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[Continued on next page]

- (54) Title:** SYNTHESIS OF ACYL-PANTETHEINE DERIVATIVES AND THE USE THEREOF IN THE SYNTHESIS OF ACYL-COENZYME A DERIVATIVES

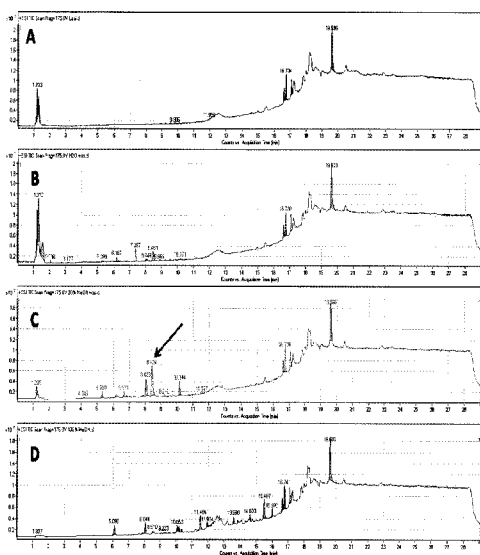
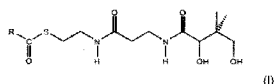


FIGURE 2



- (57) Abstract:** The present invention relates to a novel synthesis method for acyl-pantetheine derivatives. The present invention further relates to the use of said synthesized acyl-pantetheine derivatives as a starting material in the enzymatic synthesis of acyl-coenzyme A derivatives. 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), wherein R is an acyl group. The present invention also provides a method for the synthesis of acyl-coenzyme A derivatives as well as the use of a source of pantetheine and a source of acyl ester in the preparation steps of these two methods.



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**SYNTHESIS OF ACYL-PANTETHEINE DERIVATIVES AND THE USE THEREOF
IN THE SYNTHESIS OF ACYL-COENZYME A DERIVATIVES**

Technical Field

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

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Background of the Invention

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Coenzyme A esters are used as substrates by about 4% of enzymes, including several medically important enzymes. Coenzyme A dependent enzymes are involved in several fundamental biochemical reactions, including fatty acid biosynthesis and degradation, the citric acid cycle, synthesis of hormones, histone modification and the inactivation of antibiotics. The study of all of these groups of enzymes is dependent on the use of coenzyme A esters as substrates or probes of enzyme function.

20

These latter compounds are commonly either bought from a commercial supplier or synthesized using commercially obtained coenzyme A as the precursor. Coenzyme A itself is commonly isolated from yeast, but the large culture volumes that have to be processed and the extensive purification that is needed make these commercially available coenzyme A preparations both inconvenient and expensive.

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In view of these disadvantages, several chemical and combined chemical and enzymatic synthesis methods of coenzyme A and coenzyme A analogues have been developed.

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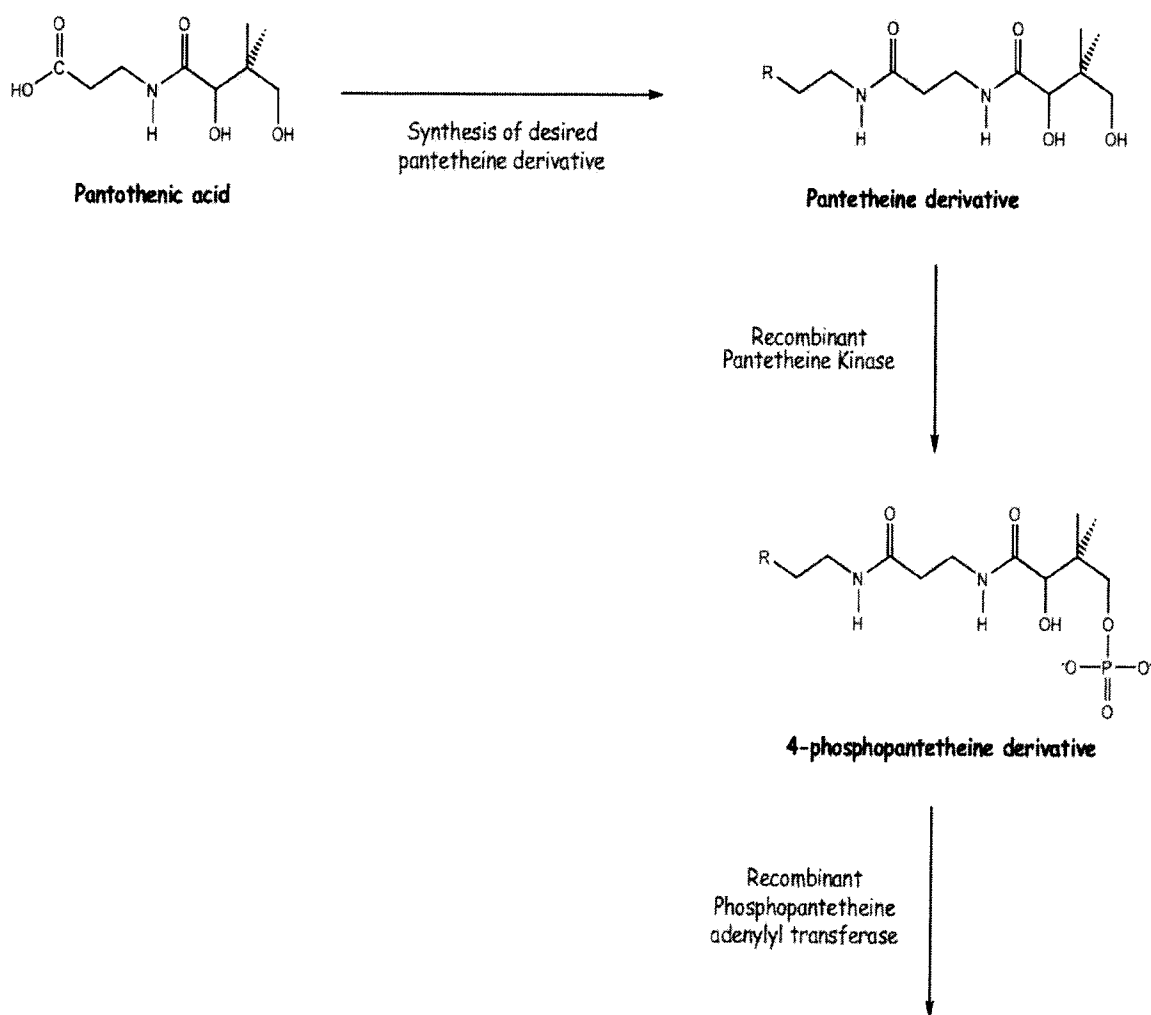
Typical chemo-enzymatic approaches to the synthesis of coenzyme A analogues employ crude enzyme extracts and use pantothenic acid derivatives chemically phosphorylated at position 4' (Martin, D. P.; Bibart, R. T.; Drueckhammer, D. G. *Synthesis of novel analogs of acetyl coenzyme A: mimics of enzyme reaction intermediates*, J. Am. Chem. Soc. 116 (1994) 4660-4668).

35

One of the most successful, recent chemo-enzymatic approaches to the synthesis of coenzyme A analogues is a "one-pot" enzymatic synthesis that uses three purified recombinant biosynthetic enzymes from *Escherichia coli* (*E. Coli*) as catalysts for the reaction, namely pantothenate kinase (PanK), phosphopantotheine adenylyltransferase (PPAT), and dephosphocoenzyme A kinase

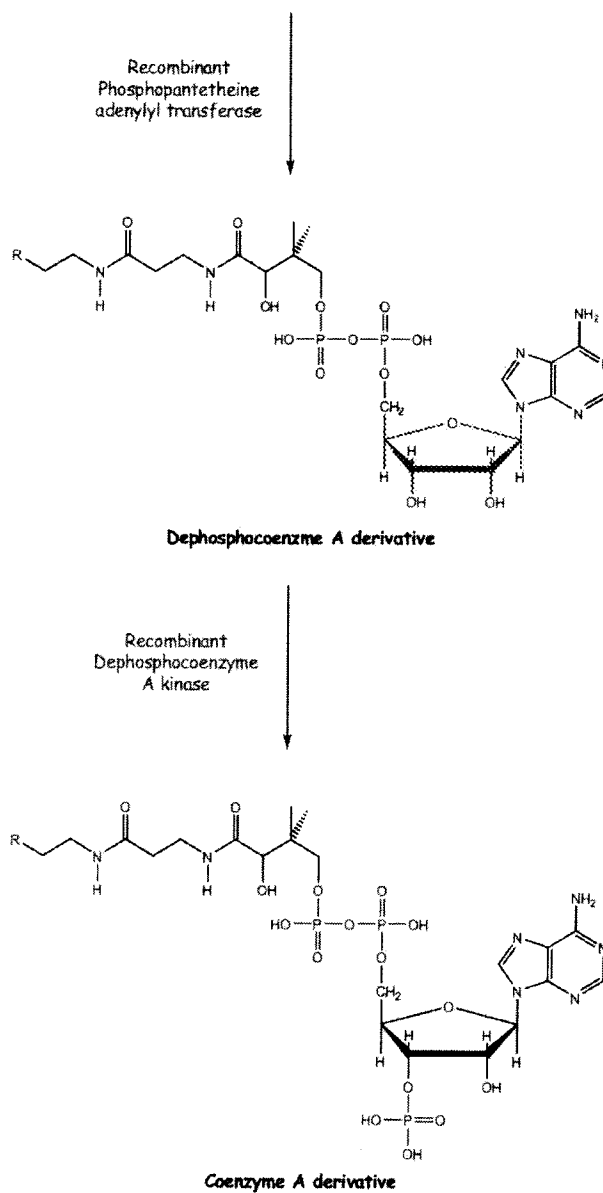
(DPCK) (Nazi, I; Koteva, K. P.; Wright, G. D. *One-pot chemo-enzymatic preparation of coenzyme A analogues*, Analytical Biochemistry 324 (2004) 100–105).

5 In terms of this method, pantothenic acid analogues are chemically synthesized followed by a one-pot enzymatic synthesis of the pantothenic acid analogues by the aforesaid purified biosynthetic enzymes to form a variety of coenzyme A analogues. The principle of this synthesis method for the synthesis of coenzyme A is illustrated in Figure 1 wherein underivatized pantetheine is chemically synthesized from underivatized pantothenic acid as a precursor.



reaction continued on next page.....

reaction continued from previous page.....



This one pot approach is unfortunately not very efficient since it is necessary to first chemically synthesize/prepare pantothenic acid (or pantothenic acid analogues) to be employed as the precursor for the synthesis of coenzyme A (or coenzyme A analogues) thereby increasing the number of process steps involved therein. Thus, the overall cost of synthesizing coenzyme A derivatives in accordance with this method is therefore not economical, particularly for commercial production purposes.

In the method described by Al-Arif (Al-Arif, A. and Blecher, M. *Synthesis of fatty acyl CoA and other thiol esters using N-hydroxysuccinimide esters of fatty acids*, Journal of Lipid Research 10(1969) 344-345), use is made of an *N*-hydroxysuccinimide ester of palmitic acid to acylate free coenzyme A, forming (S)-palmitoyl-coenzyme A. Although this method is effective as described, it has several disadvantages, especially regarding the synthesis of acyl-coenzyme A species of shorter chain length.

In the Al-Arif approach, free coenzyme A is needed. This may either be obtained from a commercial source or synthesized from suitable precursors. Because the free coenzyme A can hardly be protected from oxidation by the oxygen in air, commercial coenzyme A preparations, as well as those synthesized in the lab, are usually a combination of free coenzyme A and oxidized disulfide-linked dimers (two coenzyme A moieties linked by the sulfur atoms). Because the coenzyme A dimers cannot be acylated, and would thus negatively impact the yield of the product, a reducing agent is added to maintain the coenzyme A in the free sulfhydryl form. In the Al-Arif paper, thioglycolic acid is used as a reducing agent.

The disadvantage of this is that the thioglycolic acid used as reducing agent is also acylated, forming a substantial amount of a contaminating substance (S-palmitoyl-thioglycolic acid). In addition, the authors report the presence of (S)-palmitoyl-glutathione after acylation, which appears to be derived from impurities with respect to the coenzyme A preparation used.

To finally purify the palmitoyl-coenzyme A formed, perchloric acid is added to the water-THF mixture in order to precipitate (S)-palmitoyl-coenzyme A, (S)-palmitoyl-thioglycolic acid, (S)-palmitoyl-glutathione, and the excess palmitoyl-NHS ester. This results in a very crude preparation of the palmitoyl-coenzyme A, which must be extensively purified in order to obtain a usable product. This is done by washing the precipitate with acetone and diethyl ether, dissolving the remaining residue in water, precipitating again with perchloric acid, and washing a last time with acetone. This finally results in 87% yield of product, based on the amount of coenzyme A used. As there are no free sulfhydryl groups at the end of the synthesis, before purification,

complete conversion of coenzyme A to (S)-palmitoyl-coenzyme A can be assumed. This means that the complicated purification strategy leads to substantial loss of product.

5 There is a further, more serious limitation of this synthetic strategy. In the paper, the synthesis of other long-chain acyl-coenzyme A esters is also mentioned in the discussion. In this sense, the method appears to be as widely applicable as the authors claim. However, and to the Applicant's knowledge, it is not possible to precipitate all coenzyme A esters using perchloric acid (benzoyl-coenzyme A, for example, cannot be precipitated using high concentrations of perchloric acid). This means that the method is not applicable to the synthesis of short-chain acyl-coenzyme A
10 esters, as it would be impossible to purify the compounds by means of precipitation. In addition, use of other purification strategies would be complicated by the contaminants in the reaction, such as the (S)-acyl-thioglycolic acid. As an example, solid phase extraction would not be applicable, as the acyl-coenzyme A and the acyl-thioglycolic acid would both bind strongly to a C18 matrix.

15 In addition to the abovementioned prior art methods for synthesizing coenzyme A, the art teaches of a further conventional method of synthesizing Acyl-coenzyme A compounds using free coenzyme A and the acyl-chloride of the desired carboxylic acid. This method is fast and simple, but there are two significant disadvantages associated therewith. Firstly, acyl-chlorides are not
20 stable, thus making it impractical to store acyl-chlorides for long periods of time until use. This poses a particular problem should only small quantities of acyl-coenzyme A be prepared at a time. Secondly, acyl-chlorides are known to be very aggressive acylating reagents that will add acyl-groups not only to the desired thiol group, but also to amino- and hydroxy-groups. This results in substantial contamination by derivatives acylated at undesired functional groups.

25 Accordingly, there is a need in the art for an alternative, efficient, convenient 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.

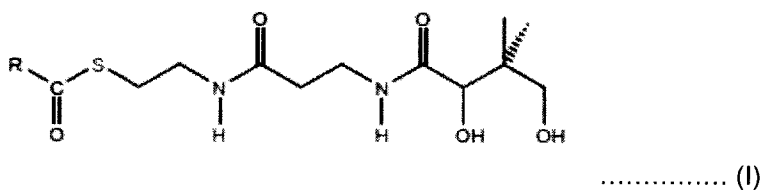
30 For purposes of the present specification, the term "derivative" and the term "analogue" may be used herein interchangeably.

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:

- 5 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)

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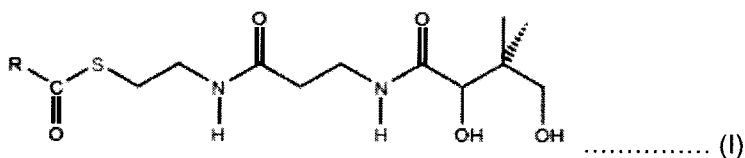
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wherein R is an acyl group.

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:

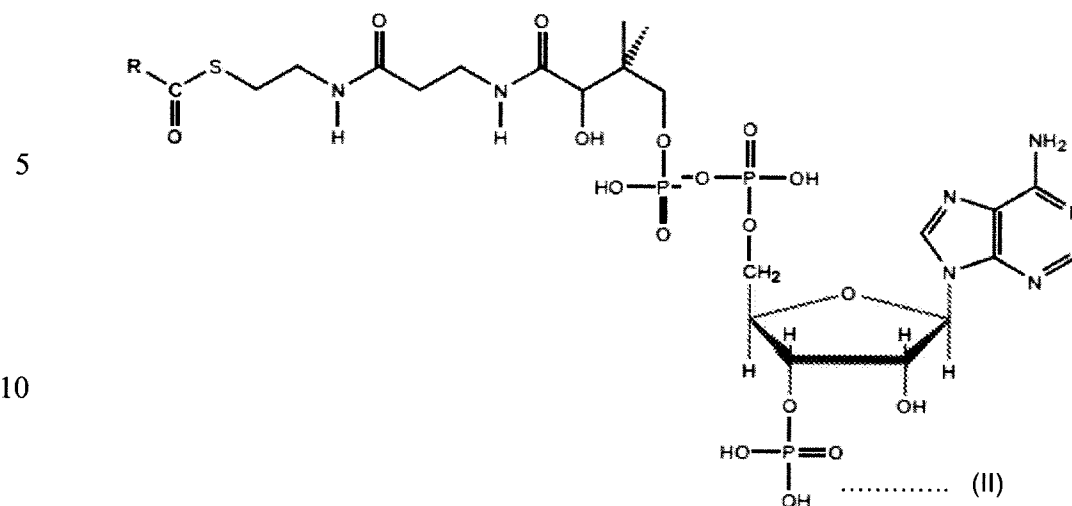
- a) providing a source of pantetheine;
 20 b) providing a source of acyl ester;
 c) contacting the source of pantetheine with the source of acyl ester to form the corresponding acyl-pantetheine derivative, having the general formula (I)

25



wherein R is an acyl group; and

- d) subjecting the acyl-pantetheine derivative of step (c) to one or more enzymatic reactions
 30 to form the corresponding acyl-coenzyme A analogue, having the general formula (II):



- 15 In an embodiment of the present invention, the source of pantetheine may be pantetheine obtained by the reduction of pantethine with dithiothreitol (DTT) and bicarbonate (NaHCO_3). It will thus be appreciated that step (a) discussed herein above may encompass the step of reducing pantethine to pantetheine.
- 20 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.
- 25 In terms of the present invention, the organic acid includes short- and medium-chain organic acids, in particular organic acids not longer than C8. 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 3-methylcrotonic acid. In a preferred embodiment of the invention, the organic acid is benzoic acid.
- 30 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 3-methylcrotonyl groups. In a preferred embodiment of the invention, the acyl group is benzoyl.

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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.

10

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.

15

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

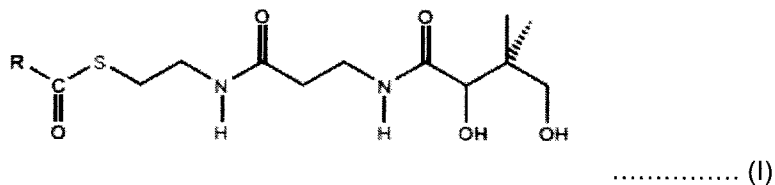
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It will, however, be appreciated that said enzymatic synthesis of step (d) may be carried out by any other suitable procedure known and/or described to one skilled in the art.

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According to a third aspect thereof, the present invention provides for an acyl-pantetheine derivative, having the general formula (I)

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10 wherein R is an acyl group, prepared according to the method as described herein before in terms of the first aspect of the invention.

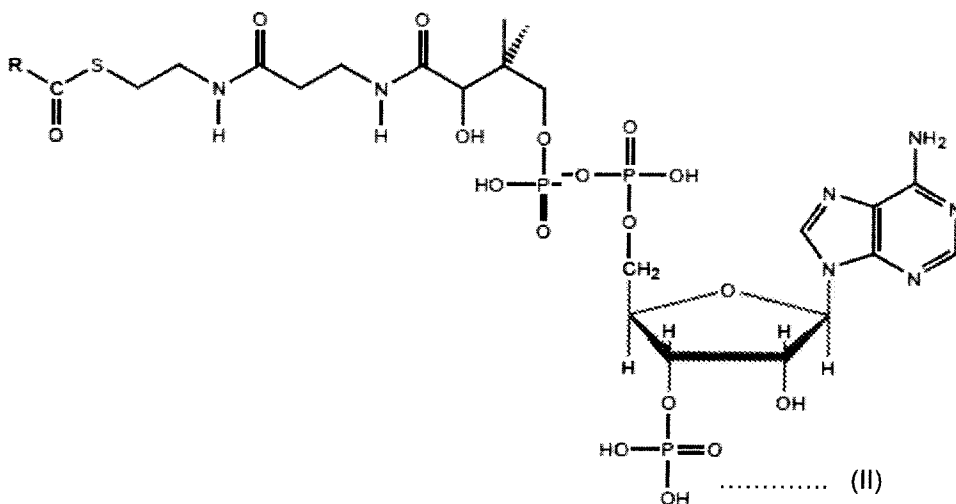
Non-limiting examples of the acyl group include benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and 3-methylcrotonyl groups. In a preferred embodiment of the
15 invention, the acyl group is benzoyl.

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

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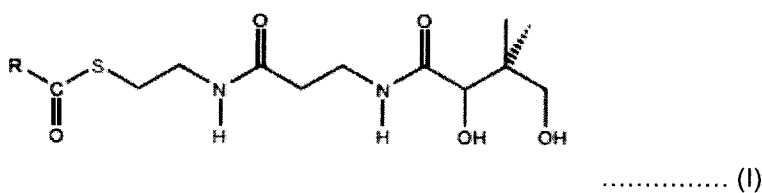
wherein R is an acyl group, prepared according to the method as described herein before in terms of the second aspect of the invention.

Non-limiting examples of the acyl group mentioned herein above include benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and 3-methylcrotonyl groups. In a preferred embodiment of the invention, the acyl group is benzoyl.

5

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)

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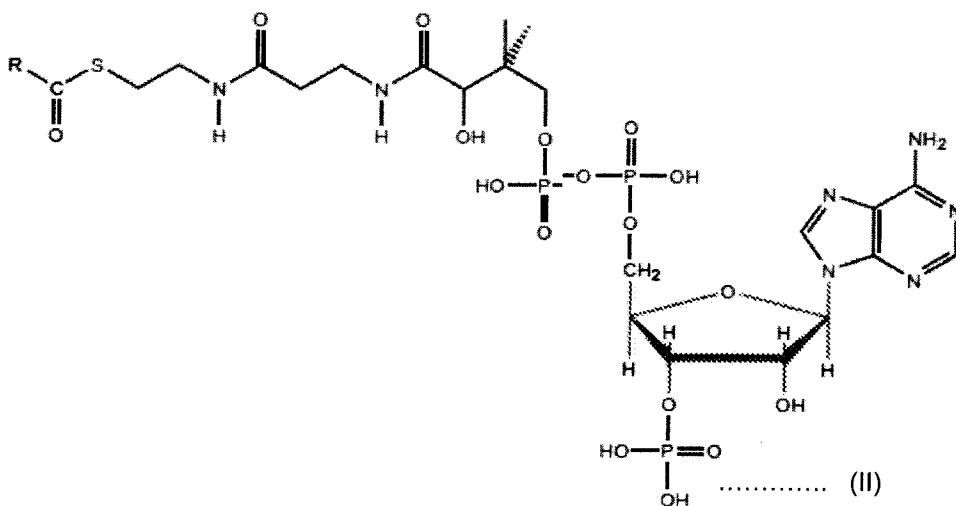
wherein R is an acyl group.

Non-limiting examples of the acyl group include benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and 3-methylcrotonyl groups. In a preferred embodiment of the invention, the acyl group is benzoyl.

20

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):

25



35

wherein R is an acyl group.

Non-limiting examples of the acyl group mentioned herein above include benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and 3-methylcrotonyl groups. In a preferred embodiment of the invention, the acyl group is benzoyl.

- 5 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.

Brief Description of the Drawings

10

Figure 1: is an agarose gel electrophoresis of PanK, PPAT and DPCK PCR amplicons. Lanes: 1) 5 µl of O'GeneRuler DNA marker; 2-4) PanK amplified with an annealing temperature of 50°C, 55°C and 60°C respectively; 5-7) PPAT amplified with an annealing temperature of 50°C, 55°C and 60°C respectively; and 8-10) DPCK amplified with an annealing temperature of 50°C, 55°C and 60°C respectively; and

15

Figures 2A to 2F: depicts the HPLC-TOF analysis of the purification of benzoyl-coenzyme A. 2A) is a chromatogram showing the flow through of the column loading step; 2B) is a chromatogram showing the water wash fraction; 2C) is a chromatogram showing the 20% methanol wash fraction; 2D) is a chromatogram showing the 100% methanol wash fraction; 2E) is a chromatogram showing only the benzoyl-coenzyme A peak; and 2F) is a mass spectrum of the peak in (2E) showing the benzoyl-coenzyme A species with an accurate mass of 872.441.

20

25

Example of the invention

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

5 Isolation of DNA from *Escherichia coli*

As template for the amplification of the coding sequences of the three biosynthetic enzymes, genomic DNA was isolated from *Escherichia coli* cells. To do this, the Genomic DNA purification kit from Fermentas was used. A 50 ml culture of LB medium was inoculated with a glycerol stock
10 of *Escherichia coli* JM109 cells, and incubated at 37 °C with vigorous shaking for 12 hours. No antibiotics were included in the medium, as no plasmid to confer antibiotic resistance was present in the cells. Of the dense culture, 2 ml was placed in a centrifuge tube, and the cells were harvested by centrifugation at 10 000 g for one minute. The supernatant was discarded and the instructions of the kit followed in order to isolate the DNA. The cells were resuspended in a buffer,
15 after which a cell lysis buffer was added to release the genomic DNA. A DNA precipitation buffer was then added to precipitate the DNA. The DNA precipitate was collected by centrifugation at 10 000 g for three minutes. The DNA was washed with 70% ethanol, dried, and resuspended in 500 µl of water.

20 PCR amplification of enzyme coding sequences

The coding sequences of the PanK, PPAT, and DPCK genes were PCR amplified from *Escherichia coli* genomic DNA using the primers listed in Table 1 herein below. The amplification reactions contained 1 x Takara ExTaq buffer, 10 nmol of each dNTP, 25 pmol of each primer,
25 50 ng of template DNA, and 2 units of Takara ExTaq DNA polymerase. The final reaction volume was 50 µl. Thermal cycling was performed using an Eppendorf thermal cycler. The cycling conditions were 94°C for 1 min, then 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by a final extension step of 10 minutes at 72°C.

30

Table 1: Oligonucleotide primers for amplification of PanK, PPAT and DPCK

Primer	Oligonucleotide sequence (5' → 3')	Manufacturer
PanK5'	GGGAATTCCATATGACCGCCAGAAACATGCTTATGAG	Inqaba Biotech
PanK3'	CGCGGATCCAAGCTTTTATTTGCGTAGTCTGACCTCTTCTACCGC	Inqaba Biotech
PPAT5'	GTCCTCGAGGGTACCATGCAAAAACGGGCGATTTATCC	Inqaba Biotech
PPAT3'	CGCGGATCCAAGCTTCTACGCTAACTTCGCC	Inqaba Biotech
DPCK5'	GGGAATTCCATATGAGGTATATAGTTGCCTTAACGGGAG	Inqaba Biotech
DPCK3'	CGCGGATCCAAGCTTTTACGGTTTTCTGTGAGACAACTGC	Inqaba Biotech

5 Cloning of enzyme coding sequences into pColdI

The PCR amplicons were cleaned up using the Machery Nagel Nucleospin II kit. For purposes of this ket, a buffer (NT) is added to the gel, which facilitates the dissolution of agarose at 50°C. The solution is then passed through a spin column containing a silica membrane. This membrane binds DNA in the presence of the chaotropic salts present in the NT buffer. Enzymes and most contaminants such as dNTPs, salts, primers and agarose do not bind and pass through the column. The membrane is then washed with a buffer (NT3) containing ethanol. DNA remains bound to the column whilst the remaining contaminants (such as ethidium) are washed off. After drying the column by centrifugation for three minutes at 10 000 g the DNA was eluted with 50 µl of water preheated to 80°C.

Plasmid DNA for sequencing and other manipulations was prepared using the PureYield plasmid midiprep kit from Promega. The instructions of the manufacturer were followed. For standard purification, 50 ml LB cultures containing 100 µg/ml ampicillin were inoculated with the desired clone and incubated at 37°C overnight with shaking. The cells were harvested by centrifugation at 2000 g for 15 minutes. The cells were then resuspended and lysed using the buffers provided. Proteins and genomic DNA are denatured by the dodecyl sulfate and high pH, while the closed-circular plasmid molecules remain in double-stranded conformation (Sambrook & Russell, 2001). Most proteins and high molecular weight chromosomal DNA were then precipitated by the addition of the ammonium acetate-acetic acid buffer, which neutralises the pH and precipitates SDS-protein complexes. After removal of the precipitate, the cleared lysate is passed through a DNA binding column (the principles are the same as for the DNA clean-up columns discussed above and the column washed with the endotoxin removal and column wash buffers. The column

is dried by centrifugation at 1500 g for 10 minutes. DNA was eluted with 600 µl of water. The plasmid preparations were analysed with the NanoDrop ND-1000 system.

5 The amplicons and pColdI DNA (isolated as described in the paragraph above) were digested with NdeI and HindIII (for PanK and DPCK) or KpnI and HindIII (for PPAT). Digested DNA was gel purified before use in ligation reactions.

10 Ligations of digested plasmid vectors and insert genes were performed as described in the literature (Sambrook & Russell, 2001). Reaction mixtures contained, in 30 µl, 18 pmol digested vector, 54 pmol digested insert DNA (a 1:3 vector to insert molar ratio), 5 Weiss units of T4 DNA ligase, and 1 x ligation buffer.

The ligation reactions were used to transform electrocompetent *Escherichia coli* (JM109) cells.

15 Transformation of electrocompetent *Escherichia coli* cells was performed as described in the literature (Sambrook & Russell, 2001). Usually, 10 µl of a 30 µl ligation reaction or 100 pico-grams of super helical plasmid DNA was used for a transformation. A BioRad GenePulser Xcell electroporator and GenePulser cuvettes were used.

20 Frozen electrocompetent cells (50 µl aliquots) were removed from storage at -80°C and thawed on ice. The DNA sample was then added and gently mixed. The cell slurry was transferred to a pre-chilled electroporation cuvette, making sure not to form any air bubbles. A pulse of 1.8 kV was applied for 1 ms. As soon as possible after pulsing, 1 ml of SOC medium was added, and the cells were allowed to recover at 37°C with gentle shaking for one hour. This allows expression of
25 the antibiotic resistance genes before selection using antibiotics is applied.

200 µl of the cell mixtures was spread out on LB agar plates containing 100 µg/ml ampicillin. The plates were left for 15 minutes to absorb the liquid and then incubated upside-down at 37°C for 16 hours.

30 Transformants were thereafter screened for the desired recombinant plasmids, and plasmid DNA was prepared for sequencing.

Screening of colonies of transformed bacteria

Colonies of transformed cells were screened for presence of the desired insert using either restriction analysis or PCR amplification.

5

For screening by means of restriction analysis, McCartney bottles containing 5 ml of LB medium (containing 100 µg/ml ampicillin) were inoculated with a colony picked from the plate. For short term preservation, the colonies were streaked onto a master plate prior to inoculation. The cultures were incubated overnight at 37°C, shaking at 180 rpm.

10

Plasmid DNA was then isolated from 2 ml of culture as follows. The cells were harvested by centrifugation at 16 000 g for 2 minutes. The supernatant was discarded, and 250 µl of STET buffer (8% sucrose, 5% TritonX-100, 50 mM EDTA, 50 mM Tris) added. The cells were resuspended by vortexing, and boiled at 98°C for one minute. The boiled lysates were immediately centrifuged for 8 minutes at 16 000 g. The pellet was removed with a toothpick and 5 µl of a 10 mg/ml ribonuclease A solution added. The mixture was incubated at room temperature for 10 minutes. DNA was precipitated by addition of 250 µl isopropanol, followed by centrifugation at 16 000 g for 10 minutes. The supernatant was discarded and the DNA washed with 600 µl of 70% ethanol. The DNA was dried in a Speed-vac and dissolved in 20 µl of 1/10 TE buffer by incubation at 65°C for 10 minutes. Of this plasmid preparation 1 µl was digested using restriction enzymes and the fragments analysed using agarose gel electrophoresis.

15

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DNA sequence determination

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To confirm that a recombinant plasmid contained the gene of interest without any sequence aberrations, Sanger sequencing was used. Samples were sent to the DNA sequencing laboratory of the Central Analytical Facility of the University of Stellenbosch. DNA sequence electrophoretograms were analysed using FinchTV version 1.40 (www.geospiza.com/finchtv) and ClustalX (Larkin et al., 2007) was used to align sequences to reference sequences.

30

Expression of the coenzyme A biosynthetic enzymes in *Escherichia coli* JM109

The recombinant proteins were expressed in the *Escherichia coli* JM109 cells into which the plasmids had been transformed. Thus *Escherichia coli* JM109 cells were used both for cloning
5 and expression of the enzymes.

Glycerol stocks were used to inoculate 150 ml cultures of LB medium containing 100 µg/ml ampicillin for plasmid selection. The cultures were grown overnight at 37°C, with vigorous shaking. In the morning, the cells were harvested by centrifugation, and resuspended in 200 ml
10 fresh LB medium containing 50 µg/ml ampicillin and incubated at 37°C for one hour. The cultures were then placed in a 15°C incubator, with gentle shaking, for 1 hour. Expression of recombinant proteins was then induced by the addition of IPTG to a final concentration of 0.5 mM and incubated at 15°C for 4 hours with vigorous shaking to ensure adequate aeration.

15 Purification of recombinant biosynthetic enzymes

Proteins were isolated using the BugBuster protein extraction reagent as follows.

Cells were harvested by centrifugation at 2000 g for 10 minutes. BugBuster protein extraction
20 reagent containing rLysozyme and Benzonase nuclease, prepared according to the manufacturer's instructions, was then added (5 ml per gram of wet cell mass). The cells were resuspended by gentle vortexing and incubated at room temperature for 5 minutes to allow for cell lysis. At this point a sample was taken for the total protein fraction. The insoluble material was then removed by centrifugation at 16 000 g for 20 minutes in a 4°C centrifuge.

25 The cells were harvested from the 200 ml culture by centrifugation, except for PPAT, where only 100 ml of culture was used. The cell pellet was then resuspended in 5 ml of BugBuster and incubated at room temperature for 1 minute. To dilute the proteins, 15 ml of ice-cold Binding buffer (50 mM TrisCl, 300 mM NaCl, 20 mM imidazole, pH 7.9) was then added. The mixture was
30 then incubated for a further 5 minutes on ice, before the insoluble material was separated by centrifugation at 12 000 g and 4°C for 30 minutes.

The proteins were then purified by nickel affinity chromatography, using the His•Bind resin from Novagen. Columns with a bed volume of 2.5 ml were packed and charged as described by the
35 manufacturer. The clarified lysates were loaded onto the columns, and allowed to drain by gravity. The columns were then washed with 3 volumes of Binding buffer, followed by 4 volumes of Wash buffer. Proteins were eluted in 15 ml of Elution buffer. The PanK and DPCK enzymes

were then dialysed at 4°C against 2 litres of storage buffer (50mM Hepes, pH 8.0, 250mM NaCl, 2mM MgCl₂), with one exchange of buffer. PPAT was not dialysed, but instead mixed immediately after elution with glycerol to a final concentration of 20% to prevent protein precipitation. Proteins were stored at 4°C in this dilute form until needed.

5

When needed, a volume of each enzyme solution containing 5 mg of protein was concentrated to less than 1000 µl using Vivaspin ultra filtration membranes. This was done immediately before use in the enzymatic synthesis. SDS-PAGE was used to monitor recombinant protein expression, as described below.

10

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS-PAGE, as described in the literature, was used for routine analysis of protein expression and purification procedures (Laemmli, 1970, Sambrook & Russell, 2001). In short, samples are

15 boiled with SDS to form complexes with a net negative charge and are then separated according to size by migration through a cross-linked polyacrylamide gel. Finally, the proteins are visualized by staining with Coomassie brilliant blue.

Separating gels generally had a final concentration of 10% acrylamide, unless indicated to be

20 15%. The composition of the separating gels was 10% acrylamide, 0.27% bisacrylamide, 375 mM TrisCl (pH 8.8) and 0.1% SDS. The composition of the stacking gels was 3.9% acrylamide, 0.1% bisacrylamide, 375 mM Tris-Cl (pH 6.8) and 0.1% SDS. Polymerization was catalysed by addition of 0.008% TEMED and 0.08% ammonium persulfate.

25 The separating gel was prepared by mixing all the components in an Erlenmeyer flask before addition of the persulfate and TEMED. The gel was then poured into an assembled Bio-Rad Mini Protean gel casting apparatus (70 x 76 mm). The gel was then overlaid with water-saturated isobutanol and left to set for about an hour at room temperature. The butanol was then poured off, and the surface of the gel dried with filter paper. The stacking gel was then prepared and

30 poured on top of the separating gel, followed by insertion of a ten well comb. Again the gel was left to set, after which it was immediately used.

Protein samples were prepared by combining 5 µl of sample with 5 µl of 4X protein loading buffer, 9 µl of water and 1 µl of 20X reducing agent. The samples were then mixed and boiled for

35 5 minutes at 98°C. Unless otherwise stated, 10 µl of this mixture was loaded onto the gel. For size estimation 5 µl of a protein molecular size marker mixture (Fermentas SM1183) was always loaded in one lane. The loaded gel was then electrophoresed in 1X TGS buffer at a constant

current of 30 mA using a Bio-Rad PowerPac Basic system. Electrophoresis was for about 40 minutes, or until the pink dye front reached the bottom of the gel.

5 The electrophoresed gels were removed from the glass plates, rinsed with water and then submerged in Coomassie gel staining solution with gentle shaking for 60 minutes. The gels were then removed from the staining solution and rinsed with a small volume of methanol-acetic acid gel destain solution before submersion in more destain solution. The destaining gel was gently shaken, with occasional exchange of the destain solution until the gels were no longer blue in colour. The stained gels were placed between two plastic sheets and digitised using an HP digital
10 document scanner.

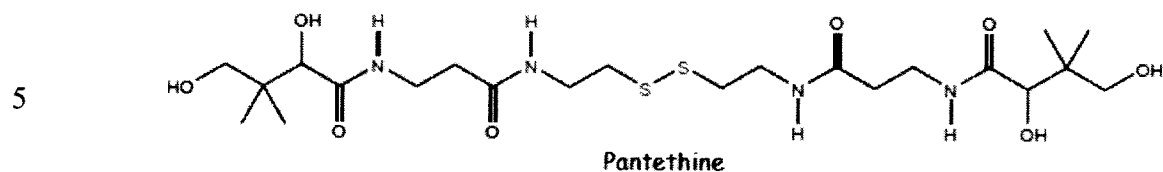
Synthesis of S-benzoyl pantetheine

As a first step, the NHS ester of benzoic acid was synthesised (Lapidot et al., 1967). This was
15 then used to acylate pantetheine, which was generated by reducing pantethine with DTT (Al Arif & Blecher, 1969). The procedure is outlined below.

The N-hydroxysuccinimide ester of benzoic acid was synthesised as follows. Benzoic acid and N-hydroxysuccinimide (10 mmol each) were dissolved in 40 ml of ethyl acetate (freshly distilled) in a
20 screw-top Erlenmeyer flask. An equimolar amount of dicyclohexyl-carbodiimide was dissolved in 10 ml ethyl acetate, and combined with the benzoic acid solution. The solution was thoroughly mixed by swirling the flask, and left to stand overnight at room temperature (in the dark). Dicyclohexylurea (the insoluble white crystals that form) was removed by filtration. The filtrate was dried under nitrogen to recover the white crystalline NHS-benzoic acid ester. The product
25 was used in the next steps as is, without further purification.

The acylation of pantetheine to form S-benzoyl pantetheine was carried out as follows. Pantethine (42 mg), sodium bicarbonate (160 mg), and DTT (80 μ mol) were dissolved in 3 ml of water, and left to stand for 10 minutes to allow reduction of the pantethine to pantetheine to take
30 place. The NHS-benzoic acid ester (0.12 grams) was dissolved in 7 ml of tetrahydrofuran (THF). The THF was distilled over sodium borohydride before use, to remove peroxides that may have formed during storage. This was done by mixing 500 ml of THF with 0.2 grams of the reducing agent before transferring to the distillation apparatus.

19

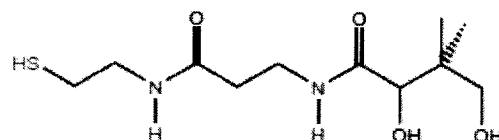


10

15

20

Dithiothreitol
bicarbonate
in H₂O



Pantetheine

- The water and THF solutions were combined in a small glass bottle and maintained as a single phase by vigorous magnetic stirring for four hours. At this point, 5 μ l of the reaction mixture was removed and tested for the presence of free thiol groups. This was done using the DTNB colour reaction for detection of sulfhydryl groups (Kolvraa & Gregersen, 1986). The 5 μ l of sample is added to 495 μ l of a 0.1 mM DTNB solution (in 100 mM TrisCl, pH 8.0). If the solution turned deep yellow, the reaction was left for another 30 minutes.
- The reaction was transferred to a glass test tube and left to settle into two phases. The upper THF layer was then removed under a stream of nitrogen gas. The remaining water was removed by freeze-drying. To dissolve the benzoyl pantetheine, 0.5 ml of absolute ethanol was added to the tube, which was then heated to 50°C in a water bath, with frequent shaking, for about 5 minutes. To this 5 ml of water was added, and the mixture was again heated to 50°C, with frequent shaking. The mixture was left to cool, and if the solution became cloudy, more water was 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 were combined, and used without further purification in the enzymatic synthesis.

Enzymatic synthesis of benzoyl-coenzyme A

5

The enzymatic synthesis reaction mixtures contained 20 mM KCl, 10 mM MgCl₂, 18 mM ATP, 50 mM TrisCl, pH 7.5 and 5 mM benzoyl-pantetheine in a final volume of 30 ml. This reaction mixture was set up by combining the benzoyl-pantetheine solution prepared in the previous step with the other components and increasing the volume to about 25 ml. The pH was then adjusted to 7.5 before filling up the volume to 30 ml.

10

The reaction was initiated by the addition of 5 mg of recombinant PanK and incubated at room temperature for 30 minutes. After 30 minutes, 5 mg of recombinant PPAT was added, followed by a further 30 minute incubation period. Finally, 5 mg of recombinant DPCK was added, followed by a 2 hour incubation period at room temperature. Gentle magnetic stirring was performed throughout the incubations. After completion, the reaction mixture was passed through a 3 ml column of His•Bind resin to remove the recombinant enzymes.

15

Partial purification of coenzyme A using solid phase extraction

20

Solid phase extraction was used for purification and concentration of the benzoyl-coenzyme A since, in contrast to 'free' coenzyme A, the product is relatively stable and resistant to oxidation. 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.

25

In terms hereof, a column packed with a resin onto which hydrophobic molecules such as C8 or C18 are immobilised is used to adsorb compounds from a solution. The column can then be washed with water to remove salts and very polar or ionic compounds. The desired compound can then be eluted from the column using methanol. If it is known at which methanol concentration the desired compound elutes, this information can be used to better purify the compound. The column can first be washed with a lower methanol concentration to elute the less hydrophobic molecules, after which the compound is eluted with the lowest possible methanol concentration. This elutes the compound of interest while leaving the more hydrophobic compounds on the column.

30

35

The reaction mixture was passed through a Zorbax XDB-C18 solid phase extraction column, which is packed with a C18 resin. The column was washed with three volumes of 18 Ω water, and the compound was then eluted with three volumes of 20% methanol. The eluate was placed under a stream of nitrogen to remove most of the methanol before being freeze-dried. The lyophilised compound was stored at -20°C until needed.

HPLC-TOF analyses of the synthesis and purification of benzoyl-coenzyme A

HPLC-TOF analysis was used to monitor the synthesis and purification of benzoyl-coenzyme A and to accurately determine the concentration of benzoyl-coenzyme A in purified samples.

The analyses were performed using an Agilent 6210 Time-of-Flight LC/MS in extended dynamic range, coupled to an Agilent 1200 SL Series LC system. The LC system consisted of a binary pump, vacuum degasser, automatic liquid sampler, thermostated column compartment and MassHunter Workstation. Burdick & Jackson LC/MS grade acetonitrile and locally produced 18.1 Ω water were used for mobile phases.

Conditions for liquid chromatography are described as follows. A Zorbax XDB-C18 column (4.6 mm x 50 mm, 1.8 μ m) was used at 25°C. Two buffers were combined in different proportions to make up the mobile phases. Buffer A contained 5 mM ammonium formate and 0.1% formic acid in water and Buffer B was composed of 0.1% formic acid in acetonitrile. A flow rate of 0.4 ml/min was used for a 6 minute analysis. A gradient program was used: the proportion of Buffer B used was initially 5%, kept at 5% for 1 minute, gradually increased to 100% over 15 minutes, kept at 100% for 10 minutes, and then decreased to 5% over 3 minutes. Sample injection volume was 1 μ l.

The conditions used for time-of-flight mass spectrometry are described as follows. Positive ionisation was used, with a nozzle voltage of 500 V. Drying gas temperature and flow rate were 320°C and 8 L/min, respectively. A nebuliser gas pressure of 30 psi, capillary voltage of 3500 V and fragmentor voltage of 175 V were used. Reference ion masses of 121.050873 and 922.009798 were employed. A scan rate of 3 hertz was used. The MassHunter Qualitative Analysis program was used for molecular feature extraction and database searching.

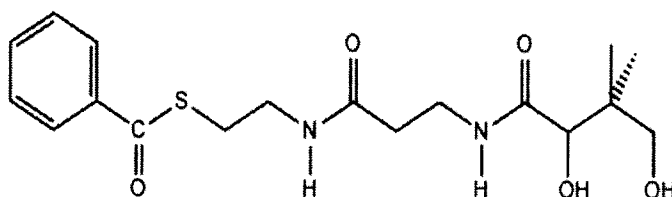
Results and discussion

Cloning of the PanK, PPAT, and DPCK coding sequences into pColdI

- 5 The agarose gel in Figure 1 shows the PCR amplicons for the PanK, PPAT, and DPCK coding sequences. Using NdeI and HindIII restriction enzymes for PanK and DPCK, and KpnI and HindIII restriction enzymes for PPAT, the PCR amplicons and pColdI vector were digested. After ligation and transformation, colonies were screened using restriction enzyme digestion.
- 10 Plasmids extracted from positive clones were sequenced using the pCold primers to confirm that the genes were cloned without any sequence aberrations. The sequences obtained for the three enzymes were identical to the reference sequences (GenBank accession numbers: gi 226957607 for PanK, gi 15804175 for PPAT and gi 91209166 for DPCK).

15 Synthesis of S-benzoyl pantetheine

- For the synthesis of benzoyl-coenzyme A, the pantetheine derivative benzoyl-pantetheine was used. It appears, from absence of literature, that S-benzoyl pantetheine has not been synthesised from benzoic acid and pantetheine before. From the structure of the compound, it appears that it
- 20 should be water soluble, due to its polar nature. In the development of the synthesis, it was attempted to extract the water phase with ethyl acetate, after the THF had been removed under a stream of nitrogen. It was thought that the benzoyl-pantetheine would remain in the water phase, while excess NHS ester and other organic residues would be extracted. However, after using the extracted water phase for the enzyme synthesis, it was found that only about 5% of the expected
- 25 yield of benzoyl-coenzyme A was obtained. The absence of benzoyl-pantetheine and the other intermediates of the synthesis indicated that there was no problem with the enzymatic synthesis and suggested that there was only a small amount of benzoyl-pantetheine to start with (HPLC-TOF results not shown). It turned out that the benzoyl-pantetheine preferentially enters the organic phase and that extraction with ethyl acetate removed the benzoyl-pantetheine from the
- 30 water phase. Similarly, S-benzyl-pantetheine, a related compound, is soluble in water saturated butanol and can be extracted from water with chloroform (Walton et al., 1954).



S-benzoyl pantetheine

Subsequently, another approach was used. After removing the THF phase under a stream of nitrogen, the water was removed by freeze-drying. If 0.5 ml of absolute ethanol was added to the residue and the mixture heated to about 50°C, some residue started to dissolve. By adding 5 ml of water, and heating this mixture to 50°C, more residue was dissolved. It was found that most residue benzoyl-pantetheine dissolves under these conditions. Less water soluble compounds, such as excess NHS ester of benzoic acid, are left behind on the walls of the glass tube. No standard was available for quantification of the benzoyl-pantetheine, but HPLC analysis indicated that the mixture contained high amounts of benzoyl-pantetheine, with only two unidentified contaminating peaks.

Enzymatic synthesis and purification of benzoyl-coenzyme A

To synthesise benzoyl-coenzyme A, the three recombinant biosynthetic enzymes were expressed and purified, and benzoyl-pantetheine was synthesised for use as the pantetheine analogue. The enzymatic synthesis was then carried out as described above. To purify the synthesised benzoyl-coenzyme A, solid phase extraction (SPE) was used.

After the synthesis was completed, HPLC-TOF analysis showed that neither benzoyl-pantetheine nor any of the intermediates of the synthesis were present in the reaction mixture. Furthermore, the amount of benzoyl-coenzyme A in the reaction mixture was what is theoretically expected, based on the amount of pantetheine started with. This indicated stoichiometric conversion of benzoyl-pantetheine to benzoyl-coenzyme A.

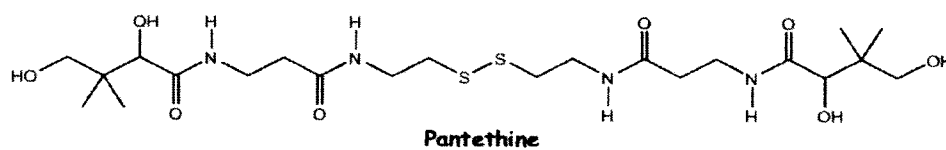
HPLC analysis was used to determine optimal methanol concentration for purification of benzoyl-coenzyme A. By eluting the HPLC column with a gradient of methanol in water, it was determined that benzoyl-coenzyme A elutes at 20% methanol. Using this information the benzoyl-coenzyme A could be purified easily. The synthesis mixture was passed through a solid phase extraction column. The column could then be washed with water to remove salts and polar compounds such

as ATP, ADP, and Tris buffer. The benzoyl-coenzyme A was then eluted with methanol at a minimal concentration of 20%. This eluted the benzoyl-coenzyme A, but left the more tightly binding contaminants on the column. These could later be removed by regenerating the column with a 100% methanol wash. The column flow through and column wash fractions contained only
5 small amounts of benzoyl-coenzyme A. The purified compound was then lyophilised as described herein before.

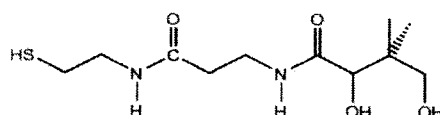
A sample of the benzoyl-coenzyme A was diluted to approximately 0.5 mg/ml for analysis. A commercial standard was also diluted to 0.5 mg/ml. Both the synthesised sample and the
10 commercial standard were analysed by HPLC-TOF, as described before. Because the benzoyl-coenzyme A could be well resolved as a narrow peak containing only benzoyl-coenzyme A and no contaminating compounds (Figure 2), the areas and ion-intensities of these peaks could be integrated and used to compare concentrations. The concentration of benzoyl-coenzyme A in the sample could be calculated by comparing the relative ion abundance readings from the TOF
15 analysis to that of the commercial standard (at the low concentrations analysed, the relationship between concentration and ion abundance is linear).

The 250 µl aliquots of benzoyl-coenzyme A were again freeze-dried, and could be stored at -20°C until needed. Based on the exact concentration determined using the HPLC-TOF analysis,
20 the volume of water needed to make up the lyophilised samples to 10 mg/ml could be calculated. Approximately 85 to 100 mg of benzoyl-coenzyme A was usually obtained for a synthesis. This is a yield of approximately 70% to 85%, which is acceptable. The synthesised benzoyl-coenzyme A could be used for enzyme assays, and in this sense is indistinguishable from the commercially obtained compound.

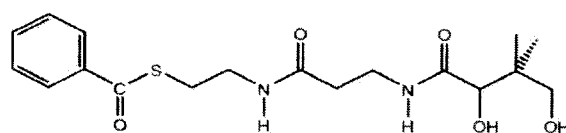
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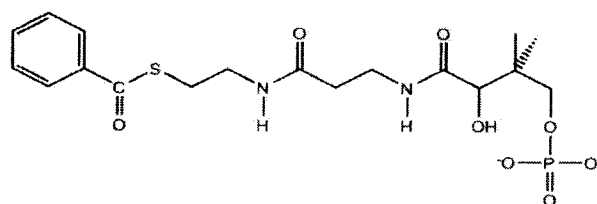
Dithiothreitol,
bicarbonate in H₂O



N-hydroxysuccinimide
ester of benzoic acid



Recombinant
Pantetheine Kinase

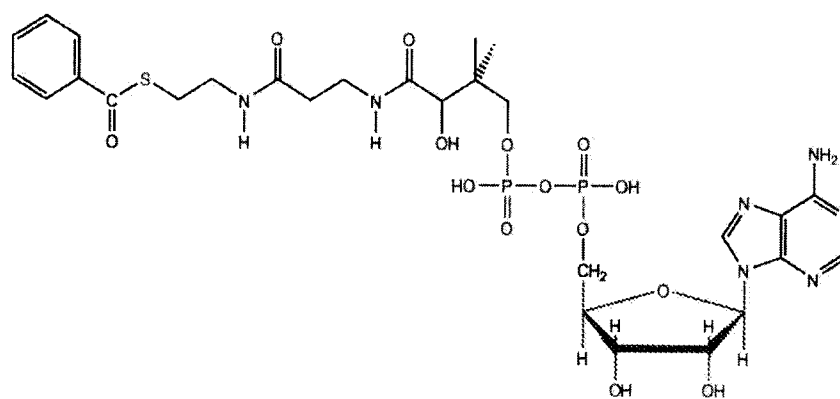


Recombinant
Phosphopantetheine
adenylyl transferase

reaction continued on next page.....

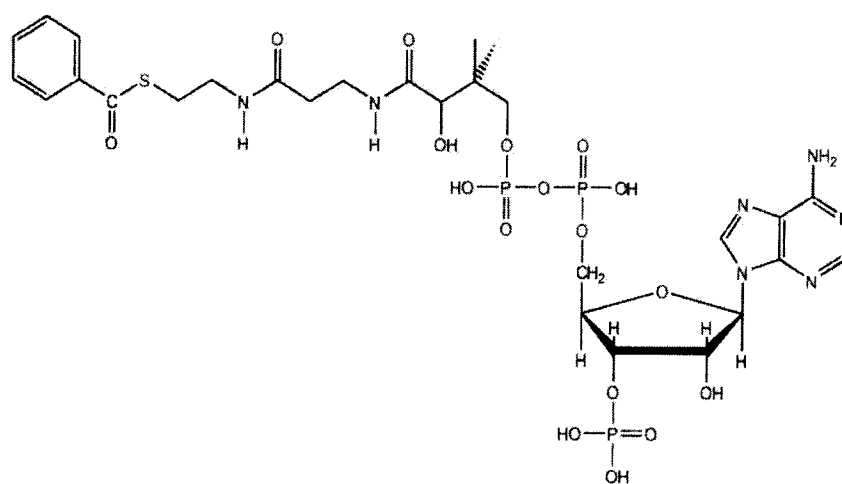
reaction continued from previous page.....

Recombinant
Phosphopantetheine
adenylyl transferase



(S)-benzoyl-dephosphocoenzyme A

Recombinant
Dephosphocoenzyme
A kinase



(S)-benzoyl-coenzyme A

In this way, the Applicant has now unexpectedly developed a novel method for the synthesis of acyl-coenzyme A, in particular benzoyl-coenzyme A.

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

As mentioned herein before, the study of certain fundamental biochemical reactions is dependent on coenzyme A. However, this compound is very expensive to purchase thus rendering research into these biochemical reactions very costly. The Applicant has now surprisingly found a method
10 of synthesizing acyl-coenzyme A analogues which presents a significantly cheaper alternative to commercial coenzyme A thereby making it affordable to perform large numbers of enzyme assays (coenzyme A is a far more expensive compound to purchase than the pantetheine and ATP needed for the method of the present invention). Whilst the exact saving is difficult to calculate, it appears that synthesis according to the method of the present invention is about two
15 orders of magnitude cheaper than purchasing the commercial compound.

In terms of the present invention, water is used to selectively dissolve acyl-pantetheine after acylation, leaving behind the water-insoluble contaminants, such as the acyl-NHS ester and the acyl-dithiothreitol esters. For this reason, no intermediate purification step is needed between
20 acylation of the pantetheine and use of the acylated product in the enzymatic synthesis reaction, thereby preventing losses due to inefficient purification.

Since no intermediate purification step is required, the overall costs and number of process steps involved in the synthesis of the resultant coenzyme A product is significantly reduced, making the
25 present invention more economically viable and convenient than the methods of the prior art. In this way, the present method affords a simple and efficient single-tube synthesis protocol without any complex, intermediate purification procedures.

Turning to the enzymatic synthesis reaction, this reaction can be considered to be a very "clean" synthesis in the sense that not many contaminating chemicals are involved. The reaction contains
30 only the acyl-pantetheine ester, certain buffer salts, excess ATP, the ADP and pyrophosphate formed by the enzymes, and the enzymes themselves. The main contaminants are then ATP and ADP, both very hydrophilic compounds. These compounds are easily washed off a C18 matrix, while the acyl-coenzyme A remains tightly bound. Thus, it is easy to achieve reasonable
35 purification using solid phase extraction.

It will be appreciated by the skilled artisan that synthesizing acyl-coenzyme A analogues according to the method of the instant invention, instead of first synthesizing coenzyme A and then acylating with benzoic acid, as was taught by the prior art, is most advantageous. Firstly, the pantethine reduction step and acylation with benzoic acid are carried out in one reaction mixture, which results in minimal loss of material. Further, the excess DTT used for reduction of pantethine is inactivated in this reaction (by the excess of NHS ester of benzoic acid). As mentioned above, it is accordingly not needed to purify the benzoyl-pantetheine before doing the enzymatic synthesis, further avoiding loss of material. Finally, the benzoyl-coenzyme A synthesized is less susceptible to oxidation and more easily purified than un-acylated coenzyme A.

In addition to the foregoing, a further advantage of the present invention resides 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.

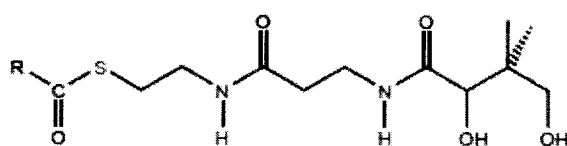
The present invention thus enables the simple, affordable, in-house synthesis of acyl-coenzyme A derivatives from the corresponding pantetheine analogue which avoids the shortcomings and disadvantages associated with the methods of the prior art.

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.

Claims

1. 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)

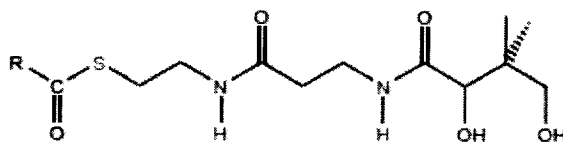


..... (I)

wherein R is an acyl group.

2. A method for the synthesis of acyl-coenzyme A analogues, the method including the steps of:

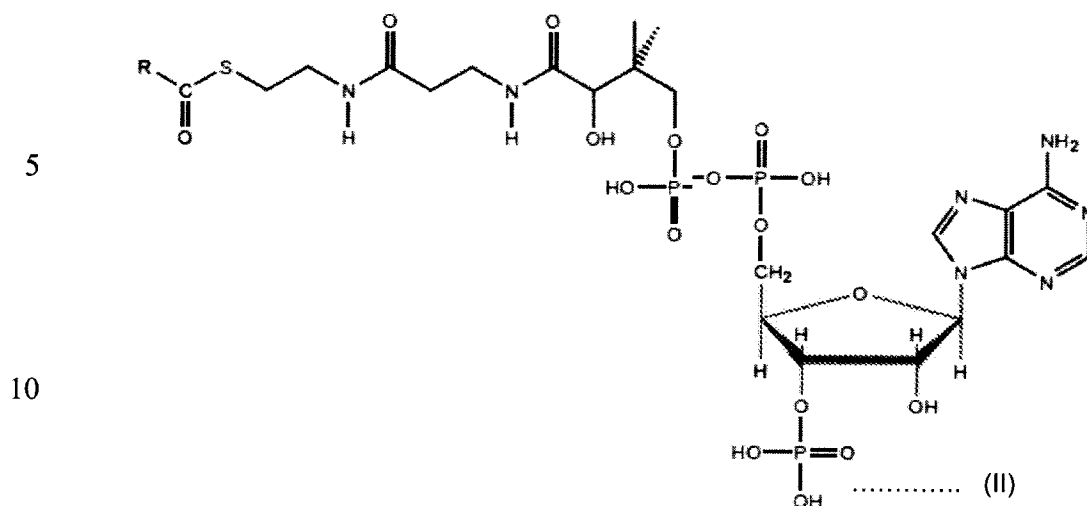
- a) providing a source of pantetheine;
- b) providing a source of acyl ester;
- c) contacting the source of pantetheine with the source of acyl ester to form the corresponding acyl-pantetheine derivative, having the general formula (I)



..... (I)

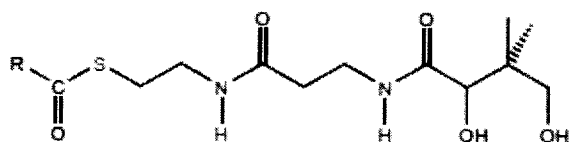
wherein R is an acyl group; and

- 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):



- 15 3. The method according to claim 1 or claim 2, wherein the source of pantetheine is obtained by the reduction of pantethine with dithiothreitol (DTT) and bicarbonate (NaHCO_3).
- 20 4. The method according to claim 1 or claim 2, wherein the source of acyl ester is prepared by contacting an organic acid, having the general formula R-COOH wherein R is any acyl group, with a suitable activating agent.
- 25 5. The method according to claim 4, wherein the organic acid is selected from the group consisting of benzoic acid, acetic acid, isovaleric acid, propionic acid, butyric acid, valeric acid, hexanoic acid, octanoic acid and 3-methylcrotonic acid.
6. The method according to claim 5, wherein the organic acid is benzoic acid.
- 30 7. The method according to claim 4, wherein the activating agent is N-hydroxysuccinimide, hydroxylbenzotriazole, N-hydroxy-5-norbornene-2,3-dicarboximide or various activated acyl-chlorides.
8. The method according to claim 7, wherein the activating agent is N-hydroxysuccinimide.
- 35 9. The method according to any one of the preceding claims, wherein the acyl group is selected from the group consisting of benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and methylcrotonyl.

10. The method according to claim 9, wherein the acyl group is benzoyl.
11. The method according to any one of claims 1, 2 or 4, wherein the source of acyl ester is a
5 N-hydroxysuccinimide (NHS) ester of benzoic acid.
12. The method according to any one of the preceding claims, wherein R is a benzoyl group.
13. The method according to claim 2, wherein the enzymatic reactions referred to in step (d)
10 is a chemo-enzymatic synthesis wherein recombinant pantothenate kinase (PanK), recombinant phosphopantotheine adenyltransferase (PPAT) and recombinant dephosphocoenzyme A kinase (DPCK) are employed as catalysts.
14. An acyl-pantetheine derivative, having the general formula (I)
15

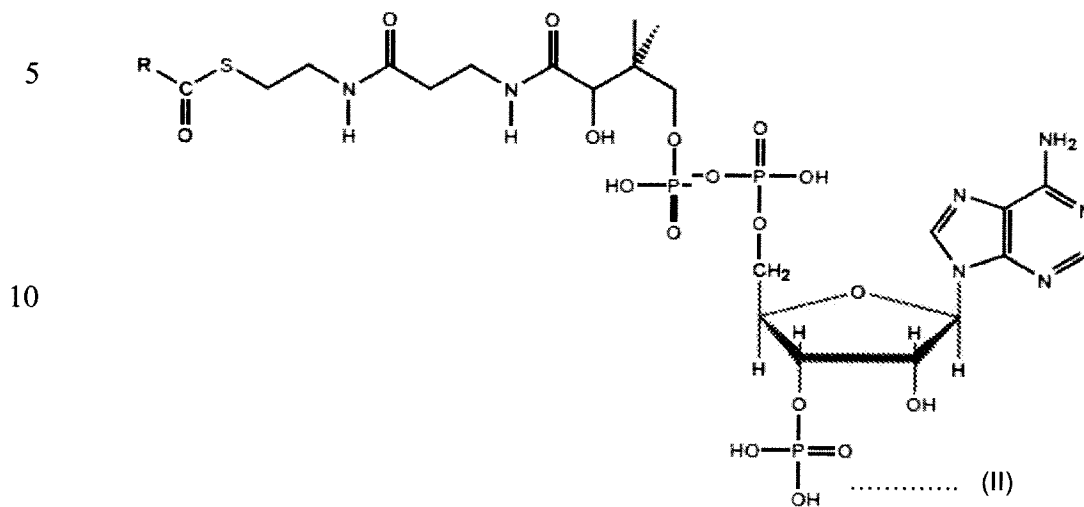


..... (I)

wherein R is an acyl group, prepared according to the method of claim 1.

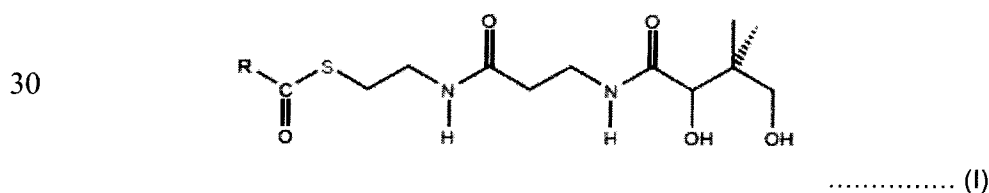
15. The acyl-pantetheine derivative according to claim 14, wherein the acyl group is selected
25 from the group consisting of benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and methacrytonyl.
16. The acyl-pantetheine derivative according to claim 14 or 15, wherein the acyl group is
30 benzoyl.

17. An acyl-coenzyme A analogue, having the general formula (II):



wherein R is an acyl group, prepared according to the method of claim 2.

18. The acyl-coenzyme A analogue according to claim 17, wherein the acyl group is selected from the group consisting of benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and methylcrotonyl.
19. The acyl-coenzyme A analogue according to claim 17 or 18, wherein the acyl group is benzoyl.
20. 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)



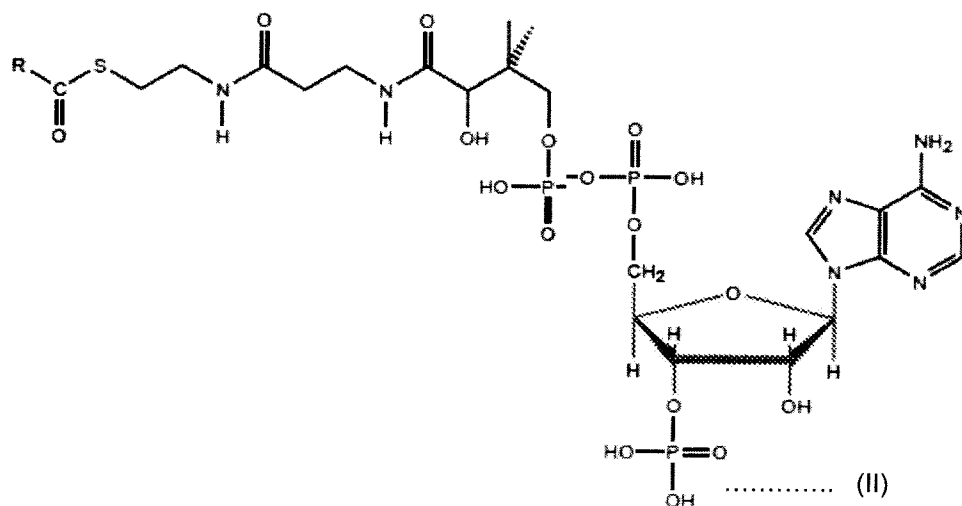
wherein R is an acyl group.

21. 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):

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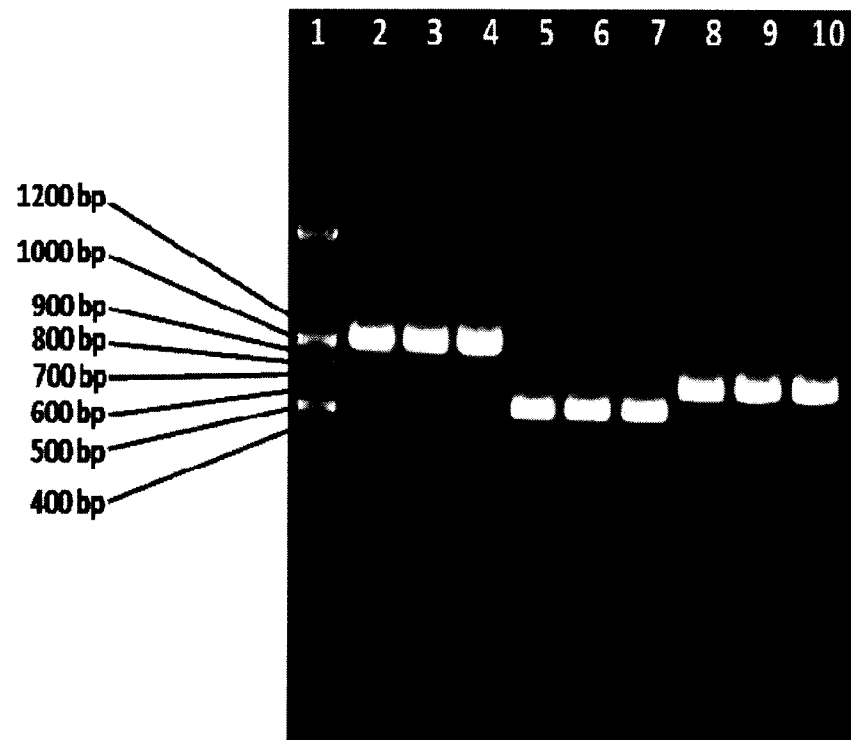
wherein R is an acyl group.

22. The use according to claim 20 or 21, wherein the acyl group is selected from the group consisting of benzoyl, acetyl, isovaleryl, propionyl, butyryl, valeryl, hexanoyl, octanoyl and methylcrotonyl group.

23. The use according to claim 22, wherein the acyl group is benzoyl.

25

1/2

FIGURE 1

2/2

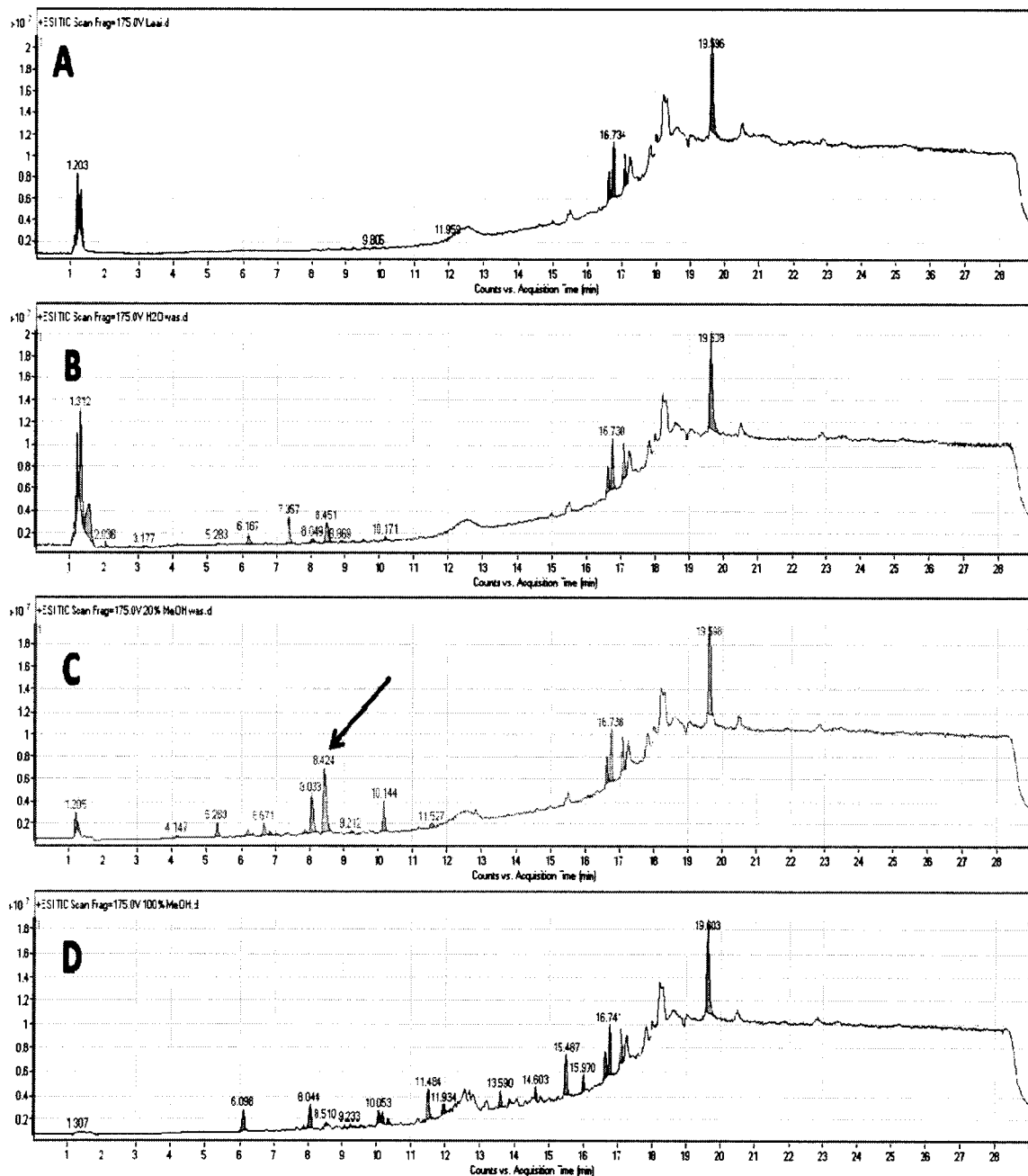


FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2011/053458

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C07C327/00 C07H19/20
 ADD.

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)
 C07C C07H

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 practical, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. SHIMIZU ET AL.: "Investigations on Panthothenic acid and its related compounds. I. Chemical studies. (1). A novel synthesis of Pantethine", CHEM. PHARM. BULL., vol. 13, 1965, pages 180-188, XP002666217, the whole document	1,14-16
X	E. WALTON ET AL.: "Synthesis of Pantetheine and S-acetylpantetheine", J. AM. CHEM. SOC., vol. 76, 1954, pages 1146-1148, XP002666218, the whole document	1,14-16
X	US 2 857 408 A (FELDER ERNST H ET AL) 21 October 1958 (1958-10-21) the whole document	1,14-16
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Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

9 January 2012

Date of mailing of the international search report

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Authorized officer

de Nooy, Arjan

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2011/053458

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	----- MYERS D E ET AL: "The enzymatic synthesis of some potential photoaffinity analogs of benzoyl-coenzyme A", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 112, no. 1, 15 March 1981 (1981-03-15), pages 23-29, XP024822728, ISSN: 0003-2697, DOI: 10.1016/0003-2697(81)90255-4 [retrieved on 1981-03-15] the whole document	17-19
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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