvate carboxylase, the DNA encoding each peptide was amplified using PCR with genomic DNA. Sequence data for *Candida albicans* pyruvate carboxylase was obtained from the Stanford Genome Technology Centre (orf6.2989 orf6-2189:1646-4594).

To amplify DNA for the 74 amino acid peptide (caPC-74) from *Candida albicans* pyruvate carboxylase, the following oligonucleotides were used:
C18/38 [5'ATCTACGGATCCAAAGCTTCAGCATCAAATGAAGTTGG] (SEQ ID No. 21;
C19/43 [5'ATCTACGAATTCATCAATGAATACTAGTAATCAAATCATTAGC] (SEQ ID No. 22)

To amplify DNA for the 93 amino acid peptide (caPC-93) from *Candida albicans* pyruvate carboxylase, the following oligonucleotides were used:
C17/34 [5'ATCTACGGATCCATGAGATCAGTTTCCGTTGAAG] (SEQ ID No. 23);
C19/43 [5'ATCTACGAATTCATCAATGAATACTAGTAATCAAATCATTAGC] (SEQ ID No. 24)

To amplify DNA for the 115 amino acid peptide (caPC-115) from *Candida albicans* pyruvate carboxylase, the following oligonucleotides were used:
C20/28 [5'GGATCCATGGCTGTTGGTGATGTTTCGG] (SEQ ID No. 25);
C19/43 [5'ATCTACGAATTCATCAATGAATACTAGTAATCAAATCATTAGC] (SEQ ID No. 26)

To amplify the above DNAs, PCR was performed with 2.5 units *PfuTurbo* DNA polymerase (Stratagene) in 200 uM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X100 and 0.1 mg/ml BSA. Between 0.1 and 0.5 uM of the required oligonucleotide primers were included along

with 1 ug of genomic DNA as the template. Thermocycling conditions were 30 cycles of 92°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

The above oligonucleotides introduced a *BamH*1 restriction site at the 5' end of the product and an *Eco*R1 site at the 3' end. The PCR products were digested with *BamH*1 and *Eco*R1 restriction endonucleases and ligated into similarly treated pGEX-4T-2 plasmid (Amersham-Biosciences).

To construct plasmids suitable for expressing the C-terminal 81 and 108 amino acids of human pyruvate carboxylase, the DNA encoding each peptide was amplified using PCR with genomic DNA. The sequence data required for cloning human pyruvate carboxylase was obtained from GenBank accession number U30891.

To construct expression vectors suitable for expressing the C-terminal 81 and 108 amino acids of human pyruvate carboxylase, the DNA encoding each peptide was amplified using PCR from plasmid pEF-PC, which contains the full-length cDNA encoding human pyruvate carboxylase, as described in Hobbs *et al.* (1998) *Biochem Biophys Res Commun.* 252(2):368-272.

To amplify DNA for the 81 amino acid peptide (hPC-81) from human pyruvate carboxylase, the following oligonucleotides were used:
81F [5'AATTGGTAGAATTCATGCACTTCCACCCCAAGGCC] (SEQ ID No. 27);
Pcrev [5'GATTAATTCTCGAGTTATCACTCGATCTCCAGGATGAGGTCG] (SEQ ID No. 28)

To amplify DNA for the 108 amino acid peptide (hPC-81) from human pyruvate carboxylase, the following oligonucleotides were used:
108F [5'AATTGGTAGAATTCGCCGGCCAGAGGCAGGTCTTC] (SEQ ID No. 29);
Pcrev [5'GATTAATTCTCGAGTTATCACTCGATCTCCAGGATGAGGTCG] (SEQ ID No. 30)

To amplify the above DNAs, PCR was performed with 2.5 units *PfuTurbo* DNA polymerase (Stratagene) in 200 uM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X100 and 0.1 mg/ml BSA.

Between 0.1 and 0.5 uM of the required oligonucleotide primers were included along with either 1 ng of pEF-PC plasmid containing the target sequence. Thermocycling conditions were 25 cycles of 92°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

The above oligonucleotides introduced an *Eco*R1 restriction site at the 5' end of the product and a *Xho*1 site at the 3' end. The PCR products were digested with *Eco*R1 and *Xho*1 restriction endonucleases and ligated into similarly treated pGEX-4T-2, essentially as described in Sambrook, J., Fritsch, E.F., and Maniatis, T., in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989).

Example 2

Analysis of peptide fragments from C. albicans and human pyruvate carboxylase as biotin protein ligase substrates

The ability of each of the *Candida* and human peptides to function as a substrate for biotin protein ligase *in vivo* was performed in *E. coli*. Each pGEX-4T-2 derived construct was transformed into *E. coli* BL21 cells by $CaCl_2$ mediated transformation and colonies grown in 2YT media until log phase. Expression of the GST fusion proteins was induced with 0.1 mM IPTG for 1 hour at 37°C before cells were harvested.

Biotinylation of the fusion proteins was assayed on whole cell lysates by streptavidin-blot. Whole cell lysates were prepared using the method essentially as described in Chapman-Smith et al. (1994) *Biochem J.* 302, 881-887. Proteins were transferred to nitrocellulose, as described by Sambrook, J., Fritsch, E.F., and Maniatis, T., in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989), and biotin containing peptides detected, essentially as described by Salto *et al.* (1999) *Mol Cell Biochem* 200:111-7. Expression of each construct was determined by anti-GST Western blot following manufacturer's instructions (Amersham Bioscience).

The results of the streptavidin and anti-GST blots for the human pyruvate carboxylase fragments are shown in Figure 2a. The results of the streptavidin and anti-GST blots for the *Candida* pyruvate carboxylase fragments are shown in Figure 2c. Products of the predicted size were detected by both Western using anti-GST antibodies or by steptavidin blot.

Quantitation of the Western Blots for the human pyruvate carboxylase fragments was performed using NIH Image software version 1.62. The relative biotinylation ratio was calculated by dividing the signal from the streptavidin blot by the signal from the anti-GST Western blot. The results are shown in Figure 2B.

As can be seen from the data shown in Figure 2, GST alone showed no evidence of biotin incorporation. However the two human peptides, hPC-81 and hPC-108, were biotinylated (Figure 2A) implying enough information was contained in the 81 amino acid fragment for correct protein folding. Quantitation of biotinylation, calculated relative to the level of fusion protein expression, revealed that both peptides were equal BPL substrates *in vivo*. (Figure 2B)

Similarly, all three peptides from *C. albicans* pyruvate carboxylase were equivalent biotin protein ligase substrates in the *in vivo* assay (Figure 2C). As can be seen from the data, the peptides from both *Candida* and human were efficiently biotinylated *in vivo*.

Example 3

Purification of biotin domains

Each peptide from *Candida* and human was expressed as a GST fusion protein permitting both high level expression in *E. coli* and rapid purification by affinity chromatography. To express the fusion proteins for purification, bacterial cultures of BL21 harbouring the pGEX-4T-2 based expression vectors were grown in shake flasks in 2YT supplemented with 100 ug/ml ampicillin. Overnight cultures were diluted 1:100 into 1 litre fresh media and grown at 37^{o}C to A_{600} 0.6-0.8 before addition of IPTG to a final concentration of 0.1 mM. After 1 h, the cells were harvested by centrifugation,

washed in phosphate buffered saline (PBS) and resuspended in 30 ml PBS. Cells were disrupted by two passages through a French Press (42 000 to 60 000 kPa) and the cellular debris removed by centrifugation at 10,000 x g for 10 minutes followed by filtration through a 0.45 μm filter.

The prepared lysate was passed over a 1ml GST-Trap column (Amersham-Biosciences) continuously overnight at 4°C. Unbound material was removed by washing with 10 column volumes of PBS containing 1 mM DTT. The column was equilibrated in 5 volumes of thrombin digestion buffer (20 mM Tris-HCL pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) before addition of 7.5 U of biotinylated thrombin (Novagen). The GST fusions were cleaved overnight at RT before the cleaved biotin domains were washed off the column in 5 volumes of thrombin digest buffer. Biotinylated thrombin and biotin domain were simultaneously removed from the solution using Streptavidin-Sepharose High Performance (Amersham Biosciences) in a pull-down reaction, following manufacturers instructions. The non-biotinylated material (biotin domain) in the supernatant was collected, dialysed against 2 mM ammonium acetate pH 7.4 and lyophilised.

Purification of the human pyruvate carboxylase biotin domain (hPC-108) is shown in Figure 3A. Material in the unbound fraction is shown in lane 1 and protein in the wash fraction is shown in lane 2. The biotin domain was released from GST by addition of thrombin directly onto the column. Cleaved material (lane 3) was washed off the column and biotin-containing material removed using Streptavidin Sepharose (lane 4). The purification was monitored by SDS-PAGE (top panel) and Streptavidin blot (lower panel). Migration of molecular mass standards (kDa) is shown on the left and the position of hPC-108 shown on the right.

Example 4

Purified biotin domains are efficient substrates for biotinylation

Fractions of purified hPC-108 prepared as described in Example 2, were fractionated on duplicate 12% polyacrylamide gels under reducing conditions using Tris-tricine running buffer essentially as decribed in Schagger & von Jagow (1987) *Anal. Biochem.* 166:

368-79. One gel was used for SDS-PAGE analysis, and the other was used for a Western transfer onto nitrocellulose membrane and probed with Streptavidin-horse radish peroxidase. Evidence of biotinylated fusion protein (38 kDa) and biotinylated biotin domain (12 kDa) were observed on the Streptavidin blot indicating these proteins can function as substrates for *E. coli* BPL *in vivo*.

Biotinylated biotin domain was removed from the preparation of the thrombin cleaved hPC-108 using streptavidin sepharose and protein concentration determined using a BCA assay kit following manufacturer's instructions (Pierce).

Purified hPC-108 was analysed in an *in vitro* biotinylation assay using recombinant human BPL as follows: BPL activity was assayed by measuring the incorporation of [^{3}H]biotin into biotin domain. The reactions contained 50 mM Tris-HCl, pH 8.0, 3 mM ATP, 5.5 mM $MgCl_2$, 50 mM KCl, 5 uM biotin, 5 pmol [^{3}H]biotin (specific activity 35-44 Ci/mmol) 0.1 mM dithiothreitol, 0.1 mg/ml BSA and varying concentrations of hPC-108. The reaction was initiated by addition of purified yeast BPL to a final concentration of 13 nM and incubated at 37^{o}C for up to 30 minutes when aliquots of the reaction were spotted onto biotin- and trichloroacetic acid-treated filters. After air-drying, the filters were washed twice in 10% ice cold trichloroacetic acid and once in ethanol, dried and the acid insoluble radioactivity measured. Values for K_m and V_{max} were determined by fitting a plot of substrate concentration against rate to the Michaelis-Menten equation using GraphPad Prism for MacIntosh (GraphPad Software Inc, San Diego, CA).

The enzyme velocity was measured with varying concentrations of hPC-108. The K_M was determined to be 1.0 ± 0.2 uM indicating that the domain is a good BPL substrate, as shown in Figure 3B.

Example 5

Cloning the C. albicans BPL gene

The sequence data for *Candida albicans* BPL was obtained from the Stanford Genome Technology Centre (accession code CAN005069; ORF YDL141w) and is designated SEQ ID. No. 31:

The gene was cloned using polymerase chain reaction (PCR) with genomic DNA from *C. albicans* (strain CBF 562) as discussed below.

Candida has a rare reassignment of the genetic code where the universal codon for leucine CUG is encoded as a serine. Therefore, it was necessary to alter four CUG codons in the *bpl* sequence to universal serine codons before recombinant expression of the enzyme in a host such as *E. coli*.

The DNA manipulations were as follows:

Three separate fragments that spanned the entire gene were initially generated and subcloned into the pGEM-T Easy cloning vector (Promega) thus allowing further modification to the DNA sequences.

A fragment spanning the 5' half of the gene, encompassing nucleotides 1 to 1016, was obtained using PCR overlap extension. The PCR was performed with 2.5 units *PfuTurbo* DNA polymerase (Stratagene) in 200 uM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X100 and 0.1 mg/ml BSA. Between 0.1 and 0.5 uM of the required oligonucleotide primers were included along with 1 ug of genomic DNA as the template. Thermocycling conditions were 30 cycles of 92°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

Two smaller genomic PCR products were initially obtained either with oligonucleotides C1/32 and C2/39, or with C3/44 and C4/44:

C1/32[5'ATCTACTCATGAATGTTTTAGTATATTCTGGC] (SEQ ID NO.32)

C2/39 [5'GACGGCTCTGGCGCCAGTACGACTTTCGTACTTGAAACC] (SEQ ID NO.33)

C3/44 [5'CGTACTGGCGCCAGAGCCGTCAAATTGAGCGTCAATACAGCTGC] (SEQ ID NO.34)

C4/44 [5'TCCGGTATACCGGCGCTAGTGAGAAAGTTGATATACTTGACTGG] (SEQ ID NO.35)

The products were purified and included as a template for PCR with oligonucleotides C1/32 and C4/44. This produced a 1027 base pair fragment which was cloned into the pGEM-T Easy producing pGEM(C1/C4)Ser. By this approach, a *BspH*1 restriction site was introduced at the initiation codon and a unique *Nar*1 restriction site engineered at nucleotide 411. In addition the CUG codons at nucleotides 402 and 432 were altered to AGU and AGC respectively.

A second fragment was obtained that spanned nucleotides 567 to 1016 using genomic PCR with oligonucleotides C5/20 [5'GTGGATTTAGAGAAGGCTGC] (SEQ ID NO.36) and C4/44. The 449 base pair fragment was subcloned into pGEM-T Easy generating pGEM(C5/C4). An *Acc*1 restriction site at nucleotide 859 and the CUG codon at position 865 were simultaneously modified using oligonucleotides C10/45 and C11/45 with the Quickchange mutatgenesis protocol (Stratagene) producing pGEM(C5/C4)Ser. A polymorphorism from the published sequence in the species used here was detected by DNA sequencing. Nucleotide 719 was found to be a T base thus introducing another *Acc*1 site into the gene and changing the codon to an alanine (published sequence contains a valine at the corresponding position). The nucleotide at this position was mutated to C with oligonucleotides C15/40:

[5'GTGGTTGACACACTTCGAGCATACGATCACAACAAAAAGG] (SEQ ID NO.37) and;

C16/40:

[5'CCTTTTTGTTGTGATCGTATGCTCGAAGTGTGTCAACCAC] (SEQ ID NO.38) producing pGEM(C5/C4)Ser-Acc.

The third of the overlapping fragments encompassed the entire 3' half of the *bpl* gene from nucleotides 943 to the termination codon at 1993. This was generated by genomic PCR with oligonucleotide C6/22: [5'ACTAGTGAGTATGTTGGTAGTG] (SEQ ID NO.39) and;

oligonucleotide C7/65: [5'GAGCTCGGTACCTAATGATGATGATGATGATGATGATGACCGGTCTT CTTATATACTAAACC] (SEQ ID NO.40) producing pGEM(C6/C7). The C7/65 primer fused the coding sequence for a glycine-threonine-$(histidine)_9$ extension onto the C-terminus of the expressed gene product (the glycine-threonine motif introduced a unique *Age*1 restriction site into the gene). *Kpn*1 and *Sac*1 restriction sites were engineered onto the end of the gene to facilitate cloning into appropriate expression vectors.

The 5' and 3' ends of the *bpl* gene were fused together by digesting pGEM(C1/C4)Ser with *Spe*1 and *Sac*1 and ligation with similarly treated fragment from pGEM(C6/C7). The sequence between nucleotides 592 to 1007 in the resulting vector was replaced with the modified sequence from pGEM(C5/C4)Ser-Acc using the *Acc*1 sites at these positions. The final construct, pGEM(caBPL-His_9) contained the full length gene with all the desired modifications. For the recombinant expression of BPL for *in vivo* complementation assays, the *BspH*1 / *Kpn*1 fragment from pGEM(caBPL-His_9) was cloned into *Nco*1 / *Kpn*1 treated vector pARA13 (as described in Cagnon *et al.* (1991) *Prot. Eng.* 4:843-847). For high-level expression of BPL for purification from *E. coli*, the *BspH*1 / *Sac*1 fragment from pGEM(caBPL-His_9) was cloned into *Nco*1 / *Sac*1 treated pET-16b (Novogen).

The nucleotide sequence of the *C. albicans bpl* gene after DNA manipulation is designated SEQ ID No. 41

Example 6

Cloning the human bpl gene

Sequence data required for cloning of the human *bpl* gene was obtained from GenBank accession number X80160. The nucleotide sequence of the human bpl gene is designated SEQ ID No.42.

The cDNA for human BPL was obtained using reverse-transcription and PCR techniques upon human liver total RNA. 5ug of RNA was heated at 65°C for 5 minutes with 2 pmol of oligo($dT)_{18}$ primer and 3 uM of dNTP mix. Samples were chilled on ice before the addition of DTT to 10 uM, and first strand cDNA synthesis buffer to 1X (Gibco BRL). 40 units of RNAsin and 200 units of Superscript II (Gibco BRL) were added and cDNA synthesis performed at 42°C for 50 minutes, followed by inactivation of the polymerase at 72°C for 15 minutes. The cDNA was subsequently employed as a template for PCR. The PCR was performed with 2.5 units *PfuTurbo* DNA polymerase (Stratagene) in 200 uM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X100 and 0.1 mg/ml BSA. Between 0.1 and 0.5 uM of the required oligonucleotide primers were included with the cDNA. Thermocycling conditions were 30 cycles of 92°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

The gene was obtained as two overlapping fragments, each of which was subcloned into pGEM-T Easy. The 5' gene fragment, encompassed nucleotides 1–1444, was produced using oligonucleotides FW1:
[5'ATATAACCATGGAAGATAGACTCCACATGGATAAT] (SEQ ID NO.43) and; oligonucleotide RW1:
[5'GGAGACGCATCGTTGTGG] (SEQ ID NO.44).

PCR was performed with 2.5 units *PfuTurbo* DNA polymerase (Stratagene) in 200 uM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X100 and 0.1 mg/ml BSA. Between 0.1 and 0.5 uM of the required oligonucleotide primers were included along with 1 ug of genomic DNA as the template. Thermocycling conditions were 30 cycles of 92°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

The 3' fragment, encompassing nucleotides 1304–2179, was produced using oligonucleotides FW2 [5'CTTGTATACCTGTGGTGACC] (SEQ ID NO.45) and RW2 [5'ATAATCCCTACTCGAGCTAATGATGATGATGATGATGCCGCCGTTTGGGG AGGATGAGGTTTCT] (SEQ ID NO.46). PCR was performed with 2.5 units *PfuTurbo* DNA polymerase (Stratagene) in 200 uM of each dNTP, 10 mM KCl, 10 mM

$(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X100 and 0.1 mg/ml BSA. Between 0.1 and 0.5 uM of the required oligonucleotide primers were included along with 1 ug of genomic DNA as the template. Thermocycling conditions were 30 cycles of 92°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

Primer RW2 introduced nine histidine codons onto the 3' end of the *bpl* gene as well as a *Xho*1 restriction site after the coding sequence. To fuse the 5' and 3' ends of the gene, the vector containing the 5' fragment was digested with *Bgl*11 and *Xho*1 and ligated to the similarly treated 3' fragment producing pGEM(hBPL-His_6). Vector pARA(hBPL-His_6) was produced by cloning the full-length gene into *Nco*1 / *Hind*111 treated pARA13.

