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(57) Abstract: The invention relates to the purification of daptomycin. More specifically, the invention relates to a simplified and optimized purification process of daptomycin from a fermentation broth by chromatographic procedures enabling high yields, selectivity and purity of the desired end product. The daptomycin is prepared in high purity of at least about 98%, and preferably at least about 96%. The present invention also relates to a process for preparing daptomycin that is scalable for commercial production.



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AMORPHOUS DAPTOMYCIN AND A METHOD OF PURIFICATION THEREOF

FIELD OF THE INVENTION

5 The invention relates to the purification of daptomycin. More specifically, the invention relates to a simplified and optimized purification process of daptomycin from a fermentation broth by chromatographic procedures enabling high yields, selectivity and purity of the desired end product. The daptomycin is prepared in high purity of at least about 98%, and preferably at least about 96%. The present invention also relates to a
10 process for preparing daptomycin that is scalable for commercial production.

BACKGROUND AND PRIOR ART OF THE INVENTION

Daptomycin, also known as LY 146032, is a cyclic lipopeptide antibiotic that can be derived from the fermentation of *Streptomyces roseosporus*. Daptomycin is a member
15 of the factor A-21978C type antibiotics of *S. roseosporus* and is comprised of a decanoyl side chain linked to the N-terminal tryptophan of a cyclic 13-amino acid peptide. Daptomycin has an excellent profile of activity because it is highly effective against most gram-positive bacteria; it is highly bactericidal and fast-acting; it has a low resistance rate and is effective against antibiotic-resistant organisms.

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A number of United States Patents describe A-21978C antibiotics and derivatives thereof including daptomycin (LY 146032) as well as methods of producing and isolating the A-21978C antibiotics and derivatives thereof.

25 United States Patent No. RE32,333, RE32,455 and 4,800,157 describe a method of synthesizing daptomycin by cultivating *Streptomyces roseosporus* NRL1 5998 under submerged aerobic fermentation conditions.

United States Patent. No. 4,885,243 describes an improved method of synthesizing
30 daptomycin by feeding a fermentation culture a decanoic fatty acid or ester or salt thereof.

United States Patent No. 4,874,843 describes a daptomycin purification method which is incorporated herein by reference.

5 U.S. Patent. No. 5,912,226 describes the identification and isolation of two impurities produced during the manufacture of daptomycin which is incorporated herein by reference.

10 US patent No. 6696412 describes commercially feasible methods to produce high levels of purified daptomycin at a purity level of 95-98%. The process chromatography method comprises sequentially using anion exchange chromatography, hydrophobic interaction chromatography (HIC) and anion exchange chromatography to purify a preparation containing daptomycin. WO02056829 disclose the 92% pure amorphous form of daptomycin which is incorporated herein by reference

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A number of different chromatographic procedures are applied to obtain the desired end result with respect to purity and yield as discussed above. There is a need in the art to develop a simple chromatographic purification procedure that may be operational on a large scale with minimal steps to isolate the purified daptomycin from a fermentation
20 broth.

The present invention has steps novel with respect to any of the above known methods and uses RP-HPLC on commercial scale to prepare highly pure daptomycin at purity levels of 96-99%. The invention further addresses the long process times as seen with
25 anion exchange method of purification by replacing 2 rounds of ion exchange chromatography with single RP-HPLC purification. The present purification process of daptomycin comprises sequentially carrying out hydrophobic interaction chromatography of microfiltered broth followed by RP-HPLC and subsequent liquid-liquid extraction, HIC or anion exchange chromatography. Finally, solvent wash for removal of endotoxin
30 prior to ultrafiltration, nanofiltration or reverse osmosis followed by lyophilization is carried out to give formulated daptomycin.

Another important advantage of the purification process according to the present invention is that they may be scaled up in a reproducible and consistent manner. Further, the process of the present invention affords products which are superior to those obtained by purification methods hitherto known and give higher yields.

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OBJECTIVES OF THE INVENTION

The main objective of the present invention is to obtain an amorphous daptomycin having at least 98% purity.

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Another main objective of the present invention is to obtain an amorphous daptomycin having at least 97% purity.

Yet another main objective of the present invention is to obtain an amorphous daptomycin having at least 96% purity.

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Still another main objective of the present invention is to obtain an amorphous daptomycin having powder XRD pattern represented in fig 2.

Still another main objective of the present invention is to obtain a method of purification of daptomycin.

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STATEMENT OF THE INVENTION

Accordingly, the present invention relates to an amorphous daptomycin having at least 98% purity; an amorphous daptomycin having at least 97% purity; an amorphous daptomycin having at least 96% purity; an amorphous daptomycin having powder XRD pattern represented in fig 2; and a method of purification of daptomycin comprising the steps of a) filtration of the fermentation broth, b) optional concentration of the filtrate from step (a) by hydrophobic interaction chromatography, ultrafiltration or nanofiltration, c) purification of daptomycin by loading the filtrate from step (a) or the concentrate from step (b) on reverse phase resin followed by elution, d) recovery of purified daptomycin from the elute of reverse phase chromatography, and e) depyrogenation and lyophilization of the recovered daptomycin to give highly pure formulated product.

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BRIEF DESCRIPTION OF THE ACCOMPANYING FIGURES

Figure1: XRD of Amorphous Daptomycin

Figure 2: Chromatogram of Daptomycin

5 **DETAILED DESCRIPTION OF THE INVENTION**

The present invention relates to an amorphous daptomycin having at least 98% purity.

The present invention also relates to an amorphous daptomycin having at least 97% purity.

10 The present invention also relates to an amorphous daptomycin having at least 96% purity.

The present invention also relates to an amorphous daptomycin having powder XRD pattern represented in fig 2.

The present invention also relates to a method of purification of daptomycin comprising the steps of:

- 15 a. filtration of the fermentation broth,
- b. optional concentration of the filtrate from step (a) by hydrophobic interaction chromatography, ultrafiltration or nanofiltration,
- c. purification of daptomycin by loading the filtrate from step (a) or the concentrate from step (b) on reverse phase resin followed by elution,
- 20 d. recovery of purified daptomycin from the elute of reverse phase chromatography, and
- e. depyrogenation and lyophilization of the recovered daptomycin to give highly pure formulated product.

25 In another embodiment of the present invention, the recovery in step (d) is carried out using the steps of:

- a. optional extraction of the elute of reverse phase chromatography with a water immiscible solvent or treatment of the elute from reverse phase chromatography by ion exchange chromatography or hydrophobic interaction chromatography,
- b. optional concentration of the elute of reverse phase chromatography or the elute of hydrophobic interaction chromatography from step (a) by evaporation,
- 30 c. subjecting the elute of reverse phase chromatography, the daptomycin-containing raffinate from step (a), the elute of ion exchange chromatography or hydrophobic interaction chromatography from step (a), or the concentrate from

step (b) to endotoxin removal followed by ultrafiltration, nanofiltration or reverse osmosis.

In yet another embodiment of the present invention, the hydrophobic interaction chromatography is performed on resin selected from the group comprising HP2MG, HP20, HP21, HP20SS, SP20, SP20SS, SP825, SP850, SP207, XAD16, XAD1600, XAD18, XAD761 and XAD7HP .

In still another embodiment of the present invention, the ultrafiltration or nanofiltration is performed using membrane selected from the group comprising polysulfone, polyether sulfone, polypropylene, polyacrylonitrile, cellulose esters, mixed cellulose esters, regenerated cellulose, polyvinylidene difluoride, nylon, teflon (PTFE) and ceramic membranes.

In still another embodiment of the present invention, the reverse phase resin is selected from C4, C8 and C18 reverse phase resin.

In still another embodiment of the present invention, the elution from the reverse phase resin is carried out with mixture of organic solvent and water or mixture of organic solvent and buffer at pH 2.5 to 8.

In still another embodiment of the present invention, the organic solvent is selected from methanol, ethanol, n-propanol, isopropanol, n-butanol, t-butanol, acetonitrile, acetone, tetrahydrofuran or mixture thereof.

In still another embodiment of the present invention, the reverse phase chromatography elution is carried out in isocratic or gradient manner.

In still another embodiment of the present invention, the gradient elution is achieved by changing the content of organic solvent, pH and/or buffer molarity during elution.

In still another embodiment of the present invention, the water-immiscible solvent is selected from ethyl acetate, propyl acetate, butyl acetate, amyl acetate, chloroform, dichloromethane, methyl-t-butyl ether, diethyl ether, butanol, hexane, heptane, cyclohexane or mixture thereof.

In still another embodiment of the present invention, the ion exchange chromatography is anion exchange chromatography or cation exchange chromatography.

In still another embodiment of the present invention, the ion exchange chromatography is anion exchange chromatography.

In still another embodiment of the present invention, the ion exchange chromatography is performed on resin selected from the group comprising diethyl aminoethyl,

quaternary aminoethyl, quaternary ammonium, polyethyleneimine and quaternized polyethyleneimine type resins.

In still another embodiment of the present invention, the ion exchange chromatography elution is carried out using a salt solution.

5 In still another embodiment of the present invention, the salt is a halogenide.

In still another embodiment of the present invention, the salt is an alkali or alkaline earth halogenide.

In still another embodiment of the present invention, the evaporation is done using a thin or wiped film evaporator, falling film evaporator, forced circulation evaporator,

10 short tube evaporator or long tube evaporator.

In still another embodiment of the present invention, the ultrafiltration or nanofiltration is performed at pH 2.5 to 8.

The term "**chromatography**" refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

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The term "**High Performance liquid chromatography**", as used herein, refers to that chromatographic procedure in which the particles (stationary phase) used in the column packing are small (between 3 and 50 microns) and regular with little variation from the selected size. Such chromatography typically employs relatively high (around 500-3500 psi) inlet pressures.

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The term "**ion-exchange**" and "**ion-exchange chromatography**" refers to the chromatographic process in which a solute of interest (such as a protein) in a mixture interacts with a charged compound linked (such as by covalent attachment) to a solid phase ion exchange material such that the solute of interest interacts non-specifically with the charged compound more or less than solute impurities or contaminants in the mixture. The contaminating solutes in the mixture elute from a column of the ion exchange material faster or slower than the solute of interest or are bound to or excluded from the resin relative to the solute of interest. "Ion-exchange chromatography" specifically includes cation exchange, anion exchange, and mixed mode chromatography.

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The object of the present invention is provided in a specifically delineated process for purification of daptomycin from fermentation broth by using chromatographic process.

In a broad aspect, the present invention relates to a chromatography process for purifying a daptomycin from fermentation broth comprising the steps of:

- a) filtration of the fermentation broth,
- b) optional concentration of the filtrate from step (a) by hydrophobic interaction chromatography, ultrafiltration or nanofiltration,
- c) purification of daptomycin by loading the filtrate from step (a) or the concentrate from step (b) on reverse phase resin followed by elution,
- d) recovery of purified daptomycin from the elute of reverse phase chromatography, and
- e) Lyophilization of the recovered daptomycin to give highly pure formulated product.

The present invention also relates to the above process, wherein the recovery of purified daptomycin from the elute of reverse phase chromatography is carried out using the following steps:

- a) optional extraction of the elute of reverse phase chromatography with a water immiscible solvent or treatment of the elute from reverse phase chromatography by ion exchange chromatography or hydrophobic interaction chromatography,
- b) optional concentration of the elute of reverse phase chromatography or the elute of hydrophobic interaction chromatography from step (a) by evaporation,
- c) subjecting the elute of reverse phase chromatography, the daptomycin-containing raffinate from step (a), the elute of ion exchange chromatography from step (a), the elute of hydrophobic interaction chromatography from step (a), or the concentrate from step (b) to endotoxin removal by solvent wash followed by ultrafiltration, nanofiltration or reverse osmosis.

In another aspect of the invention the final purified product daptomycin is amorphous in nature and 98% pure.

Those skilled in the art will recognize that there are various variables which can be adjusted during the chromatographic procedures of the present invention. Such variables include resin selection; loading, wash and eluting conditions, such as ionic strength, buffer composition, pH, temperature, addition of one or more organic
5 solvents, etc. However, such variables are routinely adjusted in this field and those skilled in the art can readily establish optimum conditions.

The present invention relates to a chromatography process for purifying a daptomycin from fermentation broth comprising the steps of:

- a) filtration of the fermentation broth,
- 10 b) optional concentration of the filtrate from step (a) by hydrophobic interaction chromatography, ultrafiltration or nanofiltration,
- c) purification of daptomycin by loading the filtrate from step (a) or the concentrate from step (b) on reverse phase resin followed by elution,
- d) recovery of purified daptomycin from the elute of reverse phase
15 chromatography, and
- e) depyrogenation and lyophilization of the recovered daptomycin to give highly pure formulated product.

The present invention also relates to the above process, wherein the recovery of purified daptomycin from the elute of reverse phase chromatography is carried out
20 using the following steps:

- a) optional extraction of the elute of reverse phase chromatography with a water immiscible solvent or treatment of the elute from reverse phase chromatography by ion exchange chromatography or hydrophobic interaction chromatography,
- b) optional concentration of the elute of reverse phase chromatography or the elute
25 of hydrophobic interaction chromatography from step (a) by evaporation,
- c) subjecting the elute of reverse phase chromatography, the daptomycin-containing raffinate from step (a), the elute of ion exchange chromatography from step (a), the elute of hydrophobic interaction chromatography from step (a), or the concentrate from step (b) to endotoxin removal followed by
30 ultrafiltration, nanofiltration or reverse osmosis.

In another aspect of the invention the final purified product daptomycin is amorphous in nature and 98% pure. The daptomycin of the present invention can be produced by fermentation. After fermentation, the daptomycin-containing extracellular solution is clarified by removing the mycelia from the fermentation broth. This clarification is performed by any standard solid-liquid separation technique, such as centrifugation or filtration. Preferably, the fermentation broth clarification is carried out using a microfiltration system, filter press, rotary drum filter, depth filter or industrial centrifuge.

The clarified solution may be directly subjected to a reverse phase chromatography. Alternately, the clarified solution is concentrated and the concentrate is subjected to reverse phase chromatography. The concentration of clarified solution can be carried out by ultrafiltration, nanofiltration, or hydrophobic interaction chromatography or combinations thereof. The ultrafiltration and nanofiltration membrane can be selected from polysulfone, polyether sulfone, polypropylene, polyacrylonitrile, cellulose esters, mixed cellulose esters, regenerated cellulose, polyvinylidene difluoride, nylon, teflon (PTFE), or ceramic membranes. The pH during ultrafiltration or nanofiltration is selected in the range 2.5 to 8. The retentate of ultrafiltration or nanofiltration gives concentrated clarified solution. Optionally, the feed for the reverse phase chromatography may be treated with activated charcoal, alumina, silica gel or may be passed through a guard column before passing it through the reverse phase chromatography column.

In hydrophobic interaction chromatography, the resin can be selected from HP2MG, HP20, HP21, HP20SS, SP20, SP20SS, SP825, SP850, SP207, XAD16, XAD1600, XAD18, XAD761, XAD7HP, etc. The clarified solution is contacted with the hydrophobic interaction chromatography resin under conditions such that daptomycin binds to the resin. This contact is carried out at pH in the range from 2.5 to 8. Preferably, the contact is carried out at pH in the range from 3.5 to 5.0. Optionally, additives including salts and/or surfactants are added to the clarified solution. Surfactants are added at small concentrations to prevent precipitation during pH adjustment and column blockage in hydrophobic interaction chromatography. The surfactant can be selected from Triton X, Tween, SDS, etc. The surfactant concentration is selected in the range from 0.001% to 1.0%. Additive salts are selected from sodium chloride, potassium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium

dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium dihydrogen phosphate, di-ammonium hydrogen phosphate, sodium acetate, ammonium acetate, ammonium chloride, sodium carbonate, sodium bicarbonate and sodium citrate. The resin is washed and eluted with water, buffer, mixture of organic solvent and water or mixture of organic solvent and buffer. The organic solvent is selected from methanol, ethanol, n-propanol, isopropanol, n-butanol, t-butanol, acetonitrile, acetone, tetrahydrofuran or mixture thereof. The pH during elution is selected in the range from 2.5 to 8. The product-containing elute from hydrophobic interaction chromatography gives concentrated clarified solution.

The clarified solution or the concentrated clarified solution is subjected to a reverse phase chromatography resin. Optionally, the feed for the reverse phase chromatography column is diluted with water. The feed is loaded onto the column at pH in the range from 2.5 to 8. Preferably, the feed pH is in the range from 3.5 to 5.0. The reverse phase resin is selected from C4, C8, C18 silica or polystyrene-divinylbenzene. Daptomycin from the column is eluted with mixture of organic solvent and water or mixture of organic solvent and buffer. The organic solvent is selected from methanol, ethanol, n-propanol, isopropanol, n-butanol, t-butanol, acetonitrile, acetone, tetrahydrofuran or mixture thereof. The pH during elution is selected in the range from 2.5 to 8. The elution is carried out in isocratic or gradient manner. In gradient elution, the content of organic solvent, pH and/or buffer molarity is changed during elution. During product elution, fractions are collected. Fractions containing product with desired purity are combined to obtain elute from reverse phase chromatography. The buffer can be prepared using phosphates of sodium, potassium or ammonium salts, acetates of sodium, potassium or ammonium salts, citrates of sodium, potassium or ammonium salts, oxalates sodium, potassium or ammonium salts, acetic acid, phosphoric acid, citric acid, oxalic acid, hydrochloric acid, sodium hydroxide, potassium hydroxide and ammonium hydroxide or mixtures thereof.

Purified daptomycin in the elute from reverse phase chromatography is then recovered.

The elute from reverse phase chromatography is optionally processed by extraction, ion exchange chromatography or hydrophobic interaction chromatography. In extraction, the elute from reverse phase chromatography is extracted with a water-immiscible organic solvent. The water-immiscible solvent is selected from ethyl acetate, propyl

acetate, butyl acetate, amyl acetate, chloroform, dichloromethane, methyl-t-butyl ether, diethyl ether, butanol, hexane, heptane, cyclohexane or mixture thereof. The extraction is carried out in batch manner in single or multiple stages. Alternately, the extraction is carried out in a co-current or counter-current continuous manner. Daptomycin remains
5 in the aqueous layer to give raffinate.

In ion exchange chromatography, the elute from reverse phase chromatography is passed through ion exchange resin column. The ion exchange resin is selected from diethyl aminoethyl, quaternary aminoethyl, quaternary ammonium, polyethyleneimine and
10 quaternized polyethyleneimine type resins. Optionally, the elute from reverse phase chromatography is diluted with water before passing through the ion exchange column. The resin is washed and eluted with water and salt solution in water. The elution is carried out in isocratic or gradient manner. The gradient elution is carried out using step or continuous gradient. During washing and elution, the salt concentration is selected in
15 the range from 0 to 1000 mM. Preferably, this concentration is selected in the range from 0 to 500 mM. The salt is selected from NaCl or KCl.

In hydrophobic interaction chromatography, the elute from reverse phase chromatography is passed through a hydrophobic interaction resin column. The
20 hydrophobic interaction resin is selected from HP2MG, HP20, HP21, HP20SS, SP20, SP20SS, SP825, SP850, SP207, XAD16, XAD1600, XAD18, XAD761, XAD7HP, etc. Optionally, the elute from reverse phase chromatography is diluted with water before passing through the hydrophobic interaction resin column. The loading is carried out at pH in the range from 2.5 to 8. Preferably, the loading is carried out at pH in the range
25 from 3.5 to 5.0. The resin is washed and eluted with water, buffer, mixture of organic solvent and water or mixture of organic solvent and buffer. The organic solvent is selected from methanol, ethanol, n-propanol, isopropanol, n-butanol, t-butanol, acetonitrile, acetone, tetrahydrofuran or mixture thereof. The pH during elution is selected in the range from 2.5 to 8.

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The elute from reverse phase chromatography or the elute from hydrophobic interaction chromatography is optionally concentrated by evaporation. Various evaporators including

thin or wiped film evaporator, falling film evaporator, forced circulation evaporator, short tube evaporator or long tube evaporator can be used for this concentration.

5 The elute of reverse phase chromatography, the daptomycin-containing raffinate from extraction, the elute of ion exchange chromatography, the elute hydrophobic interaction chromatography or the concentrate after evaporation is then subjected to depyrogenation by employing solvent wash. The organic solvent is selected from n-butanol, ethyl acetate, butyl acetate, hexane, heptane, petroleum ether or mixture thereof. The pH during solvent wash is normally selected in the range from 5 to 8.

10 Daptomycin remains in the aqueous layer to give raffinate which is subjected to ultrafiltration or nanofiltration. Optionally, the feed to the ultrafiltration or nanofiltration is diluted with water. The filtration is carried out at a pH in the range from 2.5 to 8. The ultrafiltration and nanofiltration membrane is selected from polysulfone, polyether sulfone, polypropylene, polyacrylonitrile, cellulose esters, mixed

15 cellulose esters, regenerated cellulose, polyvinylidene difluoride, nylon, teflon (PTFE), or ceramic membranes. The daptomycin containing retentate is diafiltered against water.

Finally, the daptomycin solution is sterile filtered and lyophilized to obtain amorphous

20 pure daptomycin. The purity of final product has at least 98% as measured by HPLC.

The disclosures of the prior art references referred to in this patent application are incorporated herein by reference. The invention is further defined by reference to the following examples describing in detail the process and compositions of the invention. It

25 will be apparent to those skilled in the art that many modifications, both to materials and methods, may be practiced without departing from the scope of the invention.

EXAMPLE 1

10 L fermentation broth containing daptomycin was microfiltered through 0.1 μ m filter.

30 The filtrate was mixed with 0.05% triton X100 and pH of the solution was adjusted to 4.5. The solution was passed through an HP20ss column, which was pre-equilibrated with 5 % isopropanol in sodium acetate buffer at pH 4.5. The column was washed with 10 % isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium

acetate buffer at pH 6.5. The product containing fractions were combined, diluted with water and loaded on a C8 reverse phase chromatography column. The column was eluted under isocratic conditions with acetonitrile and buffer in the ratio of 30:70. During elution, fractions were collected. The fractions with desired purity were combined, diluted with water, pH-adjusted to 6.5 and passed over weak anion exchange resin to remove organic solvent. The resin was pre-equilibrated with 30 mM Tris HCl buffer at pH 6.5. The column was eluted with 0.5 M NaCl solution. The product containing elute was concentrated and diafiltered using an ultrafiltration membrane. The concentrate was sterile filtered, filled in vials and lyophilized. The purity of daptomycin was 97.5%.

EXAMPLE 2

10 L fermentation broth containing daptomycin was microfiltered through 0.1 μ m filter. The filtrate was mixed with 0.05% triton X100 and pH of the solution was adjusted to 4.5. The solution was passed through an HP20ss column, which was pre-equilibrated with 5 % isopropanol in sodium acetate buffer at pH 4.5. The column was washed with 10 % isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium acetate buffer at pH 6.5. The product containing fractions were combined, diluted with water and loaded on a C8 reverse phase chromatography column. The column was eluted under isocratic conditions with acetonitrile and buffer in the ratio of 30:70. During elution, fractions were collected. The fractions with desired purity were combined, and subjected to liquid-liquid extraction using n-butyl acetate. Post-extraction, the aqueous layer containing daptomycin was concentrated and diafiltered by ultrafiltration. The concentrate was sterile filtered, filled in vials and lyophilized to give daptomycin of purity more than 96%.

EXAMPLE 3

10 L fermentation broth containing daptomycin was microfiltered through 0.1 μ m filter. The filtrate was mixed with 0.05% triton X100 and pH of the solution was adjusted to 4.5. The solution was passed through an HP20ss column, which was pre-equilibrated with 5 % isopropanol in sodium acetate buffer at pH 4.5. The column was washed with 10 % isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium acetate buffer at pH 6.5. The product containing fractions were combined, diluted with water and loaded on a C8 reverse phase chromatography column. The column was eluted

under isocratic conditions with acetonitrile and buffer in the ratio of 30:70. During elution, fractions were collected. The fractions with desired purity were combined, diluted with water, pH-adjusted to 4.5. This solution was passed through an HP20ss column, which was pre-equilibrated with 5% isopropanol in sodium acetate buffer at pH 4.5. The resin was washed with water and eluted with 70% methanol in buffer. The product-containing elute of purity 97.2% was diluted with water and pH of this solution was adjusted to 4.5. The pH-adjusted solution was concentrated and diafiltered using an ultrafiltration membrane. The concentrate was sterile filtered, filled in vials and lyophilized.

EXAMPLE 4

50 L broth was microfiltered through 0.1 μ m and the permeate was concentrated 10 fold on a 0.6KDa nanofiltration membrane. The concentrate was mixed with activated charcoal and filtered to remove the charcoal. The filtrate was loaded on a C8 reverse phase chromatography column. The product was eluted using a linear gradient of 30-50% acetonitrile over 90 minutes. The fractions containing product with desired purity were combined and extracted with n-butyl acetate. Post-extraction, the aqueous layer containing daptomycin was concentrated and diafiltered using an ultrafiltration membrane. The concentrate was sterile filtered, filled in vials and lyophilized.

EXAMPLE 5

50 L fermentation broth containing daptomycin was microfiltered through 0.1 μ m filter. The filtrate was mixed with 0.05% triton X100 and pH of the solution was adjusted to 4.5. The solution was passed through an HP20ss column, which was pre-equilibrated with 5 % isopropanol in sodium acetate buffer at pH 4.5. The column was washed with 10 % isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium acetate buffer at pH 6.5. The product containing fractions were combined, diluted with water and loaded on a C8 reverse phase chromatography column. The product containing fractions were combined, diluted with water and purified on a C8 reverse phase chromatography column. The product containing fractions were combined, diluted with water and loaded on a C8 reverse phase chromatography column. The column was eluted using a linear gradient from 30 to 50% acetonitrile over 90 minutes. The fractions containing product with purities ~98% were combined, pH was adjusted to 6.5 and

extracted with n-butyl acetate in multiple stages. Post-extraction, the aqueous layer containing daptomycin was concentrated and diafiltered at pH 6.5 using a nanofiltration membrane. The concentrate was sterile filtered, filled in vials and lyophilized.

5 **EXAMPLE 6**

pH of 50 L fermentation broth was adjusted to 4.5. The pH-adjusted broth was extracted with n-butanol. The butanol extract was concentrated partially and extracted with sodium phosphate buffer at pH 6.5. pH of this extract was adjusted to 4.5 and loaded onto an HP20ss column, which was pre-equilibrated with 5% isopropanol in sodium acetate
10 buffer at pH 4.5. The column was washed with 10% isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium acetate buffer. The product-containing elute was diluted with water and loaded on reverse phase C-8 resin, elution was carried as described in example 1. The fractions containing product with desired purity were combined and diluted with water. pH of the diluted solution was adjusted to 4.5 and
15 passed through an HP20ss column. The column was washed with water and eluted with 70% acetonitrile. The elute was sterile filtered and lyophilized to give the daptomycin. The purity of daptomycin obtained was 98.8%.

EXAMPLE 7

20 10 L fermentation broth containing daptomycin was microfiltered through 0.1 μ m filter. The filtrate was mixed with 0.05% triton X100 and pH of the solution was adjusted to 4.5. The solution was passed through an HP20ss column, which was pre-equilibrated with 5 % isopropanol in sodium acetate buffer at pH 4.5. The column was washed with 10 % isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium
25 acetate buffer at pH 6.5. The product containing fractions were combined, diluted with water and loaded on a C8 reverse phase chromatography column. The column was eluted using a linear gradient from 30 to 50% acetonitrile over 90 minutes. During elution, fractions were collected. The fractions with desired purity were combined, diluted with water, pH-adjusted to 4.5. This solution was passed through an HP20ss column, which
30 was pre-equilibrated with 5% isopropanol in sodium acetate buffer at pH 4.5. The resin was washed with water and eluted with aqueous acetonitrile. The product-containing elute of ~98% purity was concentrated by solvent evaporation. The concentrated solution

was diluted with water and diafiltered using an ultrafiltration membrane. The diafiltered solution was sterile filtered and lyophilized.

EXAMPLE 8

5 10 L fermentation broth containing daptomycin was microfiltered through 0.1 μ m filter. The filtrate was mixed with 0.05% triton X100 and pH of the solution was adjusted to 4.5. The solution was passed through an HP20ss column, which was pre-equilibrated with 5 % isopropanol in sodium acetate buffer at pH 4.5. The column was washed with 10 % isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium
10 acetate buffer at pH 6.5. The product containing fractions were combined, diluted with water and loaded on a C8 reverse phase chromatography column. The column was eluted under isocratic conditions with acetonitrile and buffer in the ratio of 35:65. During elution, the fractions with desired purity were combined, diluted with water, pH-adjusted to 6.5 and passed over weak anion exchange resin to remove organic solvent. The resin
15 was pre-equilibrated with 30 mM Tris HCl buffer at pH 6.5. The column was eluted with 0.5 M NaCl solution. The product containing elute was depyrogenated by contacting with n-butanol in the ratio of 1: 0.2 for 30 mins. The aqueous layer containing the product was further contacted with n-heptane in the ratio of 1: 0.4 for 30 mins. Finally the aqueous 'product containing layer' was concentrated and diafiltered using an ultrafiltration
20 membrane. The concentrate was sterile filtered, filled in vials and lyophilized. The purity of daptomycin obtained was 96.6%.

EXAMPLE 9

10 L fermentation broth containing daptomycin was microfiltered through 0.1 μ m filter.
25 The filtrate was mixed with 0.05% triton X100 and pH of the solution was adjusted to 4.5. The solution was passed through an HP20ss column, which was pre-equilibrated with 5 % isopropanol in sodium acetate buffer at pH 4.5. The column was washed with 10 % isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium acetate buffer at pH 6.5. The product containing fractions were combined, diluted with
30 water and loaded on a C8 reverse phase chromatography column. The column was eluted under isocratic conditions with acetone and buffer in the ratio of 37:63. During elution, fractions were collected. The fractions with 97% purity were combined, diluted with water, pH-adjusted to 4.5. This solution was passed through an HP20ss column, which

was pre-equilibrated with 5% isopropanol in sodium acetate buffer at pH 4.5. The resin was washed with water and eluted with 70% methanol in buffer. The product-containing elute was diluted with water and pH of this solution was adjusted to 4.5. The pH-adjusted solution was concentrated and diafiltered using an ultrafiltration membrane. The
5 concentrate was sterile filtered, filled in vials and lyophilized.

EXAMPLE 10

5 L fermentation broth containing daptomycin was microfiltered through 0.1 μ m filter. The filtrate was mixed with 0.05% triton X100 and pH of the solution was adjusted to
10 4.5. The solution was passed through an HP20ss column, which was pre-equilibrated with 5 % isopropanol in sodium acetate buffer at pH 4.5. The column was washed with 10 % isopropanol in sodium acetate buffer and eluted with 25% isopropanol in sodium acetate buffer at pH 6.5. The product containing fractions were combined, diluted with water and loaded on a C8 reverse phase chromatography column. The column was eluted
15 using 40% acetone over 300 minutes. During elution, fractions were collected. The fractions with desired purity were combined, diluted with water, pH-adjusted to 6.5 and passed over weak anion exchange resin to remove organic solvent. The resin was pre-equilibrated with 30 mM Tris HCl buffer at pH 6.5. The column was eluted with 0.5 M NaCl solution. The product containing elute was depyrogenated by contacting with n-
20 butanol in the ratio of 1: 0.2 for 30 mins. The aqueous layer containing the product was further contacted with n-heptane in the ratio of 1: 0.4 for 30 mins. Finally the aqueous 'product containing layer' was concentrated and diafiltered using an ultrafiltration membrane. The concentrate was sterile filtered, filled in vials and lyophilized to give daptomycin of 96%.

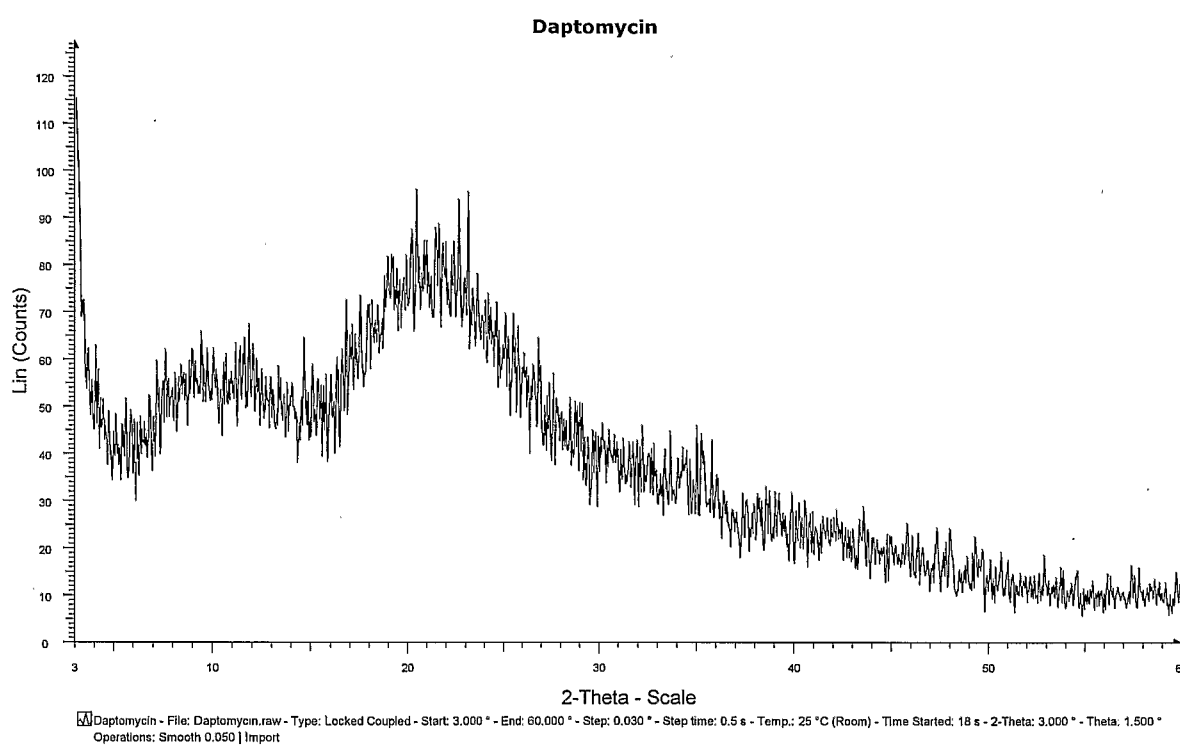
We Claim

1. Amorphous daptomycin having at least 98% purity.
2. Amorphous daptomycin having at least 97% purity.
3. Amorphous daptomycin having at least 96% purity.
- 5 4. Amorphous daptomycin having powder XRD pattern represented in fig 2
5. A method of purification of daptomycin comprising the steps of:
 - a. filtration of the fermentation broth,
 - b. optional concentration of the filtrate from step (a) by hydrophobic interaction chromatography, ultrafiltration or nanofiltration,
 - 10 c. purification of daptomycin by loading the filtrate from step (a) or the concentrate from step (b) on reverse phase resin followed by elution,
 - d. recovery of purified daptomycin from the elute of reverse phase chromatography, and
 - e. depyrogenation and lyophilization of the recovered daptomycin to give
15 highly pure formulated product.
6. The method of purification as claimed in claim 5, wherein the recovery in step (d) is carried out using the steps of:
 - a. optional extraction of the elute of reverse phase chromatography with a water immiscible solvent or treatment of the elute from reverse phase
20 chromatography by ion exchange chromatography or hydrophobic interaction chromatography,
 - b. optional concentration of the elute of reverse phase chromatography or the elute of hydrophobic interaction chromatography from step (a) by evaporation,
 - 25 c. subjecting the elute of reverse phase chromatography, the daptomycin-containing raffinate from step (a), the elute of ion exchange chromatography or hydrophobic interaction chromatography from step (a), or the concentrate from step (b) to endotoxin removal followed by ultrafiltration, nanofiltration or reverse osmosis.
- 30 7. The method of purification as claimed in claim 5 or 6, wherein the hydrophobic interaction chromatography is performed on resin selected from the group

comprising HP2MG, HP20, HP21, HP20SS, SP20, SP20SS, SP825, SP850, SP207, XAD16, XAD1600, XAD18, XAD761 and XAD7HP.

8. The method of purification as claimed in claim 5 or 6, wherein ultrafiltration or nanofiltration is performed using membrane selected from the group comprising polysulfone, polyether sulfone, polypropylene, polyacrylonitrile, cellulose esters, mixed cellulose esters, regenerated cellulose, polyvinylidene difluoride, nylon, teflon (PTFE) and ceramic membranes.
9. The method of purification as claimed in claim 5, wherein the reverse phase resin is selected from C4, C8 and C18 reverse phase resin.
10. The method of purification according to claim 5, wherein elution from the reverse phase resin is carried out with mixture of organic solvent and water or mixture of organic solvent and buffer at pH 2.5 to 8.
11. The method of purification as claimed in claim 10, wherein the organic solvent is selected from methanol, ethanol, n-propanol, isopropanol, n-butanol, t-butanol, acetonitrile, acetone, tetrahydrofuran or mixture thereof.
12. The method of purification as claimed in claim 5, wherