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Appendix I: GLYAT augmentation therapy

Recombinant therapeutics GLYAT patent application

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International application number:
PCT/IB2011/053721

The patent application was filed by:
At van Rooy, DM Kisch Inc., Pretoria, South Africa
Receipt of Electronic Submission

It is hereby acknowledged that a PCT International Application has been received via the Secure Electronic Submission Software of the IB. Upon receipt, Application Number and a Date of Receipt (Administrative Instructions, Part 7) has been automatically assigned.

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Timestamp of Receipt: 24 August 2011 16:11


/Geneva, RO/IB/
RECOMBINANT THERAPEUTIC GLYCINE N-ACYLTRANSFERASE

INTRODUCTION AND BACKGROUND TO THE INVENTION

This invention relates to a method of producing a recombinant enzyme. More particularly, this invention relates to a method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT (E.C. 2.3.1.13)).

Detoxification of toxic metabolites by the human body is an essential physiological process. The detoxification process decreases the toxicity of several endogenous metabolites, such as steroid hormones, and exogenous toxins, which could include compounds in food or industrial chemicals.

The detoxification process is divided into three main phases. Phase I detoxification activates metabolites by adding functional groups. The activated compounds generated by phase I detoxification are often more reactive and toxic than the original metabolites, and are further processed by phase II detoxification systems. In phase II detoxification, a range of conjugation reactions serve to make the activated compounds less toxic and more soluble, for excretion in the urine and bile. Phase III detoxification involves the elimination of toxins from cells.
Organic acidemias are a group of metabolic disorders caused by dysfunctional organic acid metabolism. The deficiency of certain metabolic enzymes causes the accumulation of acids which are not normally present in high levels in the human body. There are several known organic acidemias, with methylmalonic acidemia, propionic acidemia, isovaleric acidemia, glutaric aciduria, and maple syrup urine disease being some common examples.

Isovaleric acidemia is an autosomal recessive disorder. It is caused by a deficiency of isovaleryl coenzyme A dehydrogenase. A deficiency of this enzyme results in accumulation of intermediates of leucine catabolism, including isovaleric acid, 3- and 4-hydroxyisovaleric acid, isovaleryl-carnitine and isovaleryl-glycine.

Isovaleryl-glycine is formed when isovaleric acid conjugates to glycine by glycine N-acyltransferase (GLYAT). The isovaleryl-glycine is less toxic than isovaleric acid, indicating that glycine conjugation is of critical importance in the treatment of isovaleric acidemia.

Urea cycle disorder is a genetic disorder caused by an enzyme deficiency in the urea cycle responsible for eliminating ammonia from the bloodstream. In urea cycle disorders, nitrogen accumulates in the form of ammonia resulting in hyperammonemia which ultimately causes irreversible brain damage, coma and/or death.
A known method for enhancing glycine conjugation capacity in individuals suffering from organic acidemias is the administration of glycine supplements. Assays on liver samples have however shown that there is great variability in the glycine conjugation capacity in humans.

It is therefore evident that a means of augmenting the natural capacity for glycine conjugation would not only be beneficial to the general health of humans but may further present as an alternative therapeutic strategy for individuals affected by organic acidemias, urea cycle disorders, aminoacidurias, and exposure to some xenobiotic chemicals.

GLYAT is an enzyme responsible for the phase II detoxification of several toxic organic acids by means of conjugation to glycine. Several toxic compounds, both xenobiotic and endogenously derived metabolites, are detoxified by conjugation to glycine. In addition to GLYAT’s role in the detoxification of benzoic acid, the enzyme is also important in the management of certain inborn errors of metabolism.

To date, no system for the bacterial expression and purification of an enzymatically active recombinant GLYAT has been reported.

A disadvantage associated with the lack of a system for expression of an enzymatically active recombinant GLYAT is that there is no commercially
viable product currently available for directly improving the capacity of the glycine-conjugation detoxification system, particularly in the case of patients with metabolic disorders.

5 OBJECTS OF THE INVENTION

It is accordingly an object of the present invention to provide a novel method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) enzyme and to provide GLYAT produced with such a method.

10 It is a further object of the invention to provide use of a pharmaceutically effective amount of GLYAT in a method of enhancing detoxification and for treating and/or preventing metabolic disorders in mammals.

15 It is yet another object of the invention to provide a method of enhancing detoxification in mammals and for treating and/or preventing metabolic disorders with which the aforesaid disadvantage may be overcome or at least minimised.

20 SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided a method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) including the steps of:

- providing a suitable expression host;
- preparing a vector including a gene for expressing GLYAT in the expression host to form an expression plasmid;
- transforming the host with the expression plasmid to form an expression system;
- expressing the GLYAT gene in the expression system; and
- separating the expressed GLYAT from the expression system.

Further according to the invention the step of separating the expressed GLYAT from the expression system may include the steps of separating the water soluble fraction of the expression system from the insoluble material and concentrating or lyophilising the separated GLYAT.

Further according to the invention the expression host may be selected from the group consisting of eukaryotic systems, including yeast cell expression-, insect cell expression- and mammalian cell expression systems; prokaryotic systems, including Escherichia coli and Bacillus subtilis and archaeon systems.

Further according to the invention the method includes a further step of combining the separated expressed GLYAT with glycine.

According to a second aspect of the invention there is provided water soluble enzymatically active recombinant GLYAT prepared in accordance with the first aspect of the invention.
According to a third aspect of the invention there is provided a medicament comprising water soluble enzymatically active recombinant GLYAT prepared in accordance with the first aspect of the invention.

According to a fourth aspect of the invention there is provided use of a pharmaceutically effective amount of water soluble enzymatically active recombinant GLYAT prepared in accordance with the first aspect of the invention in a method of:

- improving the capacity of a glycine-conjugation detoxification system;
- enhancing detoxification; or
- treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

According to a fifth aspect of the invention water soluble enzymatically active recombinant GLYAT may be used in a method of:

- improving the capacity of a glycine-conjugation detoxification system;
- enhancing detoxification; or
- treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin,

in mammals by administering to a mammal in need thereof a biologically effective amount of between 0.1 mg and 160 mg of water soluble
enzymatically active recombinant GLYAT per kilogram of body mass depending on the demand for increased glycine conjugation.

According to a sixth aspect of the invention there is provided use of a pharmaceutically effective amount of water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of the first aspect of the invention in a method of manufacturing a medicament for use in:

- improving the capacity of a glycine-conjugation detoxification system;
- enhancing detoxification; or
- treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

Further according to the invention the metabolic disorders may be any one or more of the conditions selected from the group consisting of organic acidemias selected from propionic acidemia, isovaleric acidemia and glutaric aciduria, aminoacidurias selected from maple syrup urine disease and hyperglycinemia; and urea cycle disorder.

According to the seventh aspect of the invention there is provided a medicament prepared from water soluble enzymatically active recombinant GLYAT in accordance with the first aspect of the invention together with at least one inert pharmaceutically acceptable carrier or
diluents in a dosage form selected from the group consisting of tablets; capsules; suspension; syrup; intradermal-; intramuscular-; intravenous-; and subcutaneous injection.

5 The water soluble enzymatically active recombinant GLYAT may be administered by intravenous injection (IV) with a preparation of the enzyme in a form that is targeted to the desired sub-cellular compartments. Alternatively, water soluble enzymatically active recombinant GLYAT may be administered by using a GLYAT enzyme fused to the membrane permeating TAT (transactivator of transcription) peptide, allowing the recombinant enzyme to effectively cross cell membranes to reach the desired mitochondrial matrix. Further alternatively, water soluble enzymatically active recombinant GLYAT may be administered by using a colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures.

Further according to the invention the step of administering the biologically effective amount of water soluble enzymatically active recombinant GLYAT may include the further step of administering the water soluble enzymatically active recombinant GLYAT in combination with glycine to further stimulate glycine conjugation capacity.
BRIEF DESCRIPTION OF THE FIGURES

The invention will now be described further, by way of example only, with reference to the accompanying figures wherein:

5  figure 1: is a diagram illustrating the pColdIII expression vector used for expression of bovine GLYAT in accordance with a preferred embodiment of the invention;

figure 2: is a polymerase chain reaction (PCR) amplification of an open reading frame (ORF) encoding bovine GLYAT from a plasmid into which the ORF encoding bovine GLYAT had already been cloned (the original PCR amplification and cloning were performed using cDNA from bovine liver);

15  figure 3: is a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretogram (PAGE) illustrating the total and soluble fractions of the expression of recombinant bovine GLYAT (lanes 2 and 3, respectively);

20  figure 4: is an SDS-PAGE analysis illustrating the total and soluble fractions of the expression of recombinant bovine GLYAT (lanes 2 and 3, respectively) as well as the partially purified enzyme (nickel affinity chromatography) in lane 4;
figure 5: is an SDS-PAGE analysis illustrating the enzyme after partial purification using nickel affinity chromatography (in this purification 20 mM imidazole was added to the wash purification buffers);

figure 6: is an SDS-PAGE analysis illustrating the expression of a soluble recombinant human GLYAT gene (lanes 4 to 9), with an N-terminal fusion of the hexahistidine tag and Trx-tag;

figure 7: is an SDS-PAGE analysis illustrating the nickel-affinity purification of wild-type recombinant human GLYAT (lane 3) and single nucleotide polymorphism (SNP) variants of human GLYAT (lanes 4 to 9); and

figure 8: is a spectrophotometric assay illustrating enzyme activity of recombinant human GLYAT and bovine liver GLYAT in the presence and absence of glycine.

20 DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

According to a preferred embodiment of the invention there is provided a method for producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT).
The method includes the steps of providing a suitable expression host providing a GLYAT expressing gene; preparing a vector including a gene for expressing GLYAT in the expression host to form an expression plasmid; transforming the host with the expression plasmid to form an expression system; expressing the GLYAT in the expression system; separating the expressed GLYAT from the expression system; and combining the separated expressed GLYAT with glycine.

The expression host is selected from the group consisting of eukaryotic systems, including yeast cell expression, insect cell expression and mammalian cell expression, prokaryotic systems, including Escherichia coli and Bacillus subtilis and archaeon systems. It was found that Escherichia coli (E. coli) provided a particularly suitable host.

The gene encoding bovine GLYAT was isolated from bovine liver RNA and cloned, by means of reverse transcription and polymerase chain reaction (PCR) amplification, into a pColdIII expression vector (as illustrated in figure 1). The pColdIII expression vector allows for the expression of a protein in E. coli at 15 degrees Celsius, which enhances the expression of soluble, enzymatically active recombinant proteins.
Various other vectors could also be used for the expression of recombinant human and bovine GLYAT, or other GLYAT variants, in eukaryotic, prokaryotic and archaeon expression hosts.

In order to obtain a suitable vector, a histidine-tag (His-tag) is attached to the C-terminus of the gene. In the alternative to C-terminal histidine tags, tags are selected from the group consisting of N-terminal hexahistidine tags, maltose binding protein (MBP), glutathione S-transferase, GST tags and Strep-Tag II.

GLYAT is alternatively expressed without any purification tags, and separated from the proteins of the expression host by utilising known protein purification strategies. Owing to the fact that GLYAT is a nucleotide-cofactor binding enzyme, it may further alternatively be purified by affinity chromatography.

**Example 1: Recombinant bovine GLYAT**

Recombinant bovine GLYAT was cloned into a set of three modified pColdIII (pColdIII-E, pColdIII-A and pColdIII-EH) expression vectors encoding C-terminal histidine tags.

In order to clone the coding sequence into the expression vectors, the sequence is amplified through polymerase chain reaction (PCR) using primers containing Ndel and Xhol restriction enzyme sites to facilitate
directional cloning. The PCR reaction mixtures contained 1X Takara ExTaq buffer, 10 nmol of each dNTP, 25 pmol of each primer, approximately 50 ng of template DNA and 2 units of Takara ExTaq polymerase, in a final volume of 50 μl. Thermal cycling conditions were 94 degrees Celsius for 1 min, then 30 cycles of 94 degrees Celsius for 30 seconds, 70 degrees Celsius for 30 seconds, and 72 degrees Celsius for 1 minute, followed by a final extension at 72 degrees Celsius for 10 minutes.

After transforming E. coli with an expression plasmid containing a recombinant GLYAT coding sequence, colonies were screened for desired recombinant plasmids using either restriction analysis or PCR amplification. A colony was considered to be positive if an excised fragment of approximately 900 bp could be seen on an agarose gel, as illustrated in figure 2.

The recombinant protein was purified using a nickel affinity purification process. Upon passage through a resin with nickel ions immobilised onto it, the histidine tags fused to the recombinant GLYAT binds tightly to the column matrix, by forming coordinate bonds with the nickel ions immobilised on its surface. This enables most other proteins to be washed from the column, while the histidine tagged GLYAT remains bound. The tagged protein was eluted with a buffer containing a high concentration of imidazole, which displaced the coordinate bonds between
the histidine residues and nickel ions, resulting in a partially purified recombinant protein.

Referring to figure 3, a sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoretogram (PAGE) was used to analyse the expression of bovine GLYAT from the pColdIII vector. The proteins were visualised by staining with Coomassie brilliant blue. Lane 2 illustrates the total fraction of expressed protein and lane 3 illustrates the soluble fraction of bacterial lysate; with the soluble recombinant GLYAT expressed not being clearly visible on the background of bacterial proteins.

Referring to figure 4, recombinant bovine GLYAT was expressed from pColdIII with a C-terminal histidine tag. The soluble fraction was passed through a nickel affinity purification column, to purify the tagged recombinant GLYAT enzyme. The levels of soluble recombinant bovine GLYAT expressed were low, therefore, the final eluate of the purification was significantly concentrated. SDS-PAGE analysis revealed the total fraction of expressed protein in lane 2. Lane 3 represents the soluble fraction of the recombinant GLYAT with no significant amount of soluble recombinant GLYAT being visible against the background of bacterial proteins. Lane 4 illustrates the partially purified enzyme as a result of the nickel-affinity purification. The lower band indicates the active form of the GLYAT enzyme.
Referring to figure 5, 20 mM imidazole was added to the column wash buffers of the purification kit. The use of imidazole in the buffers resulted in the majority of the previously co-purifying proteins being lost. The lower bands, in figure 5, represent the enzymatically active bovine GLYAT and an unknown protein.

It was found that the recombinant bovine GLYAT enzyme, prepared in accordance with the invention, has similar biochemical characteristics to the GLYAT enzyme purified from bovine liver.

**Example 2: Recombinant human GLYAT**

The nucleotide sequence encoding the human GLYAT sequence was synthesised and cloned into the pET32 expression vector.

The pET32 expression vector enables the expression of human GLYAT with an N-terminal hexahistidine tag and an N-terminal Trx-tag, which respectively facilitates the purification and correct folding of the enzyme.

The expression vector encoding human GLYAT was transformed into Origami expression cells. The cells were also transformed with the pGro7 vector from Takara, which resulted in co-expression of the GroES and GroEL chaperone proteins. Chaperone proteins aid in the correct folding of proteins and increase the yield of soluble recombinant enzymes.
The Origami cells containing the plasmids for expression of recombinant human GLYAT and the chaperone proteins were grown in liquid culture. It was found that the optimal expression of soluble GLYAT occurs in the absence of IPTG (Isopropyl β-D-1-thiogalactopyranoside), thus allowing GLYAT to be expressed at slow basal rate as oppose to the known method of inducing the fusion protein with IPTG to express.

After expression, cells were harvested by means of centrifugation, and lysed using an optimised native lysis buffer containing 300 mM NaCl, 50 mM phosphate buffer, pH 8.0, 10% glycerol, 1% Triton-X, lysozyme, and protease inhibitors. The cell lysates were clarified, using centrifugation at 10,000 g for 30 minutes to remove the insoluble material and passed through Protino nickel affinity purification columns to selectively bind the hexahistidine tagged enzymes. The columns were washed, and the purified protein eluted in a final volume of 3 ml.

Referring to figure 6, soluble recombinant human GLYAT was expressed with an N-terminal hexahistidine-Trx-fusion tag. Lane 1 contains molecular size markers. Lanes 2 and 3 contain the insoluble and soluble fractions, respectively, of a culture, of which the cells were lysed using the BugBuster protein extraction reagent. It was found that this lysis reagent was not suitable for the extraction of recombinant human GLYAT, as no soluble recombinant human GLYAT was visible.
As an alternative, the optimised native lysis buffer was used to isolate the protein from cultures expressing from 0 hours to 4 hours, and the soluble fractions were loaded in lanes 5 to 9. The hexahistidine-Trx-GLYAT fusion protein is indicated by the arrow, in the 55 kDa range.

Referring to figure 7, the soluble recombinant human GLYAT fusion proteins are purified by means of nickel-affinity chromatography, using Protino Ni-TED columns. Lane 1 contains molecular weight markers, and lane 2 is empty. Lane 3 contains the wild-type recombinant human GLYAT fusion protein, after purification. Lanes 4 to 9 contain purified recombinant human GLYAT, as prepared in accordance with the invention, fusion proteins, of the known single nucleotide polymorphism (SNP) variations of the gene.

Referring to figure 8, the resultant enzyme preparation was assayed for enzyme activity using the spectrophotometric assay for GLYAT. In the assay, bovine GLYAT is used as a positive control. Reactions without glycine were run as negative controls illustrating that the enzyme activity observed is glycine dependent. The recombinant human GLYAT illustrated an increase in optical density (OD) at 412 nm confirming the enzyme activity of recombinant human GLYAT, prepared in accordance with the invention, in the presence of glycine.
In addition to using a recombinant therapeutic GLYAT enzyme in the above described manner, it is possible that novel forms of the GLYAT enzyme may be obtained by rational and semi-rational enzyme engineering strategies, and these may alternatively be used for their specialised functions. Qualities of the GLYAT enzyme that may be subjected to modification by enzyme engineering strategies include catalytic rate, substrate specificity, stability, immunological aspects, and optimal substrate concentration.

There are six known natural SNP (single nucleotide polymorphism) variants of human GLYAT and site-directed mutagenesis was used to generate these variant coding sequences from the wild-type sequence. It was found that of the six SNP variants, two have higher enzyme activity than the wild-type GLYAT, and the rest have much lower activity than the wild-type GLYAT. It is to be expected that there would be clear benefits associated with the use of variants with increased catalytic rate, for example.

Further findings and analysis
In use, a pharmaceutically effective amount of 0.1 mg to 160 mg of the recombinant GLYAT enzyme per kilogram of body weight, depending on the nature and extent of the metabolic disorder, is administered to a patient in need thereof by way of intravenous injection (IV) with a
preparation of the enzyme in a form targeting the desired sub-cellular compartments. Alternatively, the prepared recombinant GLYAT enzyme is administered by using a TAT (transactivator of transcription) peptide to act as a membrane permeating agent, which will allow the recombinant enzyme to effectively cross cell membranes to reach the desired mitochondrial matrix. Further alternatively, the prepared recombinant GLYAT enzyme is administered using a colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures to enhance detoxification and to treat and/or prevent metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

The metabolic disorders may be any one or more of the conditions selected from the group consisting of organic acidemias selected from propionic acidemia, isovaleric acidemia and glutaric aciduria; aminoacidurias selected from maple syrup urine disease, and hyperglycinemia, and urea cycle disorder.

The recombinant GLYAT is further alternatively formulated into any one of the following dosage forms comprising tablet; capsule; suspension; syrup; intradermal-; intramuscular-; intravenous-; and subcutaneous injection.

A medicament prepared from the recombinant GLYAT in combination with glycine is used to directly improve the capacity of the glycine-conjugation
detoxification system in the treatment of patients exposed to chemical and industrial solvents and in the emergency treatment of acute aspirin poisoning. Glycine conjugation of several organic acids is enhanced by the use of a recombinant therapeutic GLYAT enzyme.

It will be appreciated that in terms of the invention, variations in details in providing a novel method of producing a recombinant enzyme and more particularly relating a novel method of producing a water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) enzyme, are possible without departing from the scope of the appended claims.
CLAIMS

1. A method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) including the steps of:
   - providing a suitable expression host;
   - preparing a vector including a gene for expressing GLYAT in the expression host to form an expression plasmid;
   - transforming the host with the expression plasmid to form an expression system;
   - expressing the GLYAT gene in the expression system; and
   - separating the expressed GLYAT from the expression system.

2. A method according to claim 1 wherein the step of separating the expressed GLYAT from the expression system includes the steps of separating the water soluble fraction of the expression system from the insoluble material and concentrating or lyophilising the separated GLYAT.

3. A method according to claim 1 wherein the expression host is selected from the group consisting of eukaryotic systems, including yeast cell expression-, insect cell expression-, and mammalian cell expression systems; prokaryotic systems, including Escherichia coli, and Bacillus subtilis; and archaeon systems.
4. A method according to any one of claims 1 to 3 including the further step of combining the separated expressed GLYAT with glycine.

5. Water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of any one of claims 1 to 4.

6. A medicament comprising water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of any one of claims 1 to 4.

7. Use of a pharmaceutically effective amount of water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of any one of claims 1 to 4 in a method of:
   - improving the capacity of a glycine-conjugation detoxification system;
   - enhancing detoxification; or
   - treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

8. Water soluble enzymatically active recombinant GLYAT for use in a method of:
   - improving the capacity of a glycine-conjugation detoxification system;
enhancing detoxification; or
- treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin, in mammals by administering to a mammal in need thereof a biologically effective amount of between 0.1 mg and 160 mg of water soluble enzymatically active recombinant GLYAT per kilogram of body weight.

9. Use of a pharmaceutically effective amount of water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of any one of claims 1 to 4 in a method of manufacturing a medicament for use in:
- improving the capacity of a glycine-conjugation detoxification system;
- enhancing detoxification; or
- treating and/or preventing metabolic disorders and acute or chronic poisoning with compounds such as xylene or aspirin in mammals.

10. Use according to any one of claims 7 to 9 wherein the metabolic disorders are any one or more of the conditions selected from the group consisting of organic acidemias selected from propionic acidemia, isovaleric acidemia and glutaric aciduria; aminoacidurias
selected from maple syrup urine disease and hyperglycinemia, and urea cycle disorder.

11. Use according to any one of claims 7 to 10, wherein the water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of claims 1 to 4 is administered in combination with glycine in order to further stimulate glycine conjugation capacity.

12. A medicament comprising water soluble enzymatically active recombinant GLYAT prepared in accordance with the method of any one of claims 1 to 4 in combination with glycine, together with at least one inert pharmaceutically acceptable carrier or diluents in a dosage form selected from a group comprising tablet; capsule; suspension; syrup; TAT (transactivator of transcription) peptide; a colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures; intradermal-; intramuscular-; intravenous-; and subcutaneous injection.

13. A method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT) as herein described and exemplified, with reference to the accompanying figures.
ABSTRACT

This invention relates to a method of producing a recombinant enzyme, more particularly, this invention relates to a method of producing water soluble enzymatically active recombinant glycine N-acyltransferase (GLYAT (E.C. 2.1.3.13)), including the steps of providing a suitable expression host; preparing a vector including a gene for expressing GLYAT in the expression host to form an expression plasmid; transforming the host with the expression plasmid to form an expression system; expressing the GLYAT gene in the expression system; and separating the expressed GLYAT from the expression system.
FIGURE 1

pCold III DNA
(4,377 bp)

FIGURE 2

1200 bp
1000 bp
900 bp

900 bp amplicon
FIGURE 3
FIGURE 4

Insoluble recombinant protein

Upper band

Middle band

Lower band

No visible soluble recombinant protein

72 kDa

55 kDa

36 kDa

28 kDa
**FIGURE 5**

![Diagram with protein bands labeled 72 kDa, 55 kDa, 36 kDa, and 28 kDa.]

**FIGURE 6**

![Graph showing time course of IPTG concentration with different concentrations marked.]
FIGURE 6

FIGURE 7
FIGURE 8
Appendix II: Annotations for some important references

Here brief annotations of some of the more important references used in this thesis are provided. The purpose is to briefly explain how these references contribute to our understanding of the field of glycine conjugation. The • symbol is used to indicate that a reference is of considerable interest.

The importance of glycine conjugation in metabolism

Conti and Bickel, 1977
Of historical interest, this is a useful review of the early history of glycine conjugation and biotransformation. It discusses the discovery of hippurate in urine, the initial experiments by Ure demonstrating the conversion of benzoate to hippurate, and the finding by Dessaignes that hippurate is a peptide conjugate between glycine and benzoate.

Lees et al., 2013 •
This excellent review does not discuss the glycine conjugation pathway in detail, but offers a valuable summary of the factors that influence urinary hippurate excretion, such as the composition of the gut microbiome, age, gender, and several disease states. These include obesity, diabetes, hepatitis, and parasitic infections.

Knights et al., 2007 •
This is one of the better reviews of amino acid conjugation. It explains the formation and toxicity of acyl-CoA metabolites and suggests that the amino acid conjugations are important for inactivation of reactive acyl-
CoA thioesters. Of special interest is the suggestion that the evolutionary conservation of the substrate selectivities of the amino acid conjugations implies that they are important defences against the mitochondrial toxicities of organic acids.

*Bartlett and Gompertz, 1974*  
This paper reports a relationship between the acylglycines excreted in the urines of organic acidemia patients and the substrate selectivity of bovine GLYAT. It is of particular interest to those interested in the study and treatment of inborn errors of organic acid metabolism.

*Mitchell et al., 2008*  
In this review the concept of CASTOR disorders is introduced. It reviews the importance of CoASH in metabolism and suggests that the pathogenesis of several inherited and acquired conditions may be explained by sequestration of CoASH, the toxicity of acyl-CoAs, and abnormal ratios of different acyl-CoA metabolites.

*Zhang et al., 2007*  
This paper reports the results of a study where the pantothenate analogue hopantenate was administered to rats. This compound inhibits CoASH biosynthesis and results in fatal hypoglycaemia. It also results in transcriptional reprogramming of the liver, with increased expression of thioesterases and decreased expression of CoASH-consuming enzymes.

*Beyoglu et al., 2012*  
This review suggests homeostatic regulation of amino acid levels in the brain as an alternative role for the amino acid conjugation pathways. The authors argue that the detoxification value of amino acid conjugation is low, because of the relatively small increase in water solubility that results from these conjugation reactions.
Beyoglu and Idle, 2012 •

This review extends the argument that amino acid conjugations are neuroregulatory processes rather than detoxification mechanisms. The focus is on the relationship between glycine conjugation and the levels of glycine in plasma and CSF. The “glycine deportation system” is introduced in this paper.

Guo et al., 2013 •

In this paper a statistically significant correlation between three SNPs in or near the human GLYAT gene and musculoskeletal structure is reported, although no satisfactory explanation for this correlation is provided. The findings reported in this paper seem to suggest that the GLYAT gene influences the development of the musculoskeletal system by some mechanism that is not understood at present.

Melendez-Hevia et al., 2009

This good review of glycine metabolism compares the capacity of humans for glycine synthesis to the metabolic demands for glycine, and concludes that humans seem to have a moderate glycine deficiency. The authors suggest that although this glycine deficiency is not lethal, it can negatively impact collagen turnover and thereby contribute to the accumulation of damage to connective tissues.

Wu et al., 2013 •

This review of amino acid metabolism supports the view that glycine is a conditionally essential amino acid, and argues that there is no substantial experimental evidence supporting the assumption that some amino acids are nonessential. The “nonessential amino acids” play important roles in the regulation of gene expression, neurotransmission, and immune responses, suggesting that the traditional classification of amino acids as essential or nonessential must be abandoned and replaced by the more dynamic concept of “functional amino acids”.

A36
The factors that influence the rate of glycine conjugation

Gregus et al., 1992 •

This paper reports a gradual reduction in the rate of benzoate clearance from blood with increasing doses of benzoate. The authors demonstrate that this is the result of hepatic glycine depletion, which results in reduced rates of glycine conjugation and CoASH sequestration. Importantly, the rate of excretion of exogenous hippurate is much higher than the maximum rate of glycine conjugation, suggesting that the rate of renal hippurate excretion reflects the rate of hepatic glycine conjugation. These findings are very important for the argument that glycine conjugation facilitates the urinary excretion of benzoate (Paper II).

Gregus et al., 1993 •

This paper reports that glycine availability, which depends on glycine cleavage system activity, influences the rate of glycine conjugation. The authors demonstrate that cysteamine, an inhibitor of the glycine cleavage system, increases hepatic glycine content about twofold and increases the rate of glycine conjugation by about 50%. These results demonstrate not only that the rate of glycine conjugation depends on glycine availability, but also that other factors limit this process when excess glycine is available.

Gregus et al., 1996 •

This paper demonstrates that although normal hepatic ATP levels do not limit glycine conjugation in vivo, ATP depletion may impair glycine conjugation. This is demonstrated by the reduced rate of glycine conjugation following administration of oligomycin, an inhibitor of ATP synthesis, or fructose, which depletes hepatic ATP. These results support the suggestion that the reduced rate of glycine conjugation in hepatitis patients may be the result of hepatic ATP depletion.
**Interindividual variation in glycine conjugation capacity**

*Sakuma, 1991*

This paper reports interindividual variation in the rate of glycine conjugation following administration of benzoate. Of particular interest is the demonstration of benzoylecarnitine excretion and carnitine depletion after administration of benzoate. The author also reports an unexplained increase in urinary excretion of propionylcarnitine and acetylcarnitine after benzoate administration, and argues that we do not fully understand the glycine conjugation pathway or the metabolic consequences of benzoate administration.

*de Vries et al., 1948*

This paper is very interesting because it reports interindividual variation in glycine conjugation capacity and, more importantly, interindividual variation in the glycine depletion induced by administration of large doses of benzoate. This suggests that the rate of glycine conjugation, which depends on glycine availability, may be significantly influenced by the rate at which glycine can be synthesised.

*Temellini et al., 1993 *

This is one of the most important references used in this thesis. The authors report interindividual variation in the ability of human liver and kidney homogenates to synthesise hippurate from benzoate, CoASH, ATP, and glycine. The concentrations of the substrates used were similar for all samples, suggesting that different activities of ACSM2A and GLYAT in these samples must explain the differences in the rate of glycine conjugation. These results form an important part of the arguments in Chapters 2 and 4.

*Itoh et al., 1996*

This paper reports the treatment of two isovaleric acidemia patients by means of glycine and carnitine supplementation. Several interesting observations of relevance to this thesis, and especially Chapter 2, are described. For example, the healthier patient was also the one with higher isovalerylglycine excretion.
Despite the fact that the second patient had less residual isovaleryl-CoA dehydrogenase activity, which would suggest greater accumulation of isovaleryl-CoA, less isovalerylglycine was excreted. This suggests that differences in the rate of glycine conjugation may impact the clinical outcome of this disease, which is an important argument in Chapter 2.

Williams et al., 2010 •
This paper reports that the reduced urinary hippurate excretion observed in patients with Crohn’s disease is the result of abnormal composition of the gut microbiome, which is associated with decreased production of microbial metabolites such as phenylpropionate. Most importantly, it was demonstrated that these individuals did not have a defect of the hepatic glycine conjugation system, as indicated by normal rates of hippurate synthesis from an oral dose of benzoate. The results reported in this paper are very important for the argument that variation in urinary hippurate excretion must be clearly distinguished from variation in the rate of glycine conjugation (Paper II).

Enzymes of the glycine conjugation pathway

Schachter and Taggart, 1954 •
This paper is of historical interest. It describes the partial purification of bovine liver GLYAT and reports the ability of this enzyme to use several acyl-CoAs as substrates, including benzoyl-CoA and isovaleryl-CoA. These findings were important for the later understanding of the origin of the urinary acylglycines excreted by patients with organic acidemias (Chapter 2).

van der Westhuizen et al., 2000
This paper reports the purification of GLYAT from human and bovine liver mitochondria. What makes this paper unique is the use of mass spectrometry to detect the formation of benzoyl-conjugates with other
amino acids including glutamate and alanine. The authors conclude that the use of other amino acids as acyl-acceptor substrates, which is significantly slower than the reaction with glycine, is probably insignificant under normal physiological conditions.

*Webster et al., 1976*

The aim of this study was to investigate the molecular basis for the observation that primates conjugate arylacetic acids such as phenylacetate to glutamine, while glycine is used as the acyl-acceptor substrate in non-primates. The partial purification and characterisation of two separate N-acyltransferases from the liver of rhesus monkeys and humans is described. These are GLYAT, which synthesises hippurate, and the phenylacetyl-CoA:glutamine N-acyltransferase, which synthesises phenylacetylglutamine.

*Nandi et al., 1979 •*

This paper reports the purification and characterisation of the bovine GLYAT and phenylacetyltransferase enzymes. The results show that the GLYAT enzyme uses benzoyl-CoA and glycine to synthesise hippurate, while the phenylacetyltransferase uses phenylacetyl-CoA and glycine to synthesise phenylacetylglycine. These findings confirmed previous suggestions that the species differences in the formation of phenylacetylglutamine (primates) or phenylacetylglycine (non-primates) is a reflection of differing amino acid selectivity of the phenylacetyltransferase enzymes of these species.

*Matsuo et al., 2012 •*

This is a very important paper for at least three reasons. First, it is the first publication to report the bacterial expression of an enzymatically active recombinant human GLYAT. Second, it demonstrates that the GLYAT-L1 gene encodes the phenylacetylglutamine forming enzyme in humans. Finally, and most importantly, this paper reports the transcriptional downregulation of GLYAT expression in hepatocellular carcinoma specimens, but not in normal liver samples or hepatitis specimens.
This paper reports the first data on the allele frequencies of human GLYAT variants in a small group of Japanese individuals.

Lino Cardenas et al., 2010

This paper reports the allele frequencies of GLYAT variants in a small population of French Caucasian individuals. Of particular importance to this thesis is the suggestion that the N156S variant of human GLYAT should be considered the “wild-type” human GLYAT. This is because the N156S variant, and not the reference sequence (NM_201648.2), has the highest allele frequency.

Toxicity of the aromatic acids and detoxification by means of glycine conjugation

Beloborodova et al., 2012

This study demonstrates that phenolic acids, including benzoate and cinnamate, which accumulate in the blood of sepsis patients, stimulate the production of reactive oxygen species in isolated hepatic mitochondria, while inhibiting it in neutrophils. In contrast, the more hydrophilic phenolic acids, such as phenyllactate and 4-hydroxyphenylacetate, do not stimulate ROS production and, because of the presence of hydroxyl groups, can act as free radical scavenging antioxidants. This information forms a very important part of the arguments that the aromatic acids are toxic to mitochondria, and that this toxicity depends on lipophilicity (Paper II).

Fedotcheva et al., 2008

This paper reports that aromatic acids stimulate ROS production and inhibit complex I of the mitochondrial electron transport chain. Although the authors do not point this out, there seems to be a relationship between the lipophilicity of the acids and the extent of ROS production or complex I inhibition, with the
more lipophilic compounds being more harmful. This supports our argument that the toxicity of aromatic acids depends on lipophilicity, and that amino acid conjugation decreases the toxicity of these compounds by decreasing lipophilicity (Paper II).

_Trost and Lemasters, 1996_

This paper is valuable in making the connection between isovaleric acidemia and the detoxification of benzoate, because it demonstrates that several organic acids, including isovalerate and benzoate, are toxic to mitochondria and can induce the mitochondrial permeability transition. This common mechanism of toxicity supports our use of the analogy between glycine conjugation in isovaleric acidemia and the detoxification of benzoate by means of glycine conjugation (Paper II).

_Lakeram et al., 2007_

This study demonstrated that the ability of the parabens, alkyl esters of 4-hydroxybenzoate, to induce the mitochondrial permeability transition and cell death, is linearly related to the lipophilicity or octanol/water partition coefficient of the compound (Section 4.3 of Paper II). More importantly, it was demonstrated that diazinon, which inhibits the carboxylesterase mediated hydrolysis of parabens to the more hydrophilic 4-hydroxybenzoate, significantly increased the toxicity of propylparaben to mitochondria. This indirectly supports the argument, in Section 5.3 of Paper II, that the toxicity of organic compounds to mitochondria can be reduced by biotransformation reactions that decrease lipophilicity.

_Gatley and Sherratt, 1976_

This paper is of historical interest because it describes the localisation of the enzymes of the glycine conjugation pathway to the mitochondrial matrix. However, its greatest value to this thesis is the observation that benzoate rapidly accumulates in the mitochondrial matrix to about 50X the external concentration. The authors suggested that this is a result of the pH gradient over the inner mitochondrial membrane, with the higher pH in the matrix favouring the dissociation of benzoic acid and accumulation of
benzoate. This observation is important for explaining why the glycine conjugation pathway is situated in the mitochondrial matrix, and why conjugation to glucuronic acid, which occurs in the cytoplasm, would not be ideal for the detoxification of benzoate (Figure 4 of Paper II).

Caldwell, 1982

In this paper the conjugation reactions in metabolism are reviewed and defined as processes that require high-energy intermediates, are catalysed by specific enzymes, consume relatively “nonessential” cofactors, and that generally tend to decrease the toxicity or facilitate excretion of the parent compounds.
Appendix III: A method for the cost-effective synthesis of benzoyl-CoA and other acyl-CoA thioesters

Patent application for the synthesis of acyl-CoAs

Inventors:
Christoffel Petrus Stephanus Badenhorst and Alberdina Aike van Dijk

International application number:
PCT/IB2011/053721

The patent application was filed by:
Ursula Brunetti, DM Kisch Inc., Johannesburg, South Africa
Receipt of Electronic Submission

It is hereby acknowledged that a PCT International Application has been received via the Secure Electronic Submission Software of the IB. Upon receipt, Application Number and a Date of Receipt (Administrative Instructions, Part 7) has been automatically assigned.

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<td>PCT/AB2011/053458</td>
</tr>
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<td>Date of Receipt:</td>
<td>03 August 2011</td>
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Your Reference: P42228PC00
Applicant: NORTH-WEST UNIVERSITY
Number of Applicants: 3
Title: SYNTHESIS OF ACYL-PANTETHEINE DERIVATIVES AND THE USE THEREOF IN THE SYNTHESIS OF ACYL-COENZYME A DERIVATIVES

Documents Submitted:
- P42228PC00-vlog.xml 1740 03 August 2011 16:16:16
- P42228PC00-requ.xml 4194 03 August 2011 16:28:36
- P42228PC00-appb-P000004.pdf 698534 03 August 2011 15:45:56
- P42228PC00-appb-P000003.pdf 38121 03 August 2011 16:13:04
- P42228PC00-appb-P000002.pdf 205531 03 August 2011 16:10:40
- P42228PC00-appb-P000001.pdf 186643 03 August 2011 16:10:24
- PCT101.GML 3079 03 August 2011 16:28:45
- P42228PC00-appb.xml 845 03 August 2011 16:16:16
- P42228PC00-fees.xml 2470 03 August 2011 16:16:16

Signed by: EMail=lizm@dmkisch.com, CN=Marius Le Roux, OU=www.verisign.com/repository/CPS Incorp. by Ref.,LIAB.LTD(c)99, OU=WIPO Customer CA, O=World Intellectual Property Organization

Timestamp of Receipt: 03 August 2011 16:28

/ Geneva, RO/IB/
SYNTHESIS OF ACYL-PANTETHEINE DERIVATIVES

Technical Field

The present invention relates to a novel synthesis method for acyl-pantetheine derivatives. The present invention further relates to the synthesis of acyl-pantetheine derivatives for use as a starting material in the enzymatic synthesis of acyl-coenzyme A derivatives.

Background of the Invention

Coenzyme A esters are used as substrates by about 4% of enzymes, including several that are involved in fundamental biochemical reactions. Examples include enzymes of the Krebs cycle, β-oxidation enzymes, enzymes involved in histone modification and enzymes responsible for antibiotic resistance in bacteria. Studies of all these groups of enzymes are dependent on the use of coenzyme A esters as substrates or probes of enzyme function.

Commercially available coenzyme A preparations are very expensive. Various procedures have been developed for the chemical or combined chemical and enzymatic synthesis of coenzyme A and coenzyme A analogues. Typical methods included the use of crude enzyme extracts together with pantothenic acid derivatives chemically phosphorylated at position 4’ (Martin, D. P.; Bibart, R. T.; Drueckhammer, D. G. Synthesis of novel analogs of acetyl coenzyme A: mimics of enzyme reaction intermediates, J. Am. Chem. Soc. 116 (1994) 4660-4668).

One of the most successful, recent approaches is a “one-pot” enzymatic synthesis that uses purified recombinant biosynthetic enzymes from E. coli as the catalysts for the synthesis of coenzyme A analogues from pantetheine derivatives and adenosine triphosphate.

This method can be used to synthesize various analogues of coenzyme A by starting with the corresponding analogue of pantetheine, said pantetheine analogue being a

5 The described method uses the purified recombinant CoA biosynthetic enzymes from *Escherichia coli*, namely pantothenate kinase (PanK), phosphopantotheine adenylytransferase (PPAT), and dephosphocoenzyme A kinase (DPCK) for the modification of chemically synthesized pantothenate derivatives. Chemical synthesis of pantothenic acid derivatives followed by one-pot assembly into the CoA scaffold by the purified biosynthetic enzymes allows facile access to a variety of CoA derivatives. The principle of the synthesis method is illustrated in Figure 1 for the synthesis of coenzyme A from underivatised pantetheine.

![Figure 1](image)

Figure 1 The enzymatic synthesis of coenzyme A from pantetheine. The diagram shows the three enzymes, PanK, PPAT and DPCK, which are involved in the biosynthesis of coenzyme A from pantetheine. The symbol below PanK symbolises feedback inhibition on this enzyme by coenzyme A.

In order to synthesize various analogues of coenzyme A, the corresponding pantetheine analogue is chemically synthesized from pantothenic acid as a precursor. This approach is unfortunately not very efficient with respect to the number of process steps involved therein. Moreover, the percentage of yield obtained from this approach is not satisfactory, with yields in the range of between 50% and 95% being obtained. The overall cost of synthesizing pantetheine derivatives in accordance with this method is therefore not economical, particularly for commercial production purposes.
Accordingly, there is a need in the art for an alternative, efficient and economical method for synthesizing acyl-coenzyme A derivatives that does not suffer from the shortcomings associated with methods of the prior art, as described herein above.

Summary of the Invention

According to a first aspect thereof, the present invention provides a method for the synthesis of acyl-pantetheine derivatives, the method including the steps of:

a) providing a source of pantetheine;

b) providing a source of acyl ester; and

c) contacting the source of pantetheine with the source of acyl ester to form the corresponding acyl-pantetheine derivative, having the general formula (I)

\[
\text{R} \quad \text{(I)}
\]

wherein R is an acyl group.

According to a second aspect thereof, the present invention provides a method for the synthesis of acyl-coenzyme A analogues, the method including the steps of:

a) providing a source of pantetheine;

b) providing a source of acyl ester;

c) contacting the source of pantetheine with the source of acyl ester to form the corresponding acyl-pantetheine derivative, having the general formula (I)

\[
\text{R} \quad \text{(I)}
\]

wherein R is an acyl group; and
d) subjecting the acyl-pantetheine derivative of step (c) to one or more enzymatic reactions to form the corresponding acyl-coenzyme A analogue, having the general formula (II):

\[ (II) \]

\[
\begin{array}{c}
\text{wherein } R \text{ is an acyl group.}
\end{array}
\]

In an embodiment of the present invention, the source of pantetheine is pantetheine obtained by the reduction of pantethine with dithiothreitol (DTT) and bicarbonate (NaHCO₃). It will be thus be appreciated that step (a) discussed herein above may encompass the step of reducing pantethine to pantetheine.

The source of acyl ester may be prepared by contacting an organic acid, having the general formula R-COOH wherein R is any acyl group, with a suitable activating agent. It will be appreciated that the present invention is not limited to the aforesaid preparation method and any suitable acyl ester may thus be employed herein.

Non-limiting examples of the organic acid include benzoic acid, acetic acid, isovaleric acid, propionic acid, butyric acid, valeric acid, hexanoic acid, octanoic acid and dodecanoic acid. In a preferred embodiment of the invention, the organic acid is benzoic acid.
The activating agent may be any suitable activating agent, including, but not limited to N-hydroxysuccinimide, hydroxylbenzotriazole, N-hydroxy-5-norbornene-2,3-dicarboximide and various activated acyl-chlorides. Preferably, the activating agent is N-hydroxysuccinimide.

Non-limiting examples of the acyl group mentioned herein above include benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and dodecanoyl groups. In a preferred embodiment of the invention, the acyl group is benzoyl.

According to a further preferred embodiment, the present invention provides for the source of acyl ester to be a N-hydroxysuccinimide (NHS) ester of benzoic acid.

According to a yet further preferred embodiment, the present invention provides for R in any of the abovementioned formulae to be a benzoyl group.

It is to be appreciated by those skilled in the art of the instant invention that the acyl-pantetheine derivative, synthesized in accordance with the first aspect of the invention, may be employed in any suitable chemical process.

In terms of the second aspect of the instant invention, the invention provides a novel method for the synthesis of various acyl-coenzyme A analogues from pantetheine and the corresponding acyl ester.

In one embodiment, the enzymatic reactions referred to in step (d) of the second aspect of the present invention is a "one-pot" chemoenzymatic synthesis wherein three recombinant biosynthetic enzymes from *Escherichia coli* are employed as catalysts. In terms of this embodiment, recombinant pantothenate kinase (PanK), recombinant phosphopantotheine adenyltransferase (PPAT) and recombinant dephosphoenozyme A kinase (DPCK) are employed as the catalysts.

It will, however, be appreciated that said enzymatic synthesis of step (d) may be carried out by any other suitable procedure known and/or described to one skilled in the art.
According to a third aspect thereof, the present invention provides for an acyl-pantetheine derivative, having the general formula (I):

\[
\begin{align*}
\text{(I)} & \\
\text{\footnotesize where } R \text{ is an acyl group, prepared according to the method as described herein before in terms of the first aspect of the invention.}
\end{align*}
\]

According to a fourth aspect thereof, the present invention provides for an acyl-coenzyme A analogue, having the general formula (II):

\[
\begin{align*}
\text{(II)} & \\
\text{\footnotesize where } R \text{ is an acyl group, prepared according to the method as described herein before in terms of the second aspect of the invention.}
\end{align*}
\]
According to a fifth aspect thereof, the present invention provides for the use of a source of pantetheine and a source of acyl ester in the synthesis of an acyl-pantetheine derivative, having the general formula (I):

\[
\begin{align*}
\text{Formula (I)}
\end{align*}
\]

wherein \( R \) is an acyl group.

According to a sixth aspect thereof, the present invention provides for the use of a source of pantetheine and a source of acyl ester in the synthesis of an acyl-coenzyme A analogue, having the general formula (II):

\[
\begin{align*}
\text{Formula (II)}
\end{align*}
\]

wherein \( R \) is an acyl group.

The foregoing and other objects, features and advantages of the present invention will become more apparent from the following description of certain embodiments of the present invention by way of a specific example.
Example of the invention

The invention will now be described with reference to the following non-limiting example.

5 Preparation of pantetheine from pantethine

In this step, pantetheine is prepared by the reduction of pantethine with dithiothreitol (DTT) and bicarbonate.

10 Pantethine (42 mg), sodium bicarbonate (160 mg) and DTT (80 µmole) are dissolved in 3 ml of water, and left to stand for 10 minutes to allow reduction of the pantethine to pantetheine to take place:

30 Preparation of the N-hydroxysuccinimide (NHS) ester of benzoic acid

Benzoic acid and N-hydroxysuccinimide (10 mmoles of each) are dissolved in 40 ml of ethyl acetate (freshly distilled) in a screw-top Erlenmeyer flask. An equimolar amount of
dicyclohexylcarbodiimide is dissolved in 10 ml ethyl acetate and combined with the benzoic acid solution. The solution is thoroughly mixed and left to stand overnight at room temperature (in the dark). The insoluble white crystals of dicyclohexylurea are removed by filtration. The filtrate is dried under nitrogen to recover the white crystalline NHS-benzoic acid ester.

Synthesis of S-benzoyl pantetheine

This step involves the acylation of the already synthesized pantetheine to form S-benzoyl pantetheine, as shown in the figure below:

The above synthesized NHS-benzoic acid ester (0.12 g) is dissolved in 7 ml tetrahydrofuran (THF). The THF is distilled over sodium borohydride before use, to remove peroxides that may have formed during storage. This is done by mixing 500 ml of THF with 0.2 grams of the reducing agent before transferring to the distillation apparatus.

The water solution containing the pantetheine is combined with the NHS-benzoic acid ester THF solution in a small glass bottle and maintained as a single phase by vigorous
magnetic stirring for four hours. It is advised to test for the presence of free thiol groups at this stage, to determine the progress of the reaction.

*Test for free thiol groups: DTNB colour reaction*

A small amount (5 µl) of the reaction mixture is added to 495 µl of a 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB] solution (in 100 mM TrisCl, pH 8.0). If the solution turns deep yellow, the reaction is left for another 30 minutes.

The reaction mixture is subsequently transferred to a glass tube and left to settle into two phases. The upper THF layer is then removed under a stream of nitrogen gas. The remaining water is removed by freeze-drying. To dissolve the benzoyl pantetheine, 0.5 ml of absolute ethanol is added to the tube, which is then heated to 50°C in a water bath, with frequent shaking, for about five minutes. Water (5 ml) is added to the reaction mixture and the mixture is again heated to 50°C, with frequent shaking. The mixture is left to cool, and if the solution becomes cloudy, more water is added in 2 ml volumes, and the process repeated. This procedure dissolves the S-benzoyl pantetheine while leaving excess NHS ester behind as a white residue on the walls of the tube. The solution is poured out, and the tube rinsed with another 5 ml of water. The water fractions are combined and used without further purification for further synthesis procedures as may be required.

**Enzymatic synthesis of S-benzoyl coenzyme A**

The corresponding acyl-coenzyme A analogue may be prepared from the acyl-pantetheine derivative using procedures known to one skilled in the art and procedures forming part of the state of the art.

One such procedure includes a "one-pot" chemical enzymatic synthesis from the S-benzoyl pantetheine and adenosine triphosphate using three recombinant biosynthetic enzymes from *Escherichia coli* as the catalysts.
This procedure includes the preparation of the reaction mixture by combining 20 mM KCl, 10 mM MgCl₂, 18 mM adenosine triphosphate (ATP), 50 mM TrisCl, pH 7.5 and 5 mM benzoyl-pantetheine. [No additional purification of the previously synthesized benzoyl-pantetheine is required]. The pH is adjusted to 7.5 and the volume is increased to 30 ml with water.

The reaction is initiated by the addition of 5 mg of recombinant pantothenate kinase (PankK) and incubated at room temperature for 30 minutes. After 30 minutes, 5 mg of recombinant phosphopantotheine adenylyltransferase (PPAT) is added, followed by another 30 minute incubation. Finally, 5 mg of recombinant dephosphochoenzyme A kinase (DPCK) is added, followed by a two hour incubation at room temperature. Gentle magnetic stirring is performed throughout the incubations. After completion, the reaction mixture is passed through a 3 ml column of His-Bind resin to remove the recombinant enzymes.

Solid phase extraction can be used for purification and concentration of the benzoyl-coenzyme A. The product is relatively stable and resistant to oxidation, in contrast to 'free' coenzyme A. In addition, the inherent hydrophobicity of the benzoyl-group renders the corresponding coenzyme A product more hydrophobic, and hence much easier to purify by adsorption, using for instance a C18 solid phase extraction system.

It will be apparent by the skilled artisan from the foregoing disclosures that numerous advantages are associated with the present invention.

One such advantage is the fact that it not necessary to purify the acyl-pantetheine produced in step (c) prior to the enzymatic synthesis of step (d) in the synthesis of the corresponding acyl-coenzyme A. Since no intermediate purification step is required, the overall costs involved in the synthesis of the resultant coenzyme A product is significantly reduced, making the present invention more economically viable than the methods of the prior art. The present invention therefore provides a fast, convenient and efficient method for the synthesis of acyl-coenzyme A analogues.
Secondly, only two reaction vessels are employed for the synthesis of the acyl-coenzyme A product, thus contributing to efficiency and convenience of the present method.

Further advantages of the present invention reside in the fact that no losses of material occur, with the stoichiometric conversion of pantethine to the resultant acyl-coenzyme A analogue. This, in turn, allows for the possibility to scale up the instant method for commercial production purposes.

Whilst only certain embodiments or examples of the invention have been shown in the above description, it will be readily understood by any person skilled in the art that other modifications and/or variations of the invention are possible. Such modifications and/or variations are therefore to be considered as falling within the spirit and scope of the present invention as defined herein.

Dated this 03 day of August 2010

MARIUS LE ROUX

Patent Attorney/Agent for the Applicant
Appendix IV: List of publications and scientific posters

Peer Reviewed Publications:


Submitted Manuscript:

4. CPS Badenhorst, E Erasmus, R van der Sluis, C Nortje, and AA van Dijk. A new perspective on the importance of glycine conjugation for the detoxification of benzoic acid (Manuscript submitted to Drug Metabolism Reviews). (Paper II)
Scientific Posters Presented at Conferences

2010

- **Bacterial expression and elucidation of the catalytic mechanism of glycine N-acyltransferase**
  
  CPS Badenhorst, M Jooste, and AA van Dijk.
  
  Presented at the 2010 meeting of the Society for the Study of Inborn Errors of Metabolism in Istanbul, Turkey.

- **A novel method for the synthesis of thioester derivatives of coenzyme A**
  
  CPS Badenhorst, PJ Jansen van Rensburg, and AA van Dijk.
  
  Presented at the 2010 meeting of the Society for the Study of Inborn Errors of Metabolism in Istanbul, Turkey.

2011

- **Expression and characterisation of recombinant bovine glycine N-acyltransferase and elucidation of the GLYAT catalytic mechanism**
  
  CPS Badenhorst, M Jooste, and AA van Dijk.
  
  Presented at the 2011 joint meeting of the African and South African Societies for Human Genetics in Cape Town, South Africa.
Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase

R van der Sluis, CPS Badenhorst, FH van der Westhuizen, and AA van Dijk.

Presented at the 2012 joint South African Genetics and Bioinformatics Society Conference in Stellenbosch, South Africa.
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