The human *bpl* gene was further modified to permit recombinant expression of the enzyme with the first 79 amino acids deleted as it has been reported that this variant displays greater activity than full-length BPL.

PCR was performed with oligonucleotide B14/29 [5'AAGGAGAGCCATGGCCTCTGGGAGTGAGC] (SEQ ID NO. 47) and oligonucleotide B15/23 [5'TCCTGTCCTTGTCCTCATTCTCC] (SEQ ID NO. 48) using pGEM(hBPL-His_6) as template. PCR was performed with 2.5 units *PfuTurbo* DNA polymerase (Stratagene) in 200 uM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X100 and 0.1 mg/ml BSA. Between 0.1 and 0.5 uM of the required oligonucleotide primers were included along with 1 ug of genomic DNA as the template. Thermocycling conditions were 30 cycles of 92°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

This product encompassed nucleotides 238 to 943 and introduced an *Nco*1 restriction site at the initiator methionine codon. The PCR product was digested with *Nco*1 and *Kpn*1 and ligated into similarly treated pARA(hBPL-His_6). The product, pARA[Met^1-Ala^{80}]hBPL-His_6, was used to produce a vector for high level bacterial expression. The *Nco*1 / *Hind*111 fragment was introduced into similarly treated pET-16b yielding pET[Met^1-Ala^{80}]hBPL-His_6.

The nucleotide sequence of the human *bpl* cDNA after manipulation is designated SEQ ID. No 49.

Example 7

Assay of C. albicans and human BPL activity in vivo

In order to determine the biological activity of the *Candida* and human *bpl* clones constructed, a series of expression vectors were transformed into *E. coli* BM4062 (Barker & Campbell (1981) *J. Mol. Biol.* 146:469-492). This strain contains the *birA85* mutation in the bacterial *bpl* gene which confers a temperature-sensitive phenotype. At the restrictive temperature of 42°C only those transformed cells expressing a functional exogenous BPL survive.

Plasmids pARA(yBPL-His_6), containing the entire gene for *Sacchromyces cerevisiae* BPL and expressing a functional yeast biotin protein ligase, pARA(caBPL-His_9), pARA[Met^1-Ala^{80}]hBPL-His_6 were transformed into BM4062 and grown at both 30°C and 42°C. These vectors permit constitutive expression of BPLs from *Saccharomyces cerevisiae*, *C. albicans* and *H. sapiens* BPLs respectively.

As shown in Figure 4, as expected all strains grew at the permissive temperature. Cells harbouring the parent vector pARA13 failed to grow at the restrictive temperature. All three vectors containing a *bpl* coding sequence complemented the bacterial mutation at 42°C, indicating that these genes encode functional BPL.

Example 8

Purification of recombinant C. albicans and human BPLs

Recombinant *C. albicans* and human BPL were expressed in *E. coli* and purified by nickel-chelating affinity chromatography.

Bacterial cultures of *E. coli* BL21(DE3)pLys harbouring the pET-16b based expression vectors were grown in shake flasks in 2YT supplemented with 100 ug/ml ampicillin and 30 ug/ml chloramphenicol. Overnight cultures were diluted 1:100 into 2 l fresh media

and grown at 30°C to A_{600} 0.6-0.8 before addition of IPTG to a final concentration of 0.1 mM. After 3 h, the cells were harvested by centrifugation, washed in binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 50 mM imidazole) and resuspended in 60 ml binding buffer containing 1 mM PMSF and 100 ug/ml lysozyme. The cell suspension was incubated on ice for 15 minutes before one cycle of freezing at -80°C and thawing. The cell lysate was sonicated and centrifuged at 10,000 x g for 10 minutes. After a second centrifugation, the supernatant was filtered through a 0.45 µm filter prior to chromatography.

His-tagged material was purified on a 5 ml HiTrap Chelating HP column (Amersham-Biosciences). Once the nickel charged column was equilibrated in binding buffer the cell lysate was loaded onto the column at a flow rate of 1 ml/min over 3 hours. The column was washed with 10 column volumes of binding buffer and 10 volumes of wash buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 100 mM imidazole) before the bound material was eluted with 3 volumes of elution buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 0.25 M imidazole). Fractions containing BPL were pooled and dialysed overnight against 4 l storage buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol). Fractions containing BPL, detected by SDS-PAGE and Ni-NTA Western blot, were pooled and stored at -80°C. N-terminal sequencing of proteins by automated Edman degradation, to confirm protein purification, was performed using a Perkin-Elmer Procise 492 protein sequencer.

Induction of the protein was performed at 30°C with low concentrations of IPTG to minimise the production of insoluble inclusion bodies. The BPL, tagged with a multi-histidine sequence at the C-terminus, were rapidly purified in a single step to >90% purity. Binding of the protein extract to the column was performed slowly and in the presence of 50 mM imidazole to improve the yield of enzyme whilst reducing non-specific proteins interacting with the resin. After a wash step containing 100 mM imidazole, the enzyme was eluted with 250 mM imidazole.

Analysis of *C. albicans* BPL by SDS-PAGE (12% polyacrylamide gel under reducing conditions) and NiNTA blot revealed two bands both containing the C-terminal histidine tag, as shown in Figure 4A. The most prominant band corresponded to the 78.4

kDa full-length protein, a result confirmed by N-terminal sequencing. A minor species was determined to be a proteolytic product of the enzyme where digestion had occurred between residues phenylalanine 248 and methionine 249. Analysis of the purified human BPL by SDS-PAGE and N-terminal sequencing showed a single major species was produced (Figure 4B).

Example 9

Assay of BPL activity in vitro

One hundred ng of biotin domain was coated onto each well of Griener Lumitrac 600 White 96-well plates (Stennick Scientific) with >95% of the peptide binding. Coating was performed overnight at 4°C in 100 uL of Tris Buffered saline (TBS, pH 7.5). The sample was removed and wells blocked in 150 ul of a 1% BSA solution in TBS for 1 hr at 37°C. The blocking buffer was removed and wells washed five times in TBS buffer containing 0.1% Tween-20. Into each well 85 ul of the BPL reaction mix was added. The reaction for both *C. albicans* and human BPL are performed in 50 mM Tris-HCl pH 8.0, 100 uM ATP, 5.5 mM $MgCl_2$, 0.1 mg/ml BSA, 10 uM biotin, 0.1 uM dithiothreitol.

Ten ul of either buffer alone or buffer containing inhibitor was added to the reaction mix and pre-equilibrated at 37°C for 5 minutes. BPL reactions are initiated by the addition of 5 ul enzyme to a final concentration of 3.4 nM (1-5 nM). The reaction is allowed to proceed at 37°C for 15 minutes at which point it is terminated by the addition of 5 ul 0.5 M EDTA (final concentration 25 mM). The reaction mix was discarded and wells washed five times in TBS-TD (TBS, 0.1% Tween-20 and 100 uM diethylenetriaminepentaacetic acid (DPTA)). The quantitative analysis of biotinylated protein formed in the reaction and coupled onto the well surface was determined using time resolved fluorescence. Fifty ul of Europium labelled streptavidin (Perkin-Elmer) solution, diluted to 0.1 ug/ml in TBS-TD, was added to each well. The plates were incubated at 37°C for 1 hr before being washed five times in TBS-TD then three times in water. Fifty ul of DELFIA Enhancement solution (Perkin Elmer) was added and incubated for ten minutes at room temperature before quantitation. Quantitation was performed using a FLUOstar Galaxy plate reader (BMG Labtechnologies).

Example 10

Kinetic analysis of C. albicans BPL

The activity of BPL was investigated using steady state kinetics by assaying the velocity of the enzyme within the first 10% of the reaction. BPL activity was determined by measuring the incorporation of biotin into a biotin-accepting domain adsorbed onto the surface of a 96 well plate. The presence of magnesium ions, ATP, biotin and the non-biotinylated form of a biotin domain were necessary substrates for activity. The inclusion of EDTA inhibited the reaction. Systematic investigation of each reaction component was then performed to determine the conditions required for optimal activity for *C. albicans* BPL.

A range of buffers (TBS, PBS and Tris) at pHs in the range of 7-8.5 were examined to determine the best conditions for coupling the biotin domains onto the well surface. PBS and TBS buffers performed better than the non-salt Tris buffer, although no significant difference was observed between the buffers. Optimal pH for coating was 7.5-8.0. A TBS buffer at pH 7.5 was chosen as this allowed the use of a single buffer system throughout the entire procedure.

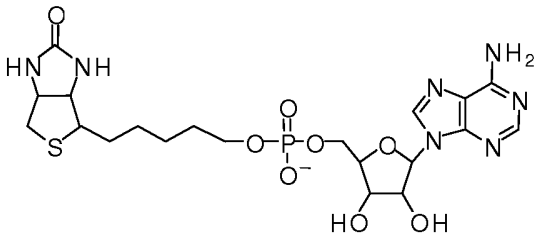
The BPL reaction with *C. albicans* enzyme was performed in sodium acetate buffer (pH 5.0-6.0), Tris buffer (pH 7.0-8.0) or sodium carbonate buffer (pH 9.0-10.0) in order to determine the optimal pH for the reaction. The *C. albicans* BPL displayed activity across a broad pH range (pH 6 – 10) with optimal activity at pH 8.0 (Figure 6). The addition of salt in the reaction, either NaCl or KCl, was omitted as both were found to be inhibitory (Figure 7). Magnesium chloride is essential in the reaction and the enzyme only poorly utilised other divalent metal ions tested (Figure 8). The enzyme requires a source of nucleotide triphosphate but when a series of compounds where assayed only ATP facilitated catalysis (Figure 9). The enzyme exhibited full activity in the assay with as little as 50 uM ATP (Figure 10). The BPL was also completely active in 2% dimethyl sulfoxide (Figure 11).

Example 11

Inhibition of BPL activity

Two potential inhibitors of the BPL reaction were tested in the *in vitro* biotinylation assay. The first, pyrophosphate, is the product of ATP hydrolysis in the first partial reaction catalysed by BPL. The addition of pyrophosphate should act as a product inhibitor in the reaction thus reducing BPL activity.

The second compound, biotinol-adenylate (BtnOH-AMP), is a derivative of biotinyl-adenylate also formed in the first partial reaction. This non-hydrolysable molecule should inhibit BPL by specifically competing with ATP and biotin for binding in the active site of the enzyme. The structure of this molecule is as follows:



Both pyrophosphate and BtnOH-AMP were potent inhibitors of the *Candida* BPL reaction.

Pyrophosphate strongly inhibited the reaction in the range of 50 uM to 1 mM (Figure 12).

Concentration response curves performed with BtnOH-AMP yielded the low IC_{50} value of 1.0 ± 0.2uM (Figure 13). Together these data show that the assay is suitable for the detection and quantitative analysis of BPL inhibitors.

BtnOH-AMP was also found to be an inhibitor of the human BPL reaction (Figure 14).

Example 12

Cloning and Purification of E. coli BPL (BirA)

A methodology for the recombinant expression and purification of the BPL from *E. coli* (BirA) is as described in Chapman-Smith *et al.* (2001) *Protein Sci* **10**(12): 2608-17.

The nucleotide sequence for the *birA* gene is designated SEQ ID. No. 50 (GenBank Accession number M15820).

To introduce the coding sequence for the birA gene into a high level expression vector, the gene will be excised from the plasmid pBA11 (Barker & Campbell (1981) *J. Mol. Biol.* 146: 469-492) using *BspH*1 and *BstY*1. The 1.17 kbp fragment will be ligated into *Nco*1 and *BamH*1 treated pET-16b (Novagen) yielding the plasmid pHBA. For protein expression this vector will be introduced into *E. coli* BL21(DE3) using calcium chloride transformation.

BirA may be purified from *E. coli* BL21(DE3) cells transformed with the plasmid pHBA, which contains the entire coding region for *E.coli* BirA, and pET16b (Novagen).

For expression, cells will be revived from storage at –80°C onto LB agar supplemented with 2% glucose and 200 ug/mL ampicillin and grown overnight at 30°C. Cells may then be harvested from an overnight culture grown at 30°C in LB containing 2% glucose and 200 ug/mL ampicillin and resuspended in fresh LB containing 200 ug/mL ampicillin or 100 ug/mL carbenicillin and 25mL aliquots may then be used to inoculate 500mL volumes of the same media. Cultures will be grown in shaker flasks at 30°C to A_{600nm} of 0.5, and then transferred to 37°C and expression induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.1mM.

After 2-3h cells are harvested, washed and resuspended in 50mM sodium phosphate, pH 6.0, 50mM KCl, 5% glycerol, 0.1mM dithiothreitol (Buffer A), and lysed with a French pressure cell. Cell-free extract may then be applied directly to a 50mL S-Sepharose Fast-Flow column (Amersham Biosciences) equilibrated in Buffer A and the BirA protein eluted with a linear gradient of 50-500mM KCl. Fractions containing enzymatic

activity elute at around 300mM KCl and coincide with the major absorbance peak at 280nm. This material is pooled and dialysed overnight against 20mM Tris-HCl, pH 8.0, 5% glycerol, 0.1mM dithiothreitol, at 4°C, and applied to a Q-Sepharose Fast-Flow column (Amersham Biosciences) equilibrated in the same buffer and the protein eluted with a linear gradient of 0-400mM KCl. Fractions containing enzymatic activity elute at around 120mM KCl and coincide with the major absorbance peak at 280nm. This material is then dialysed overnight against 20mM Tris-HCl, pH 7.5, 200mM KCl, 5% glycerol, 0.1mM dithiothreitol, at 4°C and stored at –80°C.

Example 13

Substrate for E.coli BPL

E. coli contains only a single biotinylated protein, the biotin carboxyl carrier protein (BCCP). BCCP is a component of the multi subunit enzyme acetyl CoA carboxylase (ACC).

Sequence data for the BCCP gene may be obtained from GeneBank, accession number M80458.

The biotin domain from this enzyme is contained within the 87 C-terminal residues of BCCP (BCCP-87), as follows:

M^{70}EAPAAAEISGHIVRSPMVGTFYRTPSPDAKAFIEVGQKVNVGDTLCIVEAMK MMNQIEADKSGTVKAILVESGQPVEFDEPLVVIE^{156} (SEQ ID NO. 51)

This fragment will be expressed in *E. coli* by using PCR to generate the appropriate DNA fragment for cloning into pGEX-4T-2. DNA encoding each domain will be amplified by PCR using oligonucleotides that engineer a *Bam*H1 restriction site at the 5' end of the DNA fragment and an *Eco*R1 site at the 3' end (the restriction sites are underlined in the primer sequences). By cloning the *BamH*1 and *Eco*R1 treated PCR product into similarly treated pGEX-4T-2, vectors for the expression of each peptide as a C-terminal extension to GST will be produced.

The PCR will employ plasmid pLS141 as described in Li and Cronan (1992) *J. Mol. Biol.* 267:855-863 with primer B1/32 [5' ATCTACGGATCCATGGAAGCGCCAGCAGCAGC] (SEQ ID NO.52) and primer B2/34 [5'ATCTACGAATTCATCACTCGATGACGACCAGCGG] (SEQ ID NO.53) . PCR will be performed with 2.5 units *PfuTurbo* DNA polymerase (Stratagene) in 200 uM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X100 and 0.1 mg/ml BSA. Between 0.1 and 0.5 uM of the required oligonucleotide primers will be included along with 1 ug of genomic DNA as the template. Thermocycling conditions to be employed are 30 cycles of 92°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

The peptide will be produced in *Escherichia coli* by expressing peptides encompassing the predicted biotin domain as a fusion to GST. This strategy permits rapid quantitation of both expression of the fusion protein by Western blot using anti-GST antibodies and biotinylation of the substrates by Streptavidin blot.

The biotin domain will be expressed as a GST fusion protein permitting both high level expression in *E. coli* and rapid purification by affinity chromatography. Bacterial cultures of BL21 harbouring the pGEX-4T-2 based expression vectors will be grown in shake flasks in 2YT supplemented with 100 ug/ml ampicillin. Overnight cultures are diluted 1:100 into 1 l fresh media and grown at 37°C to A_{600} 0.6-0.8 before addition of IPTG to a final concentration of 0.1 mM. After 1 h, the cells are harvested by centrifugation, washed in phosphate buffered saline (PBS) and resuspended in 30 ml PBS. Cells will be disrupted by two passages through a French Press (42 000 to 60 000 kPa) and the cellular debris removed by centrifugation at 10,000 x g for 10 minutes followed by filtration through a 0.45 µm filter.

The prepared lysate will be passed over a 1ml GST-Trap column (Amersham-Biosciences) continuously overnight at 4°C. Unbound material is removed by washing with 10 column volumes of PBS containing 1 mM Dithiothreitol. The column is then equilibrated in 5 volumes of thrombin digestion buffer (20 mM Tris-HCL pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) before addition of 7.5 U of biotinylated thrombin (Novagen). The GST fusions are cleaved overnight at RT before the cleaved biotin domains are

washed off the column in 5 volumes of thrombin digest buffer. Biotinylated thrombin and biotin domain can be simultaneously removed from the solution using Streptavidin-Sepharose High Performance (Amersham Biosciences) in a pull-down reaction, following manufacturers instructions. The non-biotinylated material, or biotin domain, in the supernatant will be collected, dialysed against 2 mM ammonium acetate pH 7.4 and lyophilised. The material will be analysed by SDS-PAGE and streptavidin blot and quantitated using *in vitro* biotinylation assay and BCA protein assay kit (Pierce).

Example 14

Cloning Arabidopsis thaliana HCS-1

A. thaliana contains two genes encoding different BPLs, *hcs-1* and *hcs-2,* known in plants as holocarboxylase synthetase (HCS). The two gene products share a high degree of identity (82%) and possess amino acid motifs conserved amongst all BPLs in the catalytic core of the enzyme. HCS-1 is the putative plastid form of the enzyme and hence is the enzyme responsible for biotinylation of acetyl-coA carboxylase in this cellular compartment. In plants, the *de novo* synthesis of fatty acids occurs primarily in the plastid, thus implicating HCS-1 as the most essential of the two BPLs.

The DNA sequence encoding the HCS-1 gene is designated in SEQ. ID. No. 54 (GenBank accession number U41369).

The *hcs-1* gene will be obtained as cDNA clone by reverse transcription (RT) and PCR, essentially as described in Sambrock, Fritsch, Maniatis (1989) Molecular Cloning, Cold Spring Harbor Laboratory Press.

Isolation of polyA mRNA from *A. thaliana* will be prepared using the Straight A's mRNA Isolation System (Novagen). First strand cDNA synthesis will be performed using 250 ng of mRNA in a final volume of 20 ul using Oligo(dT)20 primers (Thermoscript RT-PCR System, Life Technologies). The PCR will subsequently use 1 ul aliquots of RT together with oligonucleotides AbplFor:
[5' ATCTATCCATGGAAGCAGTTCGTTCAACAACAACC] (SEQ ID NO.55) and;
AbplRev:

[5'ATCCATGGATCCTAATGATGATGATGATGATGATGATGACCGGTTAT TTTTCTTCGAACCAGACC] (SEQ ID NO.56). These primers will introduce an *Nco*1 site at the 5' end of the gene and a *BamH*1 site at the 3' end respectively. In addition primer AbplRev introduces a threonine-glycine-(histidine)$_9$ sequence onto the C-terminus of the gene product, facilitating purification of the protein using nickel chelating chromatography techniques. Digestion of the PCR product with *Nco*1 and *BamH*1 will permit cloning of *hcs*-1 into similarly treated pET-16b. The enzyme will be recombinantly expressed in a bacterial host and the multi-histidine motif will permit rapid purification of the enzyme by metal ion chelating chromatography.

The predicted sequence after DNA manipulation is designated SEQ ID No. 57.

Example 15

Substrate for A. thaliana BPL

Dicotyledonous plants contain two structurally different acetyl CoA carboxylases (ACC). The type I ACC is a single polypeptide 220 kDa form, whereas type II is a multi subunit complex analogous to that of *E. coli*.

Sequence data for the BCCP gene of type II ACC may be obtained from GenBank, accession number U23155.

The biotin domain from this enzyme is contained within the 94 C-terminal residues of BCCP (BCCP-94), as follows:

P^{92}PSPPTPAKSSLPTVKSPMAGTFYRSPAPGEPPFIKVGDKVQKGQVLCIVEAMK LMNEIESDHTGTVVDIVAEDGKPVSLDTPLFVVQPVESAP^{280} (SEQ ID NO.58)

This fragment will be expressed in *E. coli* by using PCR to generate the appropriate DNA fragment for cloning into pGEX-4T-2. DNA encoding each domain will be amplified by PCR using oligonucleotides that engineer a *Bam*H1 restriction site at the 5' end of the DNA fragment and an *Eco*R1 site at the 3' end (the restriction sites are underlined in the primer sequences). By cloning the *BamH*1 and *Eco*R1 treated PCR

product into similarly treated pGEX-4T-2, vectors for the expression of each peptide as a C-terminal extension to GST will be produced.

The domain will be obtained using RT-PCR. Plant cDNA will be obtained as described in Example 14. PCR will be performed using cDNA as a template and primers AaccFor [5' ATCTACGGATCCCCACCATCCCCACCTACTCC] (SEQ ID NO.59) and AaccRev [5' ATCTACGAATTCATCATGGTGCCGATTCTACGG] (SEQ ID NO.60).

The peptide will be produced in *Escherichia coli* by expressing peptides encompassing the predicted biotin domain as a fusion to GST. This strategy permits rapid quantitation of both expression of the fusion protein by Western blot using anti-GST antibodies and biotinylation of the substrates by Streptavidin blot.

The biotin domain will be expressed as a GST fusion protein permitting both high level expression in *E. coli* and rapid purification by affinity chromatography. Bacterial cultures of BL21 harbouring the pGEX-4T-2 based expression vectors will be grown in shake flasks in 2YT supplemented with 100 ug/ml ampicillin. Overnight cultures are diluted 1:100 into 1 l fresh media and grown at 37^{o}C to A_{600} 0.6-0.8 before addition of IPTG to a final concentration of 0.1 mM. After 1 h, the cells are harvested by centrifugation, washed in phosphate buffered saline (PBS) and resuspended in 30 ml PBS. Cells will be disrupted by two passages through a French Press (42 000 to 60 000 kPa) and the cellular debris removed by centrifugation at 10,000 x g for 10 minutes followed by filtration through a 0.45 µm filter.

The prepared lysate will be passed over a 1ml GST-Trap column (Amersham-Biosciences) continuously overnight at 4^{o}C. Unbound material is removed by washing with 10 column volumes of PBS containing 1 mM Dithiothreitol. The column is then equilibrated in 5 volumes of thrombin digestion buffer (20 mM Tris-HCL pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) before addition of 7.5 U of biotinylated thrombin (Novagen). The GST fusions are cleaved overnight at RT before the cleaved biotin domains are washed off the column in 5 volumes of thrombin digest buffer. Biotinylated thrombin and biotin domain can be simultaneously removed from the solution using Streptavidin-Sepharose High Performance (Amersham Biosciences) in a pull-down reaction,

following manufacturers instructions. The non-biotinylated material, or biotin domain, in the supernatant will be collected, dialysed against 2 mM ammonium acetate pH 7.4 and lyophilised. The material will be analysed by SDS-PAGE and streptavidin blot and quantitated using *in vitro* biotinylation assay and BCA protein assay kit (Pierce).

Example 16

Cloning of Drosophila BPL

The calculated gene product for *Drosophila melanogaster* BPL is the largest member of the enzyme family so far recorded. This gene contains 1042 amino acids yielding a 120 kDa protein. Bacteria is not a favourable expression system for the production of large proteins so another expression system will be used. The baculovirus expression system will be employed using insect cells as the host together with the vector pFastBac HT (Invitrogen).

Sequence data required for the construction of a *D. melanogaster* BPL expression vector was obtained from GenBank, accession number AE003602, and is designated SEQ. ID. No. 61.

For construction, the full-length cDNA clone BcDNA:RE09732 will be obtained from ResGen (Invitrogen). This will allow amplification of the *bpl* cDNA by PCR using oligonucleotides DbplFor [5' GAATTCATGTTGACCCTGTATTACGTGAG] (SEQ ID NO.62) and DbplRev [5' AAGCTTCATTTCACTATTGATACTTGG] (SEQ ID NO.63) and the DNA-Polymerase containing proof reading activity *Pwo*. These primers introduce *Eco*R1 and *Hind*111 restriction sites (sequences underlined) at the 5' and 3' ends of the cDNA respectively. The PCR product will be subcloned into pGEM-T Easy for DNA sequencing. The product will be subcloned as a single *Eco*R1 / *Hind*111 DNA fragment into similarly treated pFastBac HT. Expression of the BPL in this system will yield a full-length product containing a MSYYHHHHHHDYDIPTTENLYFQGAMDPEF (SEQ ID NO. 64) N-terminal extension. The hexa-histidine motif will permit rapid purification of the enzyme by metal ion chelating chromatography. The nucleotide sequence of the DNA after manipulation is designated SEQ. ID. No. 65:

Example 17

Substrate for Drosophila BPL

A biotin domain from a *Drosophila* biotin-enzyme has not yet been reported so the most likely candidate to trial is a fragment containing the 125 C-terminal residues of pyruvate carboxylase.

Sequence data for the *D. melanogaster* pyruvate carboxylase gene may be obtained from GenBank, accession number NM 136683.

The biotin domain from this enzyme is contained within the 125 residues of pyruvate carboxylase (dPC-125), as follows:

G^{1057}KTLSVKALAVSADLKPNGIREVFFELNGQLRAVHILDKEAVKEIHVHPKAN KSNKSEVGAPMPGTVIDIRVKVGDKVEKGQPLVVLSAMKMEMVVQSPLAGVV KKLEIANGTKLEGEDLIMIIE^{1181} (SEQ ID NO.66)

This fragment will be expressed in *E. coli* by using PCR to generate the appropriate DNA fragment for cloning into pGEX-4T-2. DNA encoding each domain will be amplified by PCR using oligonucleotides that engineer a *Bam*H1 restriction site at the 5' end of the DNA fragment and an *Eco*R1 site at the 3' end (the restriction sites are underlined in the primer sequences). By cloning the *BamH*1 and *Eco*R1 treated PCR product into similarly treated pGEX-4T-2, vectors for the expression of each peptide as a C-terminal extension to GST will be produced.

The domain will be obtained from the full-length cDNA clone BcDNA:GH06348 ResGen (Invitrogen). This will be used as the template in a PCR with primers DpcFor [5' ATCTACGGATCCGGTAAGACGCTGAGCGTGAAAGC] (SEQ ID NO. 67) and DpcRev [5' ATCTACGAATTCCTATTCGATAATCATAATGAGGTCC] (SEQ ID NO.68).

The peptide will be produced in *Escherichia coli* by expressing peptides encompassing the predicted biotin domain as a fusion to GST. This strategy permits rapid quantitation of both expression of the fusion protein by Western blot using anti-GST antibodies and biotinylation of the substrates by Streptavidin blot.

The biotin domain will be expressed as a GST fusion protein permitting both high level expression in *E. coli* and rapid purification by affinity chromatography. Bacterial cultures of BL21 harbouring the pGEX-4T-2 based expression vectors will be grown in shake flasks in 2YT supplemented with 100 ug/ml ampicillin. Overnight cultures are diluted 1:100 into 1 l fresh media and grown at 37^{o}C to A_{600} 0.6-0.8 before addition of IPTG to a final concentration of 0.1 mM. After 1 h, the cells are harvested by centrifugation, washed in phosphate buffered saline (PBS) and resuspended in 30 ml PBS. Cells will be disrupted by two passages through a French Press (42 000 to 60 000 kPa) and the cellular debris removed by centrifugation at 10,000 x g for 10 minutes followed by filtration through a 0.45 µm filter.

The prepared lysate will be passed over a 1ml GST-Trap column (Amersham-Biosciences) continuously overnight at 4°C. Unbound material is removed by washing with 10 column volumes of PBS containing 1 mM Dithiothreitol. The column is then equilibrated in 5 volumes of thrombin digestion buffer (20 mM Tris-HCL pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) before addition of 7.5 U of biotinylated thrombin (Novagen). The GST fusions are cleaved overnight at RT before the cleaved biotin domains are washed off the column in 5 volumes of thrombin digest buffer. Biotinylated thrombin and biotin domain can be simultaneously removed from the solution using Streptavidin-Sepharose High Performance (Amersham Biosciences) in a pull-down reaction, following manufacturers instructions. The non-biotinylated material, or biotin domain, in the supernatant will be collected, dialysed against 2 mM ammonium acetate pH 7.4 and lyophilised. The material will be analysed by SDS-PAGE and streptavidin blot and quantitated using *in vitro* biotinylation assay and BCA protein assay kit (Pierce).

Example 18

Hydroxylamine as a substrate for biotin protein ligase

Biotin protein ligases also contain biotinyl-AMP synthetase activity (i.e. the first partial reaction) which can be quantitated independently of the second partial reaction.

The product of the first partial reaction, biotinyl-5'-AMP may react with a non-protein amine containing molecule such as hydroxylamine. When BPL is incubated with radiolabelled biotin, MgATP and hydroxylamine, labelled biotinyl-hydroxamate is formed. Unreactive biotin may be removed from the reaction by anion exchange resin. Product formation is measured by quantitating the amount of radioactivity in the cleared supernatant fraction.

To assay for biotinyl-AMP synthetase activity, a reaction mixture containing 50 uM Tris-HCl pH8.0, 12 mM $MgCl_2$, 12 mM ATP, 0.4 M hydroxylamine, and 33 – 52.6 Ci/mmol *d*-[8,9-^{3}H]biotin is prepared. The biotinyl-AMP synthetase activity is initiated by the addition of BPL and terminated by the addition of 100 mM EDTA or heating the sample to 80°C for 10 minutes. An excess quantity of anion exchange resin, such asAG-X2 (Bio-Rad) or Q-Sepharose (Amersham Biosciences) is added to the reaction to remove [^{3}H]biotin. After shaking the suspension and pelleting the resin by centrifugation, a sample of the supernatant is removed for quantitation of [^{3}H]biotinhydroxamate by liquid scintillation counting.

Accordingly, in determining whether a test compound is an inhibitor of a biotin protein ligase, a molecule such as hydroxylamine may be employed as a substrate for a biotin protein ligase, using the above assay.

Example 19

Determination of the extent of inhibition of biotinylation of a protein substrate in vivo by biotinol-adenylate

Human NIH3T3 fibroblasts will be grown at 37°C and 100% humidity, 5% CO_2 in 96 well plates with Dulbecco's modified Eagle's medium containing 7.5% foetal calf serum, L-glutamine and penicillin/streptomycin.

Confluent monolayers of fibroblasts will be subjected to treatment with the BPL inhibitor biotinol-adenylate by addition of the compound into the growth media for varying times from 12 - 72 hours, at concentrations ranging from 10 nM – 100 uM. After an appropriate period of time the media will be removed and cells washed with saline to remove excess inhibitor.

To determine if the inhibitor prevents *in vivo* biotinylation, cells will be lysed with Trizol reagent (Life Technologies) and the protein fraction extracted. This material will be fractionated by SDS-PAGE and used for Western transfer. Duplicate blots will be probed with both anti pyruvate carboxylase antibodies and streptavidin. The level of biotin incorporation into pyruvate carboxylase *in vivo* will be compared between treated and untreated controls.

The toxicity of the compound will be assessed using the neutral red assay (Sigma). This assay measures lysosomal membrane stability in terms of the retention time of the neutral red dye only within the lysomes of viable cells. Absorbance of converted dye is spectroscopically determined by absorbance at 540 nm.

Example 20

Inhibition of BPL from Escherichia coli

Figure 15 shows the concentration response curve of bacterial BPL activity inhibition by biotinol-adenylate. The assay for bacterial BPL was performed as described in Example 9. The activity of the enzyme was determined in the presence of varying concentrations of biotinol-adenylate.

Example 21

Assay for inhibitors of BPL activity in vitro

The assay for Candida BPL was performed as described in Example 9. A proprietary library consisting of 329 different compounds was obtained. Each compound in the screening library was dissolved in 100% DMSO, added to the reactions to a final concentration of 10 μM or 50 μM and their effect on BPL activity measured. The graph shows the average of the reactions performed in triplicate. The inhibitory activity of the compounds was determined relative to the negative control reactions containing only DMSO. The graph shows the activity of each compound assayed at 10 uM (Grey bars) and 50 uM (black bars).

As can be seen from the results shown in Figure 16, no significant inhibitory activity was observed for most compounds tested at both concentrations.

A small number of compounds showed significant inhibitory activity with Candida BPL at both 10 μM and 50 μM concentrations, exemplified by compounds 69 and 296.

Example 22

Differential inhibition of Candida and human BPL

The assay for Candida and human BPLs was performed as described in Example 9. Compounds 69 and 296 were dissolved in 100% DMSO, added to the reactions to a final concentration of 10 μM or 50 μM and their effect on BPL activity measured. The data for compound 69 is shown in Panel A of Figure 17 and the data for compound 296 is shown in Panel B.

The graphs shows the average and standard error from reactions performed in triplicate. The inhibitory activity of the compounds was determined relative to the negative control reactions containing only DMSO (blue bars). Biotinol-AMP was included in the reactions as a control for inhibition.

Compound 69 inhibited Candida BPL to 23% of full activity at a concentration of 50 uM. At the same concentration human BPL was not affected by this compound. Likewise compound 296 reduced the pathogen's enzyme activity by 81% at a concentration of 10 uM without affecting the human enzyme. These levels of inhibition are comparable to those observed using the potent BPL inhibitor biotinol-AMP at 100 uM.

Example 23

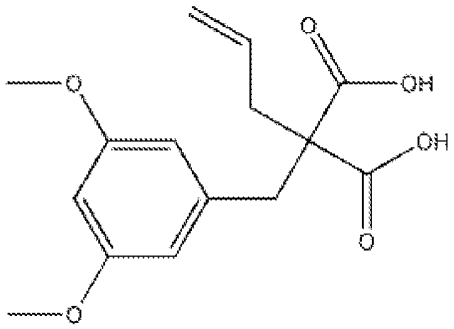
Methodology for in silico drug screening

The X-ray structures of BPL from *Escherichia coli*, in the absence (Wilson *et al* (1992) "Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains" *Proc. Natl. Acad. Sci. U S A.* 89; 9257-9261) and presence of biotin (Weaver *et al* (2001) "Corepressor-induced organization and assembly of the biotin repressor: A model for the allosteric activation of a transcriptional regulator" *Proc Natl. Acad. Sci. USA.* 98; 6045-6050) were used to identify potential inhibitory compounds using *in silico* technology. Missing amino acids

and hydrogen atoms in the structure (PDB: 1BIB) were modelled using the Biopolymer module of SYBYL v7.0 (Tripos, Inc. SYBYL, Version 7.0, St. Louis, MO 2004). Modelled sections of the protein (encompassing amino acids at positions 2, 18, 24, 27, 33, 38, 51, 56, 77, 140, 141, 211, 223, 267 and 294) were refined by conjugate gradient energy minimisation in explicit water using the program NAMD (Linge *et al* (2003) "Refinement of protein structures in explicit solvent" *Proteins* 50; 496-506; Kale *et al.* (1999) "NAMD2: greater scalability for parallel molecular dynamics" *J. Comput. Phys.* 151: 283-312). Partial charges for BPL and the 24/07/2004 "lead like" compound library of ZINC (Zinc is not Commercial) were calculated using Biopolymer and the SYBYL Programming Language (SPL) respectively. Amino acid residues 91, 112, 116, 117, 118, 119, 121, 175, 183, 188, 189 and 205 were defined as the active site of BirA and a negative image constructed with ATPTS 2001 (Moreno *et al.* (2002) "Geometric and chemical patterns of interaction in protein--ligand complexes and their application in docking" *Proteins* 47: 1-13). The ZINC compound library was then docked with DOCK v5.1.0. (Ewing *et al.* (2001) "DOCK 4.0: search strategies for automated molecular docking of flexible molecule databases" *J. Comput. Aided Mol. Des.* 15: 411-428). Poses were scored using Scorer v1.3 and ranked with a threshold of 10% in an in-house consensus scoring program based on the CScore module of SYBYL. Compounds with a score of 6 and the top compounds based on the internal Dock Energy Score were visually inspected and non-viable candidates filtered out.

Using this *in silico* methodology, eight compounds were identified that inhibited *Escherichia coli* BPL, four with low nanomolar affinity (data shown for compounds 2-5 below), and another in the micromolar range. Compounds 2-4 also demonstrated inhibitory properties when assayed against Staphylococcus aureus BPL. Enzymes from humans and the yeast *Saccharomyces cerevisiae* were not inhibited by compounds 2-5 when assayed at concentrations less than 50 μM. Together these data demonstrate that it is possible to identify differential inhibitions of biotin protein ligase.

Compound 4 identified in the screen was 2-allyl-2-(3,5-dimethoxybenzyl)malonic acid:



Example 24

Differential inhibition of BPLs

The activity of BPL from *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae* and *Homo sapiens* was measured using an *in vitro* biotinylation assay in the presence of increasing concentrations of Compounds 2 to 5. The data is shown in Figure 18.

The compounds were found to differentially inhibit the two bacterial enzymes without any significant effect on the mammalian and yeast enzymes. The half maximal inhibitory concentrations (IC_{50}) were as follows:

Compound 2:

E. coli IC_{50}: 1.0 nM

S. aureus IC_{50}: 2.8 µM

S. cerevisiae IC_{50}: > 50 µM

Human IC_{50}: > 50 µM

Compound 3:

E. coli IC_{50}: 1.7 nM

S. aureus IC_{50}: 0.9 µM

S. cerevisiae IC_{50}: >50 µM]

Human IC_{50}: > 50 µM

Compound 4:

E. coli IC_{50}: 0.9 nM

S. aureus IC_{50}: 2.7 μM

S. cerevisiae IC_{50}: > 50 μM

Human IC_{50}: > 50 μM

Compound 5:

E. coli IC_{50}: 15 nM

S. aureus IC_{50}: > 50 μM]

S. cerevisiae IC_{50}: > 50 μM

Human IC_{50}: > 50 μM

Example 24

Antibiotic Activity of Compound 2

The antibacterial properties of Compound 2 were assessed against *Staphylococcus aureus* grown on both solid media (A-C) and in liquid culture (D). The data is shown in Figure 19. Log phase culture was plated onto L-agar and filter discs soaked in A) DMSO (vehicle control), B) 100 μM ampicillin or C) 100 μM compound #2. A single filter was placed in the centre of a bacterial plate before being placed at 37°C overnight. The clear zone surrounding the disc in B & C represents sensitivity of *S. aureus* to the treatment. D) This sensitivity was quantitated in liquid culture by seeding 10^4 cells in a 96 well plate followed by overnight growth at 37°C. The growth of the culture was measured by absorbance at 600nm. The half maximal effective dose (ED50) was determined to be 11.6 ± 1.3 μM.

Example 25

Toxicity Data on Compounds 2 to 5

Hep3B2-1-17 cells were seeded with 1000 cells in 50µL cell culture medium in 96-well microtiter plates. 50µL of cell culture medium containing the test compound(s) was added to each well twenty four hours after seeding. Stock solutions of compounds 2-5 in DMSO, were serially diluted in culture medium and added to triplicate wells, producing final concentrations ranging from 40µM to 0.15625 µM. The final DMSO concentration in the incubation mixture was less than 0.05% (v/v) for the highest concentration test article and respectively lower for the lower concentration test articles. Proliferation of cells was assessed 72 hours post compound addition, using the fluorimetric Alamar Blue (CellTiter Blue) test. This assay indicates cell viability by measuring the ability of cells in culture to reduce resazurin to resorufin, whereby the intensity of the fluorescence signal is directly proportional to the number of live cells and hence an indirect indicator of cell proliferation. Following addition of Cell Titer-Blue Reagent (20 µL/well) and brief mixing, cells were incubated for another 4 hours before fluorescence was measured (Ex/Em of 560/590 nm) using a Spectramax Gemini XPS microplate reader (Molecular Devices, Surrey Hills, VIC, Australia).

The data is shown in Figure 20. Compounds 2 to 5 were well tolerated by HEP3B2-1-7 cells, however compound 2 demonstrated moderate toxicity at the highest concentration tested. The other compounds did not appear to demonstrate any toxicity at the specified concentrations.

Finally, it will be appreciated that various modifications and variations of the described methods and compositions of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in the art are intended to be within the scope of the present invention.

Claims

1. A method for identifying an inhibitor of a biotin protein ligase, the method including the steps of:

(a) providing a substrate, wherein the substrate may be biotinylated;

(b) contacting the substrate with biotin and a biotin protein ligase in the presence of a test compound;

(c) determining the extent of biotinylation of the substrate by the biotin protein ligase in the presence of the test compound; and

(d) identifying the test compound as an inhibitor of the biotin protein ligase by a reduction in the biotinylation of the substrate in the presence of the test compound as compared to the extent of biotinylation of the substrate in the absence of the test compound.

2. A method according to claim 1, wherein the biotin protein ligase is a biotin protein ligase of a pathogenic organism of a human, animal or a plant.

3. A method according to claim 2, wherein the biotin protein ligase is a bacterial or fungal biotin protein ligase.

4. A method according to any one of claims 1 to 3, wherein the substrate is selected from the group consisting of pyruvate carboxylase, acetyl CoA carboxylase, propionyl CoA carboxylase, B-methylcrotonyl CoA carboxylase, methylmalonyl-CoA carboxyltransferase, oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, glutaconyl-CoA decarboxylase, urea carboxylase, geranoyl-CoA carboxylase and geranoyl-CoA transcarboxylase, a variant of any of the aforementioned proteins, a polypeptide fragment including the biotin domain of any of the aforementioned protein substrates or variants of the proteins, and hydroxylamine.

5. A method according to any one of claims 1 to 4, wherein the test compound is selected by an *in silico* selection method.

6. A method according to claim 5, wherein the *in silico* selection method utilises the structure of a biotin protein ligase in the absence and presence of biotin to select the test compound.

7. A method according to any one of claims of 1 to 6, wherein the inhibitor has an IC_{50} of less than 1 μM.

8. A method according to any one of claims 1 to 7, wherein the method is used to identify an inhibitor in a cell free system.

9. An inhibitor identified by the method according to any one of claims 1 to 8.

10. An inhibitor according to claim 9, wherein the inhibitor is a differential inhibitor of a biotin protein ligase from a first organism as compared to a second organism.

11. An inhibitor according to claim 10, wherein the first organism is a pathogen of a human, animal or plant and the second organism is a host organism for the pathogen.

12. An inhibitor of a biotin protein ligase of a pathogenic organism of a human, animal or plant.

13. An anti-bacterial agent, wherein the agent inhibits biotinylation of a substrate by a bacterial biotin protein ligase.

14. An anti-bacterial agent according to claim 13, wherein the agent does not substantially inhibit biotinylation by a biotin protein ligase from one or more of a human, animal or plant.

15. An anti-bacterial agent according to claims 13 to 14, wherein the inhibitor has an IC_{50} for the bacterial biotin protein ligase of less than 1 μM.

16. An anti-fungal agent, wherein the agent inhibits biotinylation of a substrate by a fungal biotin protein ligase.

17. An anti-fungal agent according to claim 16, wherein the agent does not substantially inhibit biotinylation by a biotin protein ligase from one or more of a human, animal or plant.

18. An isolated biotin protein ligase suitable for use as a target for identifying an inhibitor of biotinylation.

19. An isolated biotin protein ligase according to claim 18, wherein the biotin protein ligase is a biotin protein ligaseof a pathogenic organism of a human, animal or plant.

20. An isolated biotin protein ligase according to claim 18, wherein the biotin protein is suitable for the identification of a differential inhibitor of a biotin protein ligase from a first organism as compared to a second organism.

21. An isolated biotin protein ligase according to claim 20, wherein the first organism is a pathogen of a human, animal or plant and the second organism is a host organism for the pathogen.

22. An inhibitor of a biotin protein ligase of a pathogenic organism of a human, animal or plant.

23. A method for identifying an agent that inhibits growth and/or survival of a pathogenic organism, the method including the step of identifying an agent that inhibits a biotin protein ligase.

24. A method according to claim 23, wherein the pathogenic organism is a bacterium or fungus.

25. A method according to claims 23 or 24, wherein the biotin protein ligase is a biotin protein ligase from from a bacterium or a fungus.

26. A method according to any one of claims of 24 to 25, wherein the inhibitor has an IC_{50} for the biotin protein ligase of less than 1 μM.

27. A method according to any one of claims 23 to 26, wherein the method is used to identify an agent that inhibits a biotin protein ligase in a cell free system.

28. An agent identified by the method according to any one of claims 23 to 27.

29. An agent identified according to claim 28, wherein the agent is a differential inhibitor of a biotin protein of the pathogenic organism as compared to inhibition of a biotin protein ligase from one or more of a human, animal or plant.

30. Use of a biotin protein ligase as a target for identifying an agent that inhibits growth and/or survival of an organism.

31. Use according to claim 30, wherein the biotin protein ligase is a biotin protein ligase of a pathogenic bacterium of a human, animal or plant.

32. Use according to claim 30, wherein the biotin protein ligase is a biotin protein ligase of a pathogenic fungus of a human, animal or plant.

33. An isolated biotin protein ligase suitable for use as a target for identifying an agent that inhibits growth and/or survival of an organism.

34. An isolated biotin protein ligase according to claim 33, wherein the biotin protein ligase is a biotin protein ligase of a pathogenic organism of a human, animal or plant.

35. A method for identifying an inhibitor of biotinylation in a biological system, the method including the steps of:

(a) identifying a test compound as an inhibitor of a biotin protein ligase;

(b) determining the ability of the test compound to inhibit biotinylation in a biological system; and

(c) identifying the test compound as an inhibitor of biotinylation in the biological system.

36. An inhibitor identified by the method according to claim 35.

37. A method for inhibiting growth and/or survival of a pathogenic organism, the method including the step of exposing the pathogen to an agent that inhibits biotinylation in the pathogenic organism.

38. A composition for inhibiting growth and/or survival of a pathogenic organism, the composition including an effective amount of an agent that inhibits biotinylation in the pathogenic organism.

39. A method for identifying a compound that differentially inhibits a first biotin protein ligase as compared to inhibition of a second biotin protein ligase, the method including the steps of:

(a) identifying a test compound that inhibits a first biotin protein ligase;

(b) determining the ability of the test compound to inhibit a second biotin protein ligase; and

(c) identifying the test compound as a compound that differentially inhibits the first biotin protein ligase as compared to the inhibition of the second biotin protein ligase.

40. An inhibitor identified by the method according to claim 39.

41. A method for identifying a compound that differentially inhibits biotinylation in a first biological system as compared to inhibition of biotinylation in a second biological system, the method including the steps of:

(a) identifying a test compound that inhibits biotinylation in a first biological system;

(b) determining the ability of the test compound to inhibit biotinylation in a second biological system; and

(c) identifying the test compound as a compound that differentially inhibits biotinylation in the first biological system as compared to the inhibition of biotinylation in the second biological system.

42. An inhibitor identified by the method according to claim 41.

43. An agent that differentially inhibits a first biotin protein as compared to a second biotin protein ligase.

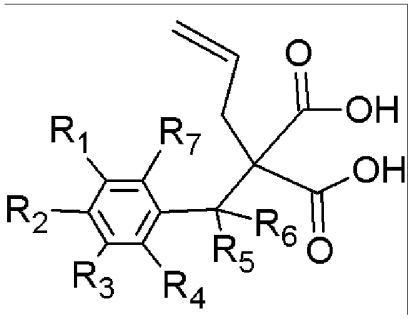
44. An inhibitor of a biotin protein ligase of a pathogen of a human, animal or plant, wherein the inhibitor does not substantively inhibit a human, plant or animal biotin protein ligase

45. A method for preventing and/or treating an infection by a pathogenic organism of a subject, the method including the step of administering to the subject an effective amount of an agent that inhibits a biotin protein ligase of the pathogenic organism.

46. A method according to claim 45, wherein the pathogenic organism is a pathogen of a human, animal or plant subject.

47. A method according to claim 45, wherein the pathogenic organism is a bacterial or fungal pathogen.

48. A method according to any one of claims 45 to 47, wherein the agent is a compound with the formula:

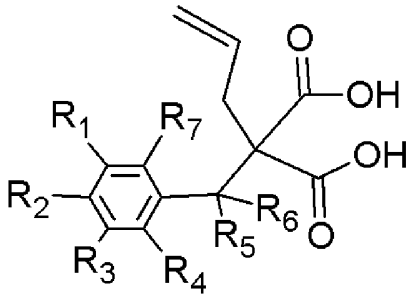


or a salt or ester thereof, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl, arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino, sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-, $R_9N(R_{10})SO_2R_{11}$-, $R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

49. A method according to claim 48, wherein the agent is 2-allyl-2-(3,5-dimethoxybenzyl)malonic acid, or a salt or ester thereof.

50. A composition for preventing and/or treating method an infection by a pathogenic organism, the composition including an effective amount of an agent that inhibits a biotin protein ligase of the pathogenic organism.

51. A composition according to claim 50, wherein the agent is a compound with the formula:



or a salt or ester thereof, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl, arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino, sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-, $R_9N(R_{10})SO_2R_{11}$-, $R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

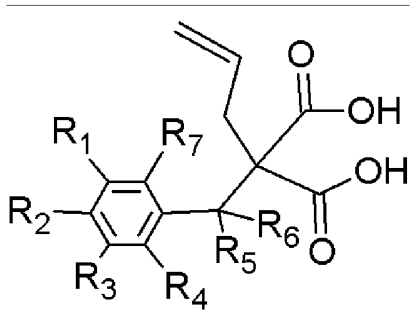
52. A composition according to claim 51, wherein the compound is 2-allyl-2-(3,5-dimethoxybenzyl)malonic acid, or a salt or ester thereof.

53. A method for preventing and/or treating an infection by a pathogenic organism of a subject, the method including the step of administering to the subject an effective amount of an agent that inhibits biotinylation in the pathogenic organism.

54. A composition for preventing and/or treating an infection of a host by a pathogenic organism, the composition including an effective amount of an agent that inhibits biotinylation in the pathogenic organism.

55. Use of an agent that inhibits a biotin protein ligase of a pathogenic organism in the preparation of a medicament for preventing and/or treating an infection by a pathogenic organism.

56. Use according to claim 55, wherein the agent is a compound with the formula:



or a salt or ester thereof, wherein R_1 to R_7 are each independently selected from the group consisting of: H, halogen, alkyl, alkenyl, alkynyl, haloalkyl, haloalkenyl, heteroalkyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, heterocycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl, arylalkenyl, cycloalkylheteroalkyl, heterocycloalkylheteroalkyl, heteroarylheteroalkyl, arylheteroalkyl, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, alkoxyaryl, alkenyloxy, alkynyloxy, cycloalkylkoxy, heterocycloalkyloxy, aryloxy, arylalkyloxy, phenoxy, benzyloxy, heteroaryloxy, amino, alkylamino, aminoalkyl, acylamino, arylamino, sulfonylamino, sulfinylamino, COOH, COR_8, $COOR_8$, $CONHR_8$, NHCOR, $NHCOOR_8$, $NHCONHR_8$, alkoxycarbonyl, alkylaminocarbonyl, sulfonyl, alkylsulfonyl, alkylsulfinyl, arylsulfonyl, arylsulfinyl, aminosulfonyl, SR_8, $R_9S(O)R_{10}$-, $R_9S(O)_2R_{10}$-, $R_9C(O)N(R_{10})R_{11}$-, $R_9SO_2N(R_{10})R_{11}$-, $R_9N(R_{10})C(O)R_{11}$-, $R_9N(R_{10})SO_2R_{11}$-,

$R_9N(R_{10})C(O)N(R_{10})R_{11}$- and acyl, each of which may be optionally substituted; each R_8, R_9, R_{10} and R_{11} is independently selected from the group consisting of a bond, H, alkyl, alkenyl, alkynyl, haloalkyl, heteroalkyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, cycloalkylalkyl, heterocycloalkylalkyl, arylalkyl, heteroarylalkyl and acyl, each of which may be optionally substituted.

57. Use according to claim 56, wherein the compound is 2-allyl-2-(3,5-dimethoxybenzyl)malonic acid, or a salt or ester thereof.

Figure 1

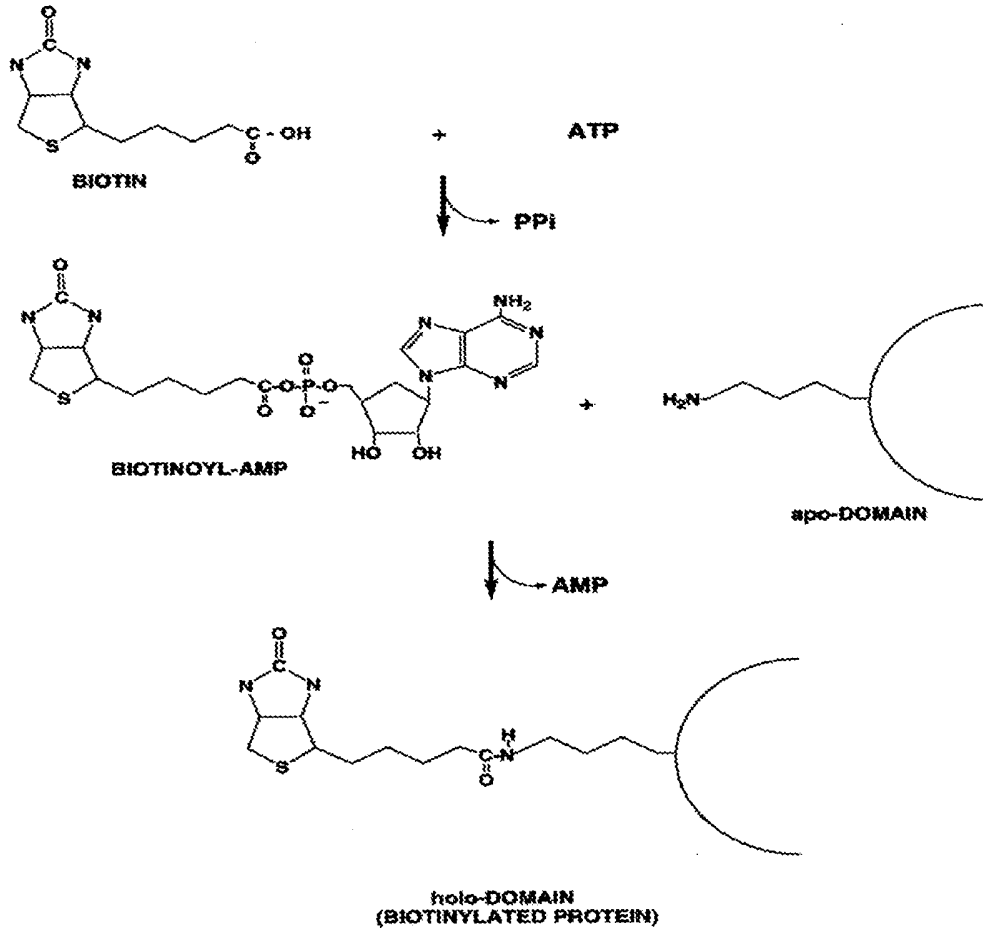
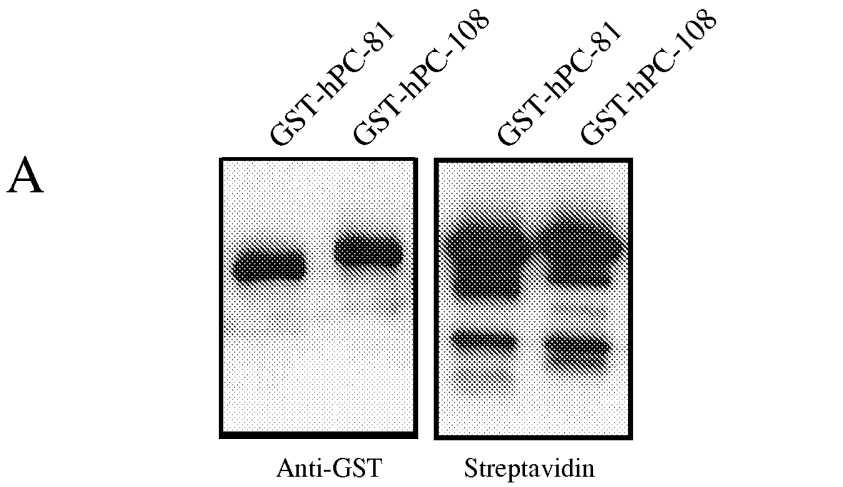
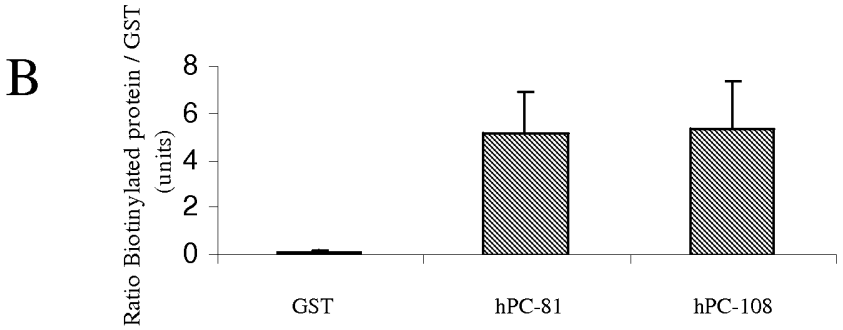

BIOTIN
ATP
PPi
BIOTINOYL-AMP
apo-DOMAIN
AMP
holo-DOMAIN
(BIOTINYLATED PROTEIN)

Figure 2


A
GST-hPC-81
GST-hPC-108
GST-hPC-81
GST-hPC-108
Anti-GST
Streptavidin


B
Ratio Biotinylated protein / GST (units)
8
6
4
2
0
GST
hPC-81
hPC-108

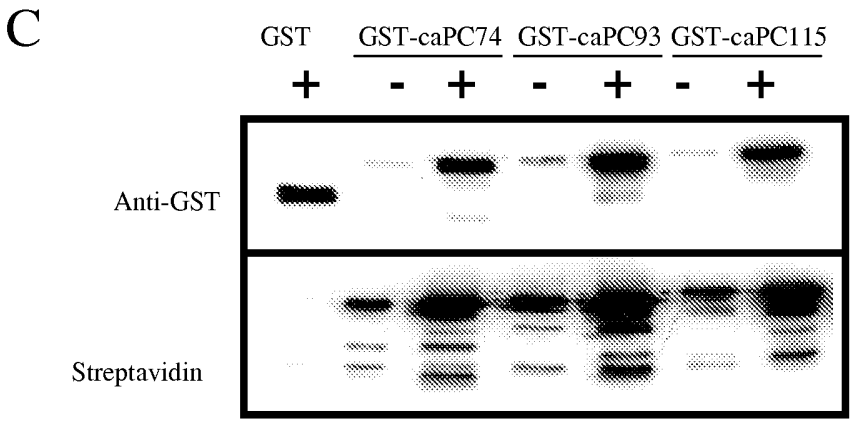

C
GST
GST-caPC74
GST-caPC93
GST-caPC115
+ - + - + - +
Anti-GST
Streptavidin

Figure 3

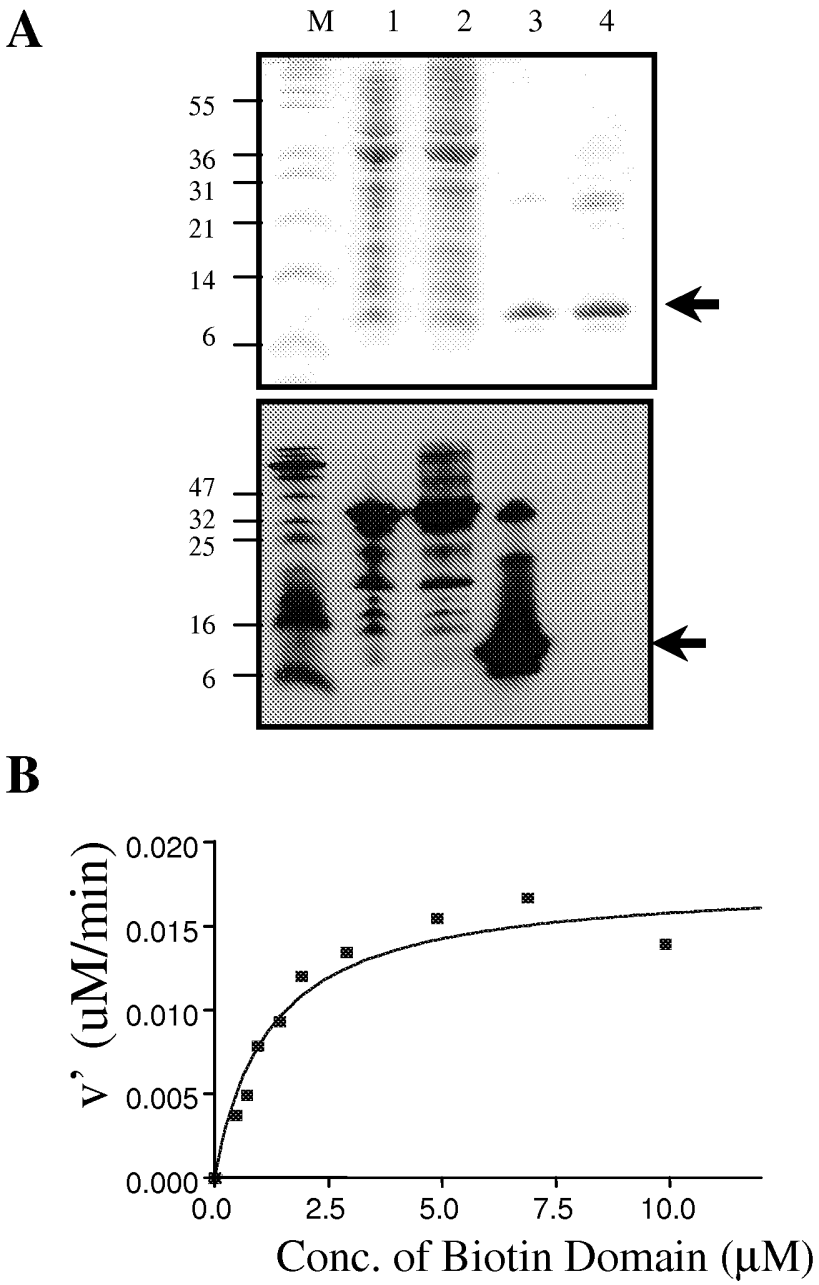

A
M
1
2
3
4
55
36
31
21
14
6
47
32
25
16
6
B
v' (uM/min)
0.020
0.015
0.010
0.005
0.000
0.0
2.5
5.0
7.5
10.0
Conc. of Biotin Domain (μM)

Figure 4

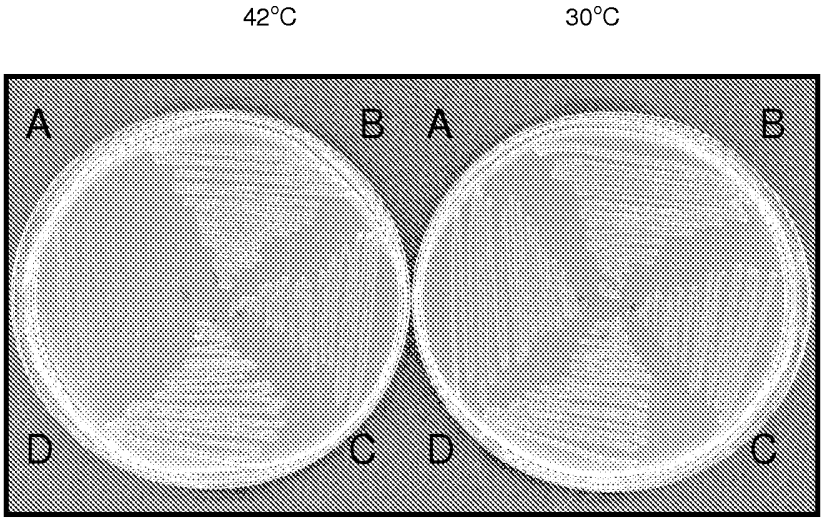


Figure 5

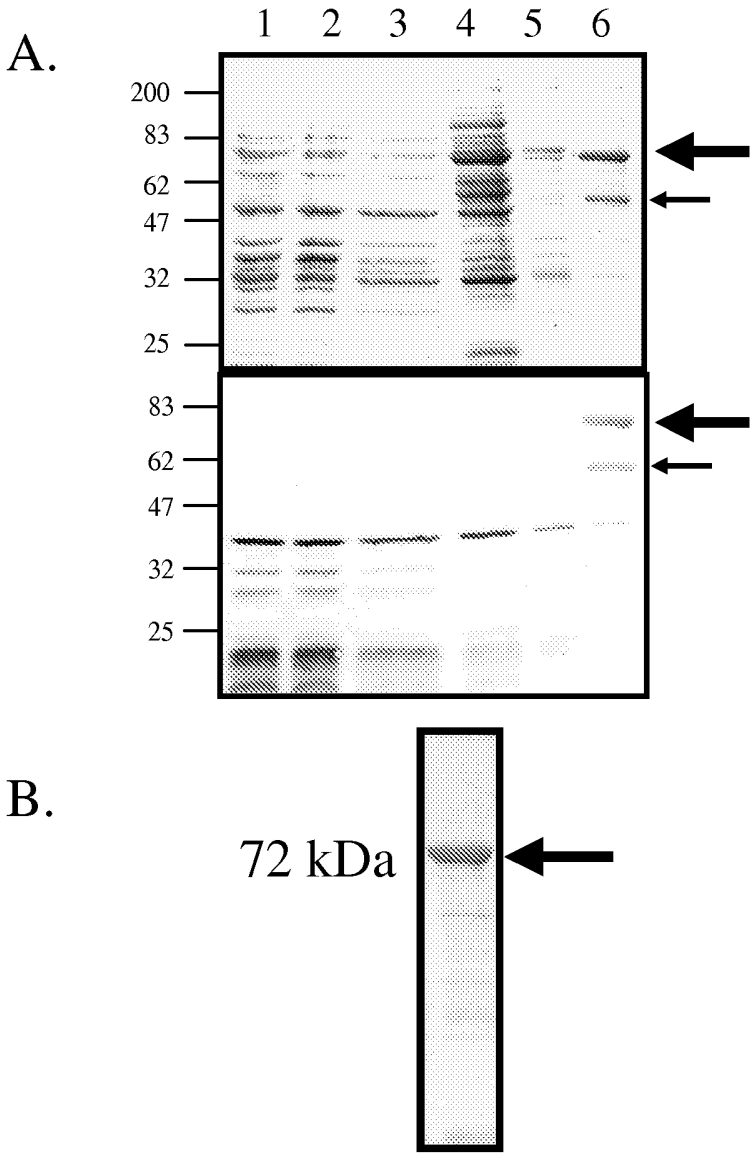


Figure 6

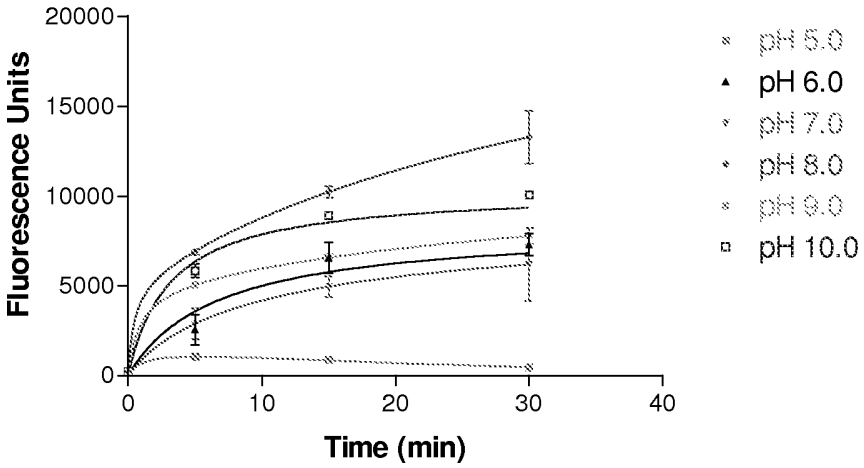

20000
15000
10000
5000
0
Fluorescence Units
0
10
20
30
40
Time (min)
pH 5.0
pH 6.0
pH 7.0
pH 8.0
pH 9.0
pH 10.0

Figure 7

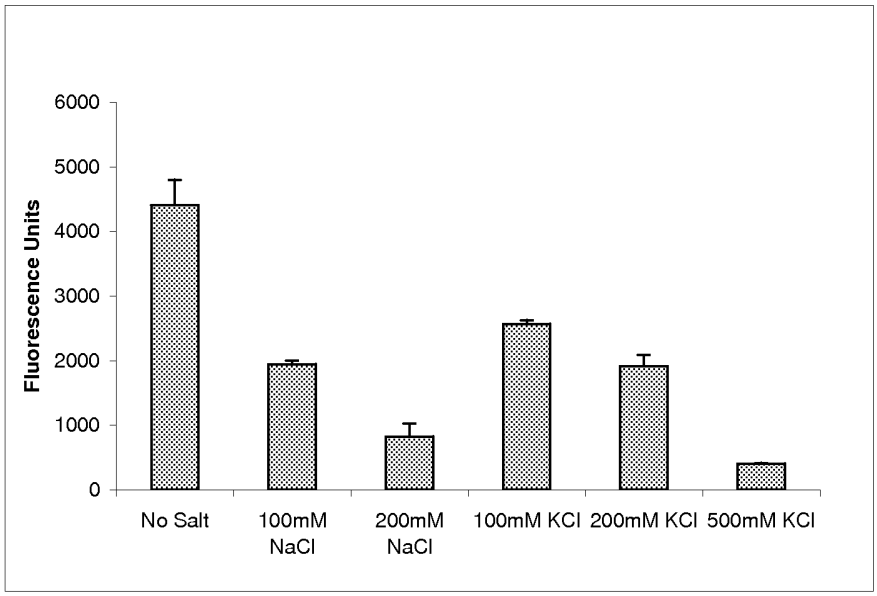

Fluorescence Units
6000
5000
4000
3000
2000
1000
0
No Salt
100mM NaCl
200mM NaCl
100mM KCl
200mM KCl
500mM KCl

Figure 8

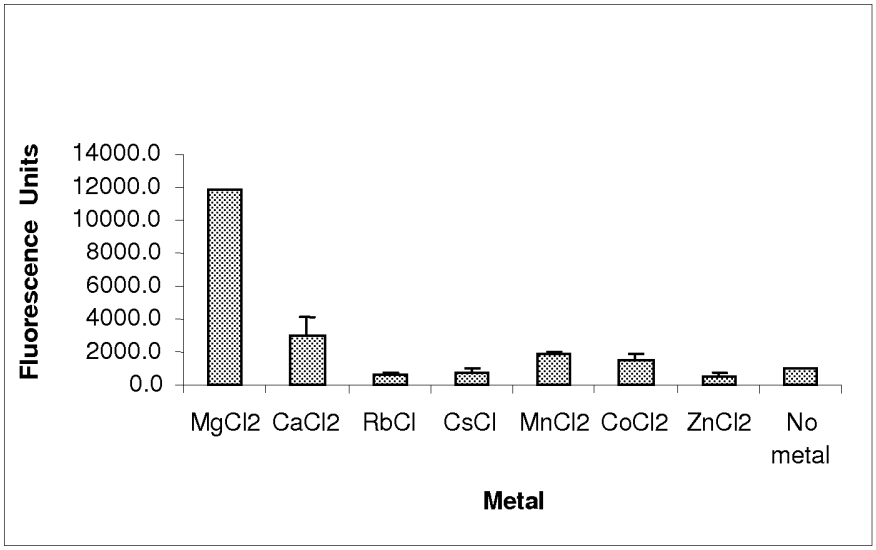

Fluorescence Units
14000.0
12000.0
10000.0
8000.0
6000.0
4000.0
2000.0
0.0
MgCl2
CaCl2
RbCl
CsCl
MnCl2
CoCl2
ZnCl2
No metal
Metal

Figure 9

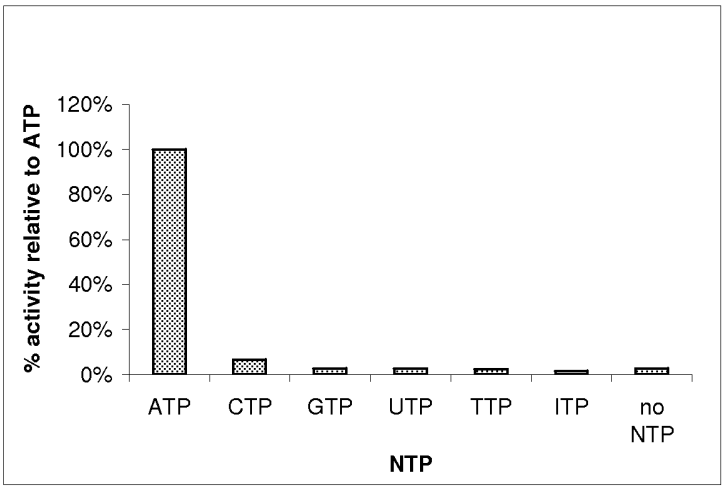

% activity relative to ATP
120%
100%
80%
60%
40%
20%
0%
ATP
CTP
GTP
UTP
TTP
ITP
no NTP
NTP

Figure 10

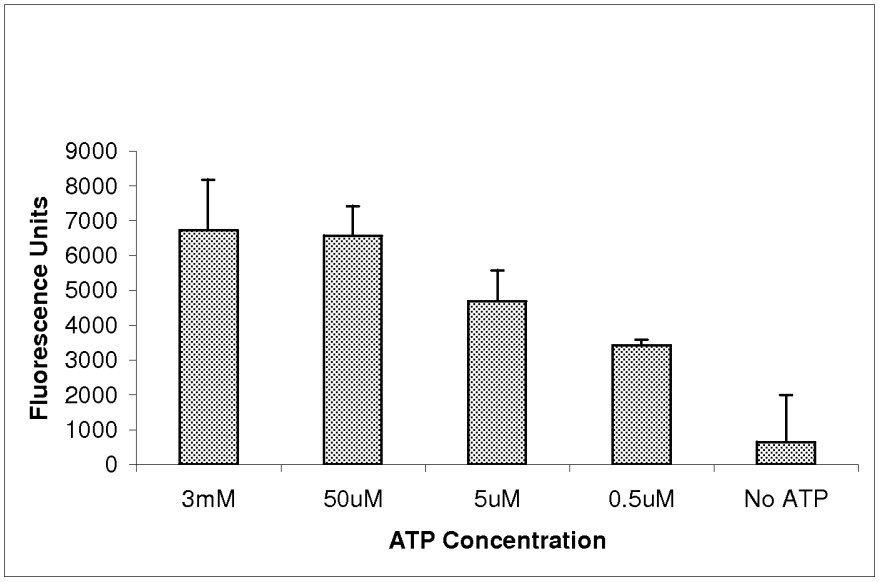

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Fluorescence Units
3mM
50uM
5uM
0.5uM
No ATP
ATP Concentration

Figure 11

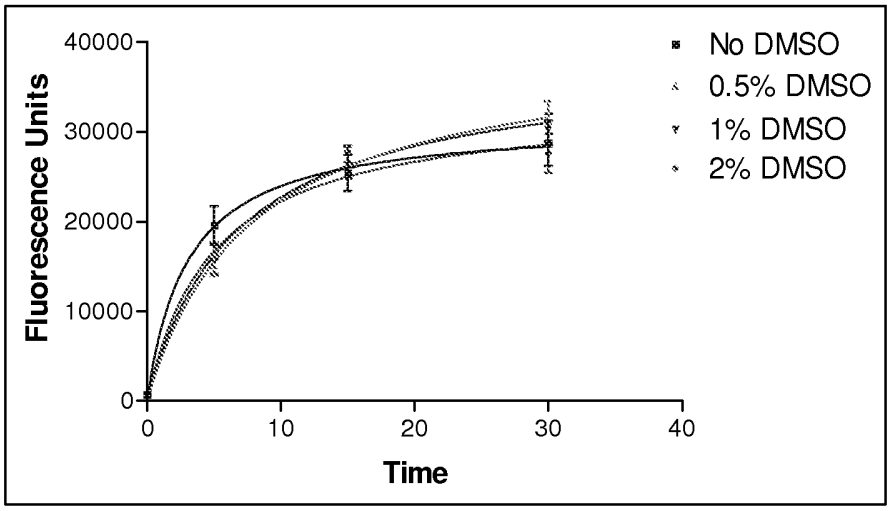

40000
30000
20000
10000
0
Fluorescence Units
0
10
20
30
40
Time
No DMSO
0.5% DMSO
1% DMSO
2% DMSO

Figure 12

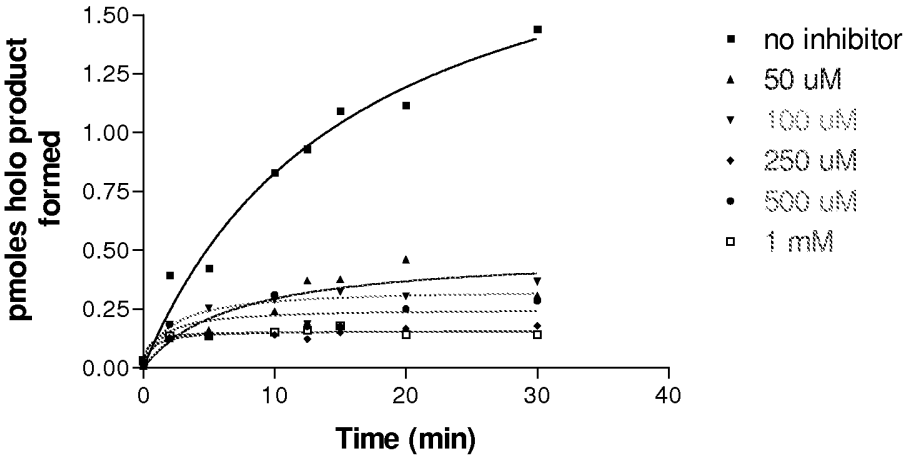

pmoles holo product formed
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
Time (min)
no inhibitor
50 uM
100 uM
250 uM
500 uM
1 mM

Figure 13

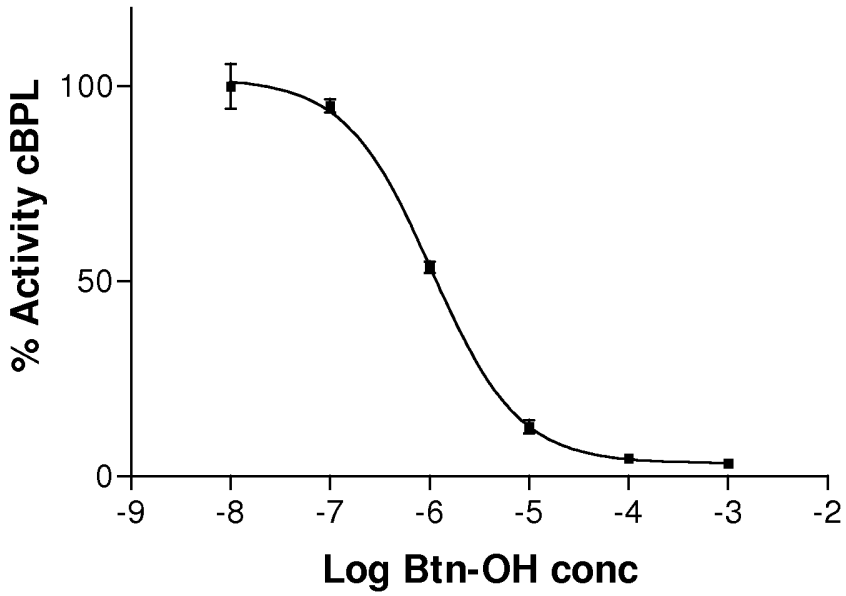


Figure 14

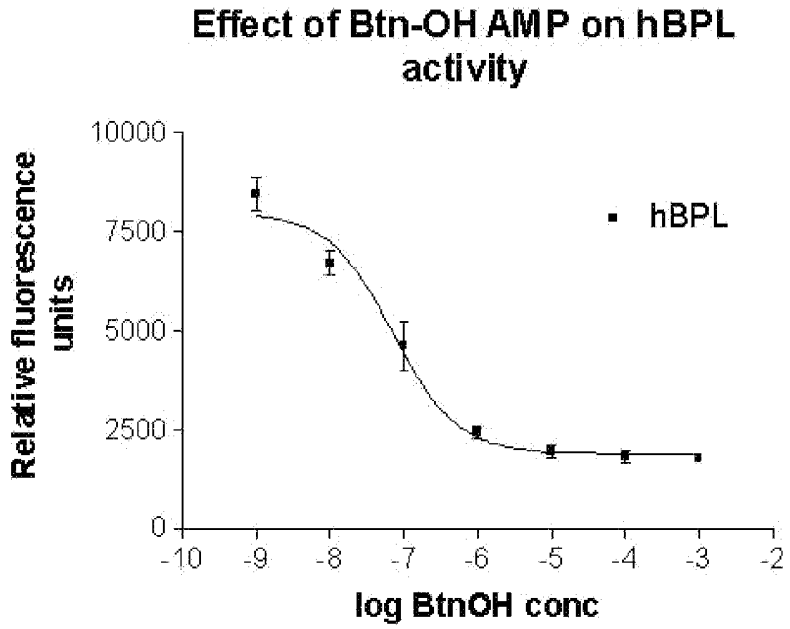

Effect of Btn-OH AMP on hBPL activity
Relative fluorescence units
10000
7500
5000
2500
0
-10
-9
-8
-7
-6
-5
-4
-3
-2
log BtnOH conc
hBPL

Figure 15

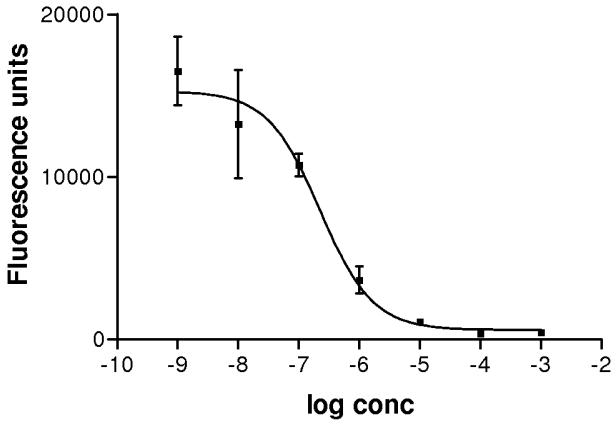


Figure 16

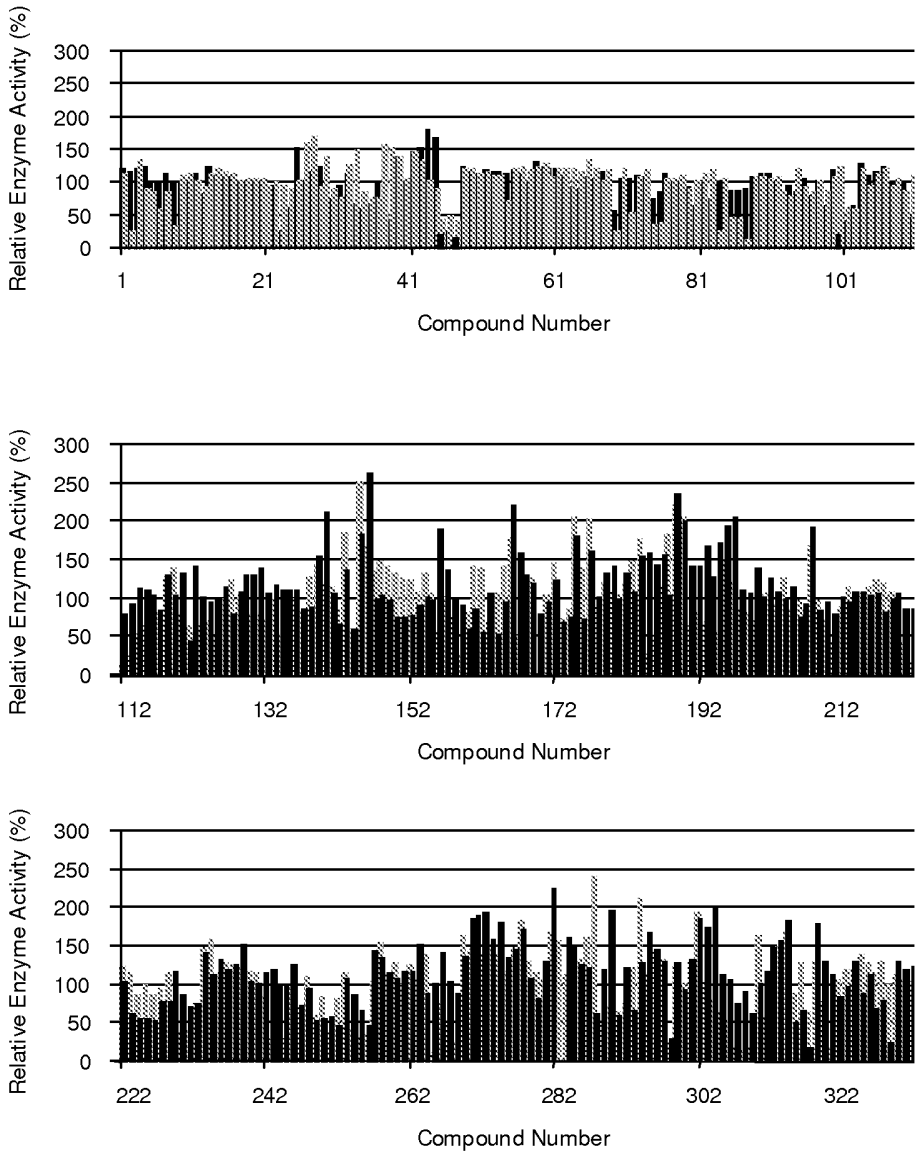
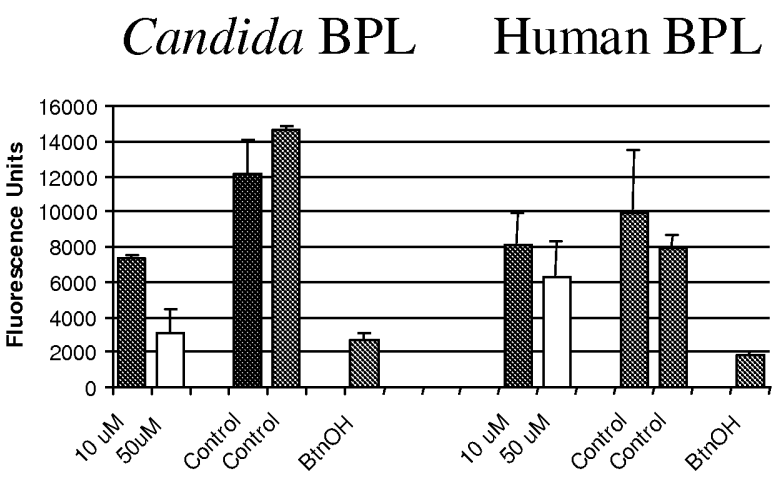

Relative Enzyme Activity (%)
300
250
200
150
100
50
0
1
21
41
61
81
101
Compound Number
Relative Enzyme Activity (%)
300
250
200
150
100
50
0
112
132
152
172
192
212
Compound Number
Relative Enzyme Activity (%)
300
250
200
150
100
50
0
222
242
262
282
302
322
Compound Number

Figure 17

A. Compound 69



B. Compound 296

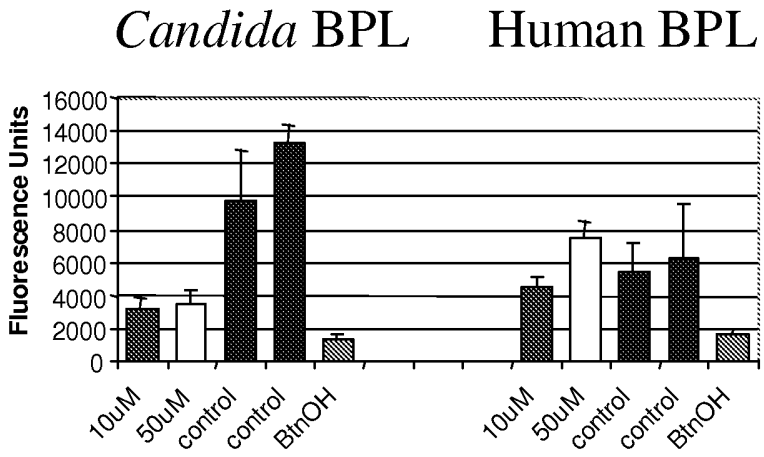


Figure 18

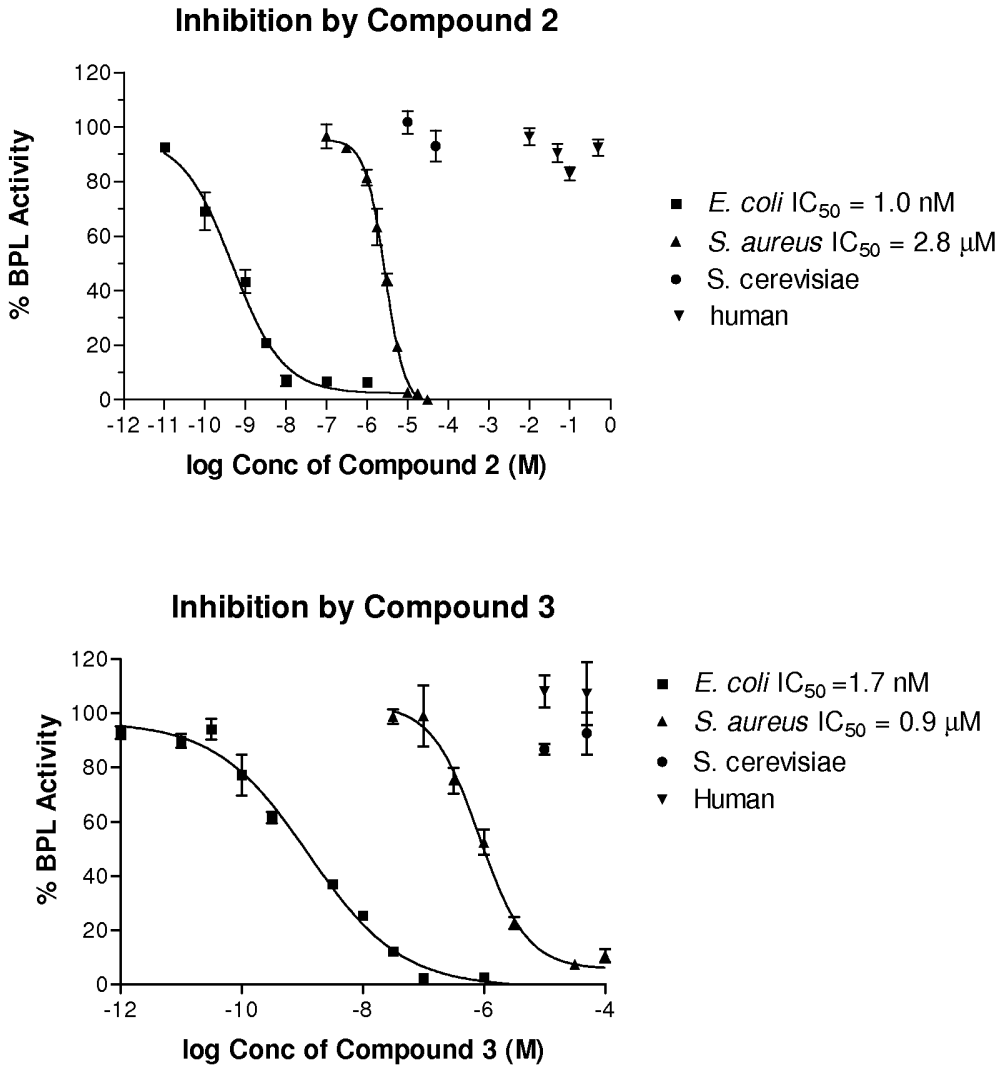
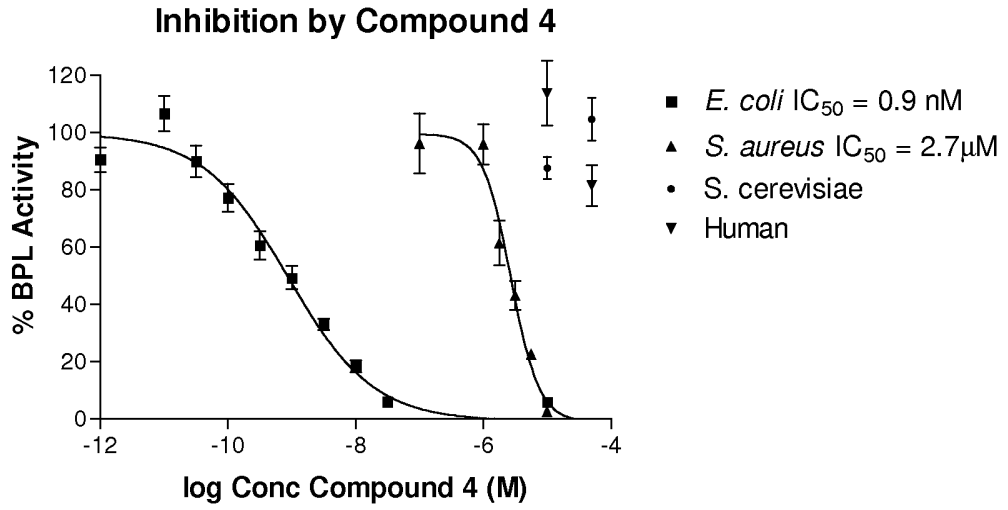

Inhibition by Compound 2
% BPL Activity
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0
log Conc of Compound 2 (M)
E. coli IC50 = 1.0 nM
S. aureus IC50 = 2.8 µM
S. cerevisiae
human
Inhibition by Compound 3
% BPL Activity
120
100
80
60
40
20
0
-12 -10 -8 -6 -4
log Conc of Compound 3 (M)
E. coli IC50 =1.7 nM
S. aureus IC50 = 0.9 µM
S. cerevisiae
Human

Figure 18 (cont.)



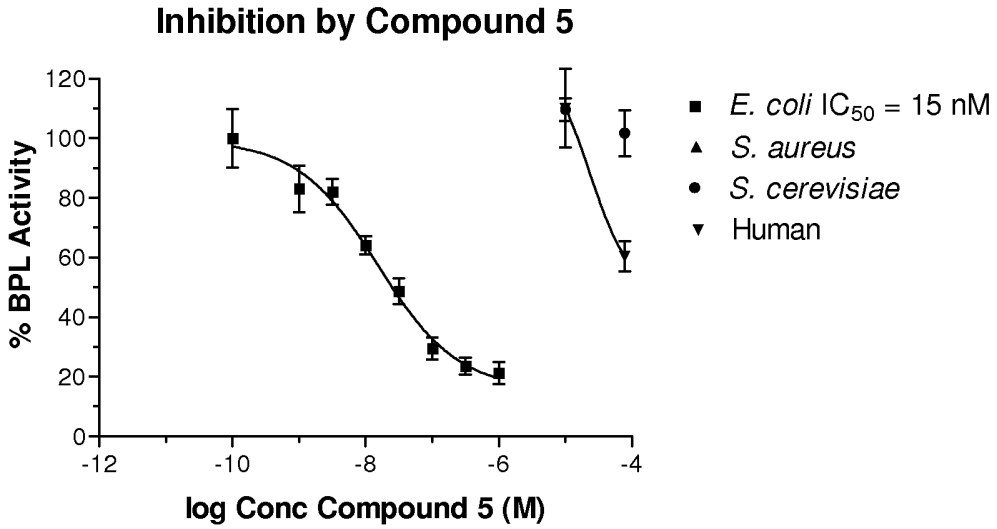
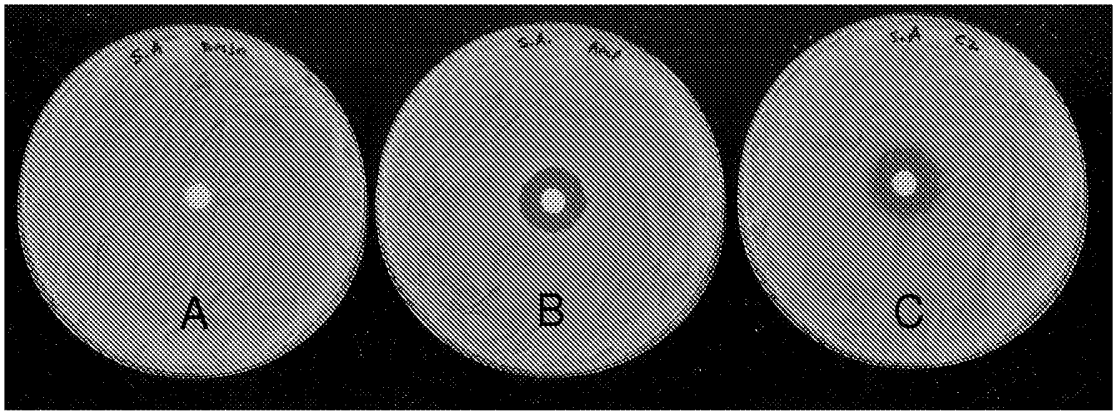
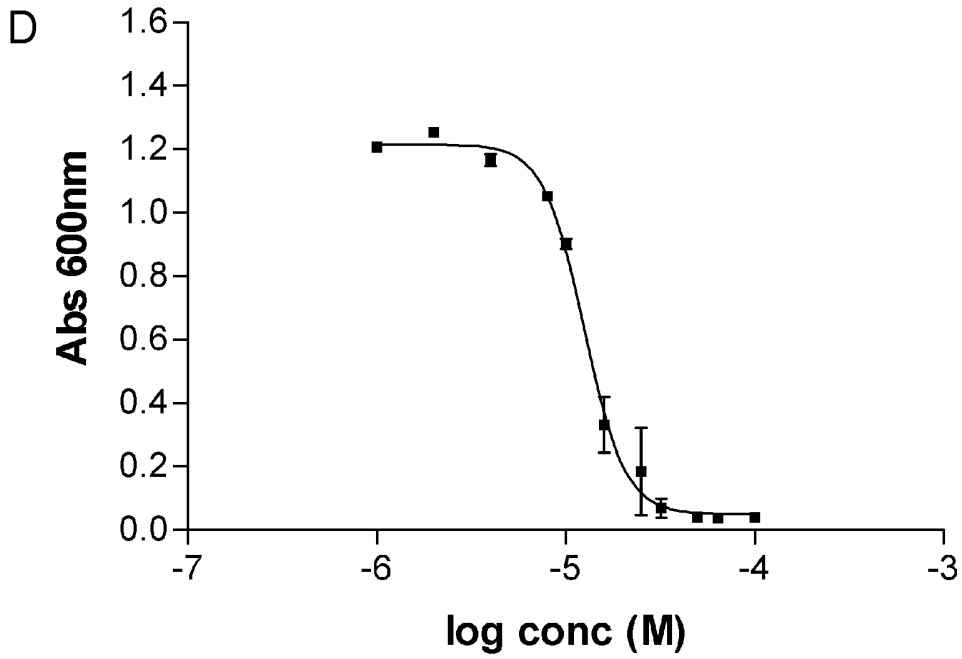


Figure 19





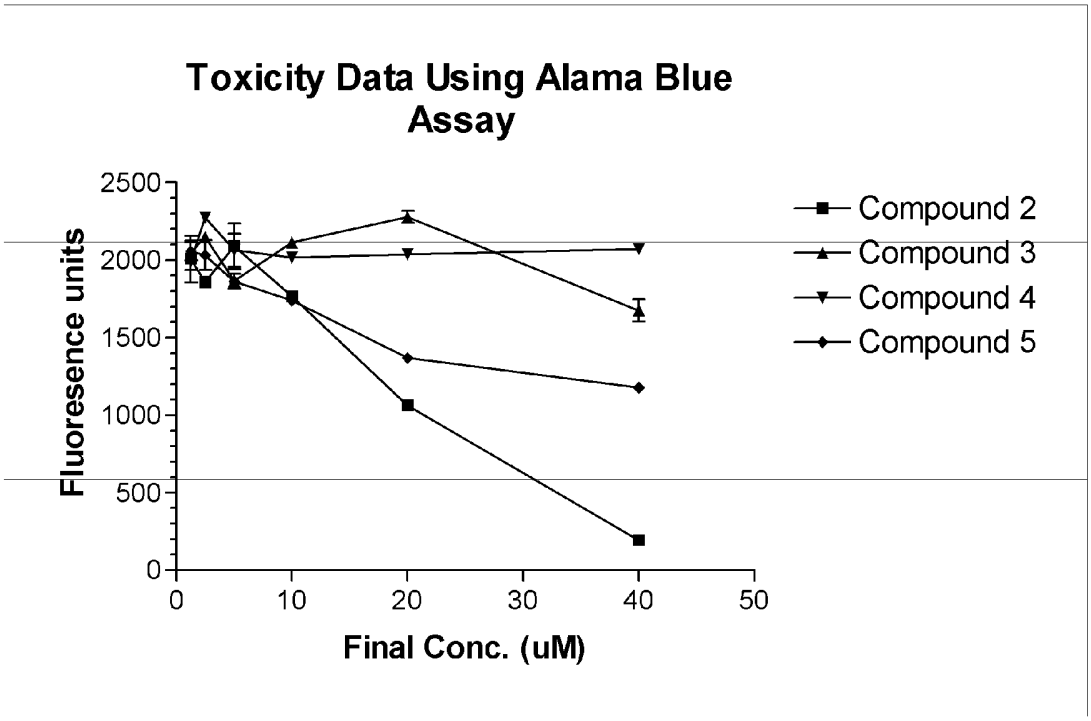

Toxicity Data Using Alama Blue Assay
Fluoresence units
2500
2000
1500
1000
500
0
0
10
20
30
40
50
Final Conc. (uM)
Compound 2
Compound 3
Compound 4
Compound 5

Figure 20

SEQUENCE LISTING

<110> Adelaide Research & Innovation Pty Ltd

<120> Inhibitors of Biotin Protein Ligase

<130> 2004906688

<160> 68

<170> PatentIn version 3.3

<210> 1

<211> 114

<212> PRT

<213> Candida albicans

<400> 1

Ala Val Gly Asp Val Ser Glu Lys Thr Gly Thr Arg Glu Val Phe Phe

1 5 10 15

Glu Leu Asn Gly Glu Met Arg Ser Val Ser Val Glu Asp Lys Thr Val

20 25 30

Ser Val Glu Leu Lys Thr Arg Pro Lys Ala Ser Ala Ser Asn Glu Val

35 40 45

Gly Ala Pro Met Ala Gly Val Val Ile Glu Ile Arg Ala His Lys His

50 55 60

Gln Gln Ile Ala Lys Gly Asp Pro Ile Ala Val Leu Ser Ala Met Lys

65 70 75 80

Met Glu Met Val Ile Ser Ala Pro Cys Ser Gly Glu Ile Gly Asp Ile

85 90 95

Leu Ile His Glu Gly Asp Ser Val Asp Ala Asn Asp Leu Ile Thr Ser

100 105 110

Ile His

<210> 2

<211> 92

<212> PRT

<213> Candida albicans

<400> 2

Arg Ser Val Ser Val Glu Asp Lys Thr Val Ser Val Glu Leu Lys Thr
1 5 10 15

Arg Pro Lys Ala Ser Ala Ser Asn Glu Val Gly Ala Pro Met Ala Gly
20 25 30

Val Val Ile Glu Ile Arg Ala His Lys His Gln Gln Ile Ala Lys Gly
35 40 45

Asp Pro Ile Ala Val Leu Ser Ala Met Lys Met Glu Met Val Ile Ser

50 55 60

Ala Pro Cys Ser Gly Glu Ile Gly Asp Ile Leu Ile His Glu Gly Asp

65 70 75 80

Ser Val Asp Ala Asn Asp Leu Ile Thr Ser Ile His

85 90

<210> 3

<211> 73

<212> PRT

<213> Candida albicans

<400> 3

Ala Ser Ala Ser Asn Glu Val Gly Ala Pro Met Ala Gly Val Val Ile

1 5 10 15

Glu Ile Arg Ala His Lys His Gln Gln Ile Ala Lys Gly Asp Pro Ile

20 25 30

Ala Val Leu Ser Ala Met Lys Met Glu Met Val Ile Ser Ala Pro Cys

35 40 45

Ser Gly Glu Ile Gly Asp Ile Leu Ile His Glu Gly Asp Ser Val Asp

50 55 60

Ala Asn Asp Leu Ile Thr Ser Ile His

65 70

<210> 4

<211> 124

<212> PRT

<213> Drosophila melanogaster

<400> 4

Lys Thr Leu Ser Val Lys Ala Leu Ala Val Ser Ala Asp Leu Lys Pro
1 5 10 15

Asn Gly Ile Arg Glu Val Phe Phe Glu Leu Asn Gly Gln Leu Arg Ala
20 25 30

Val His Ile Leu Asp Lys Glu Ala Val Lys Glu Ile His Val His Pro
35 40 45

Lys Ala Asn Lys Ser Asn Lys Ser Glu Val Gly Ala Pro Met Pro Gly
50 55 60

Thr Val Ile Asp Ile Arg Val Lys Val Gly Asp Lys Val Glu Lys Gly
65 70 75 80

Gln Pro Leu Val Val Leu Ser Ala Met Lys Met Glu Met Val Val Gln
85 90 95

Ser Pro Leu Ala Gly Val Val Lys Lys Leu Glu Ile Ala Asn Gly Thr
100 105 110

Lys Leu Glu Gly Glu Asp Leu Ile Met Ile Ile Glu
115 120

<210> 5

<211> 86

<212> PRT

<213> Escherichia coli

<400> 5

Glu Ala Pro Ala Ala Ala Glu Ile Ser Gly His Ile Val Arg Ser Pro
1 5 10 15

Met Val Gly Thr Phe Tyr Arg Thr Pro Ser Pro Asp Ala Lys Ala Phe
20 25 30

Ile Glu Val Gly Gln Lys Val Asn Val Gly Asp Thr Leu Cys Ile Val
35 40 45

Glu Ala Met Lys Met Met Asn Gln Ile Glu Ala Asp Lys Ser Gly Thr
50 55 60

Val Lys Ala Ile Leu Val Glu Ser Gly Gln Pro Val Glu Phe Asp Glu
65 70 75 80

Pro Leu Val Val Ile Glu
85

<210> 6

<211> 14

<212> PRT

<213> Escherichia coli

<400> 6

Leu Gly Gly Ile Phe Glu Ala Met Lys Met Glu Leu Arg Asp
1 5 10

<210> 7

<211> 21

<212> PRT

<213> Escherichia coli

<400> 7

Leu Phe Leu His Asp Phe Leu Asn Ala Gln Lys Val Glu Leu Tyr Pro
1 5 10 15

Val Thr Ser Ser Gly
20

<210> 8

<211> 23

<212> PRT

<213> Escherichia coli

<400> 8

Met Ala Gly Gly Leu Asn Asp Ile Phe Glu Ala Gln Lys Ile Glu Trp
1 5 10 15

His Glu Asp Thr Gly Gly Ser
20

<210> 9

<211> 80

<212> PRT

<213> Homo sapiens

<400> 9

His Phe His Pro Lys Ala Leu Lys Asp Val Lys Gly Gln Ile Gly Ala
1 5 10 15

Pro Met Pro Gly Lys Val Ile Asp Ile Lys Val Val Ala Gly Ala Lys

20 25 30

Val Ala Lys Gly Gln Pro Leu Cys Val Leu Ser Ala Met Lys Met Glu

35 40 45

Thr Val Val Thr Ser Pro Met Glu Gly Thr Val Arg Lys Val His Val

50 55 60

Thr Lys Asp Met Thr Leu Glu Gly Asp Asp Leu Ile Leu Glu Ile Glu

65 70 75 80

<210> 10

<211> 107

<212> PRT

<213> Homo sapiens

<400> 10

Gly Gln Arg Gln Val Phe Phe Glu Leu Asn Gly Gln Leu Arg Ser Ile
1 5 10 15

Leu Val Lys Asp Thr Gln Ala Met Lys Glu Met His Phe His Pro Lys
20 25 30

Ala Leu Lys Asp Val Lys Gly Gln Ile Gly Ala Pro Met Pro Gly Lys
35 40 45

Val Ile Asp Ile Lys Val Val Ala Gly Ala Lys Val Ala Lys Gly Gln
50 55 60

Pro Leu Cys Val Leu Ser Ala Met Lys Met Glu Thr Val Val Thr Ser
65 70 75 80

Pro Met Glu Gly Thr Val Arg Lys Val His Val Thr Lys Asp Met Thr
85 90 95

Leu Glu Gly Asp Asp Leu Ile Leu Glu Ile Glu

100 105

<210> 11

<211> 80

<212> PRT

<213> Homo sapiens

<400> 11

His Phe His Pro Lys Ala Leu Lys Asp Val Lys Gly Gln Ile Gly Ala

1 5 10 15

Pro Met Pro Gly Lys Val Ile Asp Ile Lys Val Val Ala Gly Ala Lys

20 25 30

Val Ala Lys Gly Gln Pro Leu Cys Val Leu Ser Ala Met Lys Met Glu

35 40 45

Thr Val Val Thr Ser Pro Met Glu Gly Thr Val Arg Lys Val His Val
50 55 60

Thr Lys Asp Met Thr Leu Glu Gly Asp Asp Leu Ile Leu Glu Ile Glu
65 70 75 80

<210> 12

<211> 73

<212> PRT

<213> Candida albicans

<400> 12

Ala Ser Ala Ser Asn Glu Val Gly Ala Pro Met Ala Gly Val Val Ile
1 5 10 15

Glu Ile Arg Ala His Lys His Gln Gln Ile Ala Lys Gly Asp Pro Ile
20 25 30

Ala Val Leu Ser Ala Met Lys Met Glu Met Val Ile Ser Ala Pro Cys
35 40 45

Ser Gly Glu Ile Gly Asp Ile Leu Ile His Glu Gly Asp Ser Val Asp
50 55 60

Ala Asn Asp Leu Ile Thr Ser Ile His
65 70

<210> 13
<211> 114
<212> PRT
<213> Candida albicans

<400> 13

Ala Val Gly Asp Val Ser Glu Lys Thr Gly Thr Arg Glu Val Phe Phe
1 5 10 15

Glu Leu Asn Gly Glu Met Arg Ser Val Ser Val Glu Asp Lys Thr Val

20 25 30

Ser Val Glu Leu Lys Thr Arg Pro Lys Ala Ser Ala Ser Asn Glu Val

35 40 45

Gly Ala Pro Met Ala Gly Val Val Ile Glu Ile Arg Ala His Lys His

50 55 60

Gln Gln Ile Ala Lys Gly Asp Pro Ile Ala Val Leu Ser Ala Met Lys

65 70 75 80

Met Glu Met Val Ile Ser Ala Pro Cys Ser Gly Glu Ile Gly Asp Ile

85 90 95

Leu Ile His Glu Gly Asp Ser Val Asp Ala Asn Asp Leu Ile Thr Ser

100 105 110

Ile His

<210> 14

<211> 92

<212> PRT

<213> Candida albicans

<400> 14

Arg Ser Val Ser Val Glu Asp Lys Thr Val Ser Val Glu Leu Lys Thr

1 5 10 15

Arg Pro Lys Ala Ser Ala Ser Asn Glu Val Gly Ala Pro Met Ala Gly

20 25 30

Val Val Ile Glu Ile Arg Ala His Lys His Gln Gln Ile Ala Lys Gly

35 40 45

Asp Pro Ile Ala Val Leu Ser Ala Met Lys Met Glu Met Val Ile Ser

50 55 60

Ala Pro Cys Ser Gly Glu Ile Gly Asp Ile Leu Ile His Glu Gly Asp

65 70 75 80

Ser Val Asp Ala Asn Asp Leu Ile Thr Ser Ile His

85 90

<210> 15

<211> 73

<212> PRT

<213> Candida albicans

<400> 15

Ala Ser Ala Ser Asn Glu Val Gly Ala Pro Met Ala Gly Val Val Ile

1 5 10 15

Glu Ile Arg Ala His Lys His Gln Gln Ile Ala Lys Gly Asp Pro Ile
20 25 30

Ala Val Leu Ser Ala Met Lys Met Glu Met Val Ile Ser Ala Pro Cys
35 40 45

Ser Gly Glu Ile Gly Asp Ile Leu Ile His Glu Gly Asp Ser Val Asp
50 55 60

Ala Asn Asp Leu Ile Thr Ser Ile His
65 70

<210> 16

<211> 107

<212> PRT

<213> Homo sapiens

<400> 16

Gly Gln Arg Gln Val Phe Phe Glu Leu Asn Gly Gln Leu Arg Ser Ile
1 5 10 15

Leu Val Lys Asp Thr Gln Ala Met Lys Glu Met His Phe His Pro Lys
20 25 30

Ala Leu Lys Asp Val Lys Gly Gln Ile Gly Ala Pro Met Pro Gly Lys
35 40 45

Val Ile Asp Ile Lys Val Val Ala Gly Ala Lys Val Ala Lys Gly Gln
50 55 60

Pro Leu Cys Val Leu Ser Ala Met Lys Met Glu Thr Val Val Thr Ser
65 70 75 80

Pro Met Glu Gly Thr Val Arg Lys Val His Val Thr Lys Asp Met Thr
85 90 95

Leu Glu Gly Asp Asp Leu Ile Leu Glu Ile Glu
100 105

<210> 17

<211> 80

<212> PRT

<213> Homo sapiens

<400> 17

His Phe His Pro Lys Ala Leu Lys Asp Val Lys Gly Gln Ile Gly Ala
1 5 10 15

Pro Met Pro Gly Lys Val Ile Asp Ile Lys Val Val Ala Gly Ala Lys
20 25 30

Val Ala Lys Gly Gln Pro Leu Cys Val Leu Ser Ala Met Lys Met Glu
35 40 45

Thr Val Val Thr Ser Pro Met Glu Gly Thr Val Arg Lys Val His Val
50 55 60

Thr Lys Asp Met Thr Leu Glu Gly Asp Asp Leu Ile Leu Glu Ile Glu
65 70 75 80

<210> 18
<211> 114
<212> PRT
<213> Candida albicans

<400> 18

Ala Val Gly Asp Val Ser Glu Lys Thr Gly Thr Arg Glu Val Phe Phe
1 5 10 15

Glu Leu Asn Gly Glu Met Arg Ser Val Ser Val Glu Asp Lys Thr Val

20 25 30

Ser Val Glu Leu Lys Thr Arg Pro Lys Ala Ser Ala Ser Asn Glu Val

35 40 45

Gly Ala Pro Met Ala Gly Val Val Ile Glu Ile Arg Ala His Lys His

50 55 60

Gln Gln Ile Ala Lys Gly Asp Pro Ile Ala Val Leu Ser Ala Met Lys

65 70 75 80

Met Glu Met Val Ile Ser Ala Pro Cys Ser Gly Glu Ile Gly Asp Ile

85 90 95

Leu Ile His Glu Gly Asp Ser Val Asp Ala Asn Asp Leu Ile Thr Ser

100 105 110

Ile His

<210> 19

<211> 92

<212> PRT

<213> Candida albicans

<400> 19

Arg Ser Val Ser Val Glu Asp Lys Thr Val Ser Val Glu Leu Lys Thr
1 5 10 15

Arg Pro Lys Ala Ser Ala Ser Asn Glu Val Gly Ala Pro Met Ala Gly
20 25 30

Val Val Ile Glu Ile Arg Ala His Lys His Gln Gln Ile Ala Lys Gly
35 40 45

Asp Pro Ile Ala Val Leu Ser Ala Met Lys Met Glu Met Val Ile Ser

50 55 60

Ala Pro Cys Ser Gly Glu Ile Gly Asp Ile Leu Ile His Glu Gly Asp

65 70 75 80

Ser Val Asp Ala Asn Asp Leu Ile Thr Ser Ile His

85 90

<210> 20

<211> 73

<212> PRT

<213> Candida albicans

<400> 20

Ala Ser Ala Ser Asn Glu Val Gly Ala Pro Met Ala Gly Val Val Ile

1 5 10 15

Glu Ile Arg Ala His Lys His Gln Gln Ile Ala Lys Gly Asp Pro Ile

20 25 30

Ala Val Leu Ser Ala Met Lys Met Glu Met Val Ile Ser Ala Pro Cys

35 40 45

Ser Gly Glu Ile Gly Asp Ile Leu Ile His Glu Gly Asp Ser Val Asp

50 55 60

Ala Asn Asp Leu Ile Thr Ser Ile His

65 70

<210> 21

<211> 38

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 21

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<210> 22

<211> 43

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 22

atctacgaat tcatcaatga atactagtaa tcaaatcatt agc 43

<210> 23

<211> 34

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 23

atctacggat ccatgagatc agtttccgtt gaag 34

<210> 24

<211> 43

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 24

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<211> 28

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 25

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<210> 26

<211> 43

<212> DNA

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<400> 26

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<211> 35

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 27

aattggtaga attcatgcac ttccacccca aggcc 35

<210> 28

<211> 42

<212> DNA

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<220>

<223> primer sequence

<400> 28

gattaattct cgagttatca ctcgatctcc aggatgaggt cg 42

<210> 29

<211> 35

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 29

aattggtaga attcgccggc cagaggcagg tcttc 35

<210> 30

<211> 42

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 30

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<210> 31

<211> 1992

<212> DNA

<213> Candida albicans

<400> 31

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ttaccatact gtaatgtact tgatggaaat ggcacgagaa agatttccaa gtatgtcaaa 240

caaggtggga agtttttagg gctttgtgct ggtgggtatt ttggatctgc taggtgtgag 300

tttgaagttg gcaatcctac catggaggtt actggaccta gggaacttgg gtttttccca 360

ggaacggcca agggatgtgc atttaaaggt ttcaagtacg aactgcgtac tggtgcaaga 420

gccgtcaaat tgctggtcaa tacagctgca ttacctggat gtgcaagtca catttataat 480

tattacgatg gcggggcggt gtttgccaat gctgaaaagt ataaagatgt tgaaatcttg 540

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aaggtcggta agggtggtgt tattttgagt ggtactcatc ctgaatttgc acctcacttg 660

ttgcatccac gagatgaaga tggggcaggt tattttatag tggttgacac acttcgagca 720

tacgatcaca acaaaaaggt atttatgaga gactgtttga agaaattggg cttgcgagtt 780

gccgagagtg ttgatacaac gattccaaga gttacaccaa tgtatgttgt atctccgttc 840

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gatagtaatg atgcttttta ttttgctgac gaaacgcagg aaactagtga gtatgttggt 960

agtgaagaag atccagtcaa gtatatcaac tttctcactc tggccggtat accggatttg 1020

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aaacggtggt tccataccaa ccagaaagtg gacgttgaca atggcagtgg gaaacaaaga 1860

acatgtataa tcaagggtat taccccagac tatggattgc ttattgctga agatgtagag 1920

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gtatataaga ag 1992

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<211> 32

<212> DNA

<213> Artificial

<220>

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<400> 32

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<210> 33

<211> 39

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 33

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<210> 34

<211> 44

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 34

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<210> 35

<211> 44

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 35

tccggtatac cggcgctagt gagaaagttg atatacttga ctgg 44

<210> 36

<211> 20

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 36

gtggatttag agaaggctgc 20

<210> 37

<211> 40

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 37

gtggttgaca cacttcgagc atacgatcac aacaaaaagg 40

<210> 38

<211> 40

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 38

ccttttgtt gtgatcgtat gctcgaagtg tgtcaaccac 40

<210> 39

<211> 22

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 39

actagtgagt atgttggtag tg 22

<210> 40

<211> 65

<212> DNA

<213> Artificial

<220>

<223> primer sequence

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aaacc 65

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<211> 2035

<212> DNA

<213> Candida albicans

<400> 41

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atttaccata ctgtaatgta cttgatggaa atggcacgag aaagatttcc aagtatgtca 240

aacaaggtgg gaagttttta gggctttgtg ctggtgggta ttttggatct gctaggtgtg 300

agtttgaagt tggcaatcct accatggagg ttactggacc tagggaactt gggtttttcc 360

caggaacggc caagggatgt gcatttaaag gtttcaagta cgaaagtcgt actggcgcca 420

gagccgtcaa attgagcgtc aatacagctg cattacctgg atgtgcaagt cacatttata 480

attattacga tggcggggcg gtgtttgcca atgctgaaaa gtataaagat gttgaaatct 540

tggcaaggta cgacgacaaa actgatattg tggatttaga gaaggctgct gttgtataca 600

gaaaggtcgg taagggtggt gttattttga gtggtactca tcctgaattt gcacctcact 660

tgttgcatcc acgagatgaa gatggggcag gttattttat agtggttgac acacttcgag 720

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ttgccgagag tgttgataca acgattccaa gagttacacc aatgtatgtt gtgtctccgt 840

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aggatagtaa tgatgctttt tattttgctg acgaaacgca ggaaactagt gagtatgttg 960

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caattatgga taagaatccg caatggttag agcatcttcc taatgggttt accattactg 1200

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gaacatgtat aatcaagggt attaccccag actacggatt gcttattgct gaagatgtag 1920

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<210> 42

<211> 2181

<212> DNA

<213> Homo sapiens

<400> 42

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ctagagccga agcctgaacc ttctcttgag attaagcctg agcaggacgg tatggagcat 180

gttggcagag atgacccaaa ggctcttggt gaagaaccca aacaaaggag aggcagtgcc 240

tctgggagtg agcctgctgg ggacagtgac aggggagggg gccccgttga gcattatcac 300

ctccatctgt ctagttgcca cgagtgtctg gaacttgaga acagcaccat tgagtcagtc 360

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accaacatgg aggccttctc atcagaacat ttcaacttag agatctatcg ccaaaatctg 1380

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cggcagaccg agggcaaagg acggggaggg aatgtgtggc tgagccctgt gggatgtgct 1560

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<211> 35

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 43

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<211> 18

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<213> Artificial

<220>

<223> primer sequence

<400> 44

ggagacgcat cgttgtgg 18

<210> 45

<211> 20

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 45

cttgtatacc tgtggtgacc 20

<210> 46

<211> 64

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 46

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ttct 64

<210> 47

<211> 29

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 47

aaggagagcc atggcctctg ggagtgagc 29

<210> 48

<211> 23

<212> DNA

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<220>

<223> primer sequence

<400> 48

tcctgtcctt gtcctcattc tcc 23

<210> 49

<211> 1973

<212> DNA

<213> Homo sapiens

<400> 49

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gaaacctcat cctccccaaa cggcggcatc atcatcatca tcattagctc gag 1973

<210> 50

<211> 966

<212> DNA

<213> Escherichia coli

<400> 50

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cagacactgc gtgactgggg cgttgatgtc tttaccgttc cgggtaaagg atacagcctg 180

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aaataa 966

<210> 51

<211> 86

<212> PRT

<213> Escherichia coli

<400> 51

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20 25 30

Ile Glu Val Gly Gln Lys Val Asn Val Gly Asp Thr Leu Cys Ile Val
35 40 45

Glu Ala Met Lys Met Met Asn Gln Ile Glu Ala Asp Lys Ser Gly Thr
50 55 60

Val Lys Ala Ile Leu Val Glu Ser Gly Gln Pro Val Glu Phe Asp Glu

65 70 75 80

Pro Leu Val Val Ile Glu
85

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<211> 32

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 52

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<210> 53

<211> 34

<212> DNA

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<220>

<223> primer sequence

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<210> 54

<211> 1104

<212> DNA

<213> Arabidopsis thaliana

<400> 54

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aagggtttga agaacaagaa ctctcttaaa ctcccagata ataccaaagt ttcactcatt 240

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ggtctggttc gaagaaaaat atga 1104

<210> 55

<211> 35

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 55

atctatccat ggaagcagtt cgttcaacaa caacc 35

<210> 56

<211> 68

<212> DNA

<213> Artificial

<220>

<223> primer sequence

<400> 56

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accagacc 68

<210> 57

<211> 1144

<212> DNA

<213> Arabidopsis thaliana

<400> 57

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<211> 93

<212> PRT

<213> Arabidopsis thaliana

<400> 58

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Pro Met Ala Gly Thr Phe Tyr Arg Ser Pro Ala Pro Gly Glu Pro Pro

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Phe Ile Lys Val Gly Asp Lys Val Gln Lys Gly Gln Val Leu Cys Ile
35 40 45

Val Glu Ala Met Lys Leu Met Asn Glu Ile Glu Ser Asp His Thr Gly
50 55 60

Thr Val Val Asp Ile Val Ala Glu Asp Gly Lys Pro Val Ser Leu Asp
65 70 75 80

Thr Pro Leu Phe Val Val Gln Pro Val Glu Ser Ala Pro
85 90

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<211> 32

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<223> primer sequence

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<210> 60

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<212> DNA

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<220>

<223> primer sequence

<400> 60

atctacgaat tcatcatggt gccgattcta cgg 33

<210> 61

<211> 3126

<212> DNA

<213> Drosophila melanogaster

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<210> 64

<211> 30

<212> PRT

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<223> six histidine extension

<400> 64

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<211> 3227

<212> DNA

<213> Drosophila melanogaster

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Val His Ile Leu Asp Lys Glu Ala Val Lys Glu Ile His Val His Pro
35 40 45

Lys Ala Asn Lys Ser Asn Lys Ser Glu Val Gly Ala Pro Met Pro Gly
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Thr Val Ile Asp Ile Arg Val Lys Val Gly Asp Lys Val Glu Lys Gly
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Gln Pro Leu Val Val Leu Ser Ala Met Lys Met Glu Met Val Val Gln
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<212> DNA

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<400> 68

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/001768

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12Q 1/25 (2006.01) ***C12N 9/00*** (2006.01)
A61K 31/00 (2006.01) ***C12N 9/99*** (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CAPLUS, BIOSIS, WPIDS (biotin ligase, biotin protein ligase, bpl, bira, biotin holoenzyme synthetase, dhbh, bior, inhibit?, modulat?, down regulate, antifung?, antibiotic, biocid?), REGISTRY (structure search)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004053485 A1 (OXFORD GLYCOSCIENCES (UK) LTD) 24 June 2004. See whole document.	1 – 8, 23 – 25, 26, 27, 30, 32, 35, 37, 39, 41, 45 – 47, 53, 55
X	WO 2000043418 A1 (SMITHKLINE BEECHAM CORPORATION) 27 July 2000. See pg's 16 – 21.	1 – 8, 23 – 25, 26 – 27, 30, 31, 35, 37, 45 – 47, 53, 55

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 January 2006	0 8 FEB 2006
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**TERRY MOORE** Telephone No : (02) 6283 2632

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/001768

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STN File CA 127:358699 & Zhao, Y.J. *et al.* 1997. Chinese Commercial Letters 8(9): 769 – 772. See Abstract and CAS RN 198713 – 15 – 4.	9 – 17, 22, 28, 29, 36, 38, 40, 42 – 44, 50, 51, 54
X	STN File CA 79:28893 & Byers, L.D. *et al.* 1973. Biochemistry 12(11): 2070 – 2078. See Abstract and CAS RN 4354 – 95 – 4.	9 – 17, 22, 28, 29, 36, 38, 40, 42 – 44, 50, 51, 54
X	STN File CA 67:11357 & Canonne *et al.* 1967. Canadian Journal of Chemistry 45(11): 1267 – 74. See Abstract and CAS RN 14558 – 15 – 7.	9 – 17, 22, 28, 29, 36, 38, 40, 42 – 44, 50, 51, 54
A	Brown, P.H. *et al.* 2004. The Biotin Repressor: Modulation of Allostery by Corepressor Analogs. Journal of Molecular Biology. 337(4): 857 – 869. See whole document.	1 – 57
A	Jitrapakdee, S. and Wallace, J.C. 2003. The Biotin Enzyme Family: Conserved Structural Motifs and Domain Rearrangements. Current Protein and Peptide Science. 4(3): 217 – 229.	1 – 57
A	Chapman-Smith, A. *et al.* 2001. The C-terminal domain of biotin protein ligase from *E. coli* is required for catalytic activity. Protein Science. 10(12): 2608 – 2617.	1 – 57

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/001768

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **18 – 21, 33, 34 (completely) 9 – 17, 22, 28 – 29, 36, 38, 40, 42 – 44, 50, 54 (partially)**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See Supplemental Box.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: II

The applicant's invention appears to reside in methods of screening for inhibitors of a biotin protein ligase by measuring the extent of biotinylation of a substrate by BPL in the presence and absence of a test compound, specific inhibitors identified using this process and the use of said inhibitors as anti-pathogenic agents. Thus the International search has been directed towards the applicant's screening method, methods of inhibiting a pathogenic organism by treating said organism with an identified inhibitor and the specific identified inhibitors (based on the structure presented on pg. 5 of the specification) when used to inhibit biotin protein ligase.

Claims 9 – 17, 22, 28 – 29, 36, 38, 40, 42 – 44, 50 and 54 have only been the subject of a partial International Search.

Claims 9 – 11, 28 – 29, 36, 40 and 42 are directed towards any inhibitor/composition identified by the applicant's screening assay. Said claims therefore define a broad range of both known and unknown compounds *per se*, which are not restricted to being produced/isolated by or used in the methods of the invention.

Claims 12 – 17, 22, 38, 43 – 44, 50 and 54 are directed to compositions and inhibitors *per se* which owe nothing to the methods of the invention.

There is no disclosure in the specification of the wide range of compounds defined in said claims. As such, the scope of said claims is so broad that no full meaningful search could be conducted. Therefore, said claims have only been searched to the extent of the disclosure provided in the specification, i.e. compositions that correspond to the molecular structure presented on pg. 5 of the specification (said structure being the generic structure of the inhibitors identified by the applicant's invention), when used to inhibit biotin protein ligase.

Claims 18 – 21 and 33 – 34 have not been the subject of an International Search.

Claims 18 – 21 and 33 – 34 define Biotin Protein Ligase *per se*. The applicant's invention resides in screening for inhibitors of Biotin Protein Ligase, a protein which is well known in the art (see specification pg's 22 – 30), for use as an antifungal and antibacterial agent. As such, claims to Biotin Protein Ligase *per se*, are not defined in terms of the technical features of the invention. Therefore, said claims have not been the subject of an International Search.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2005/001768

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	2004053485	AU	2003295091	EP	1573320
WO	2000043418	US	6448038		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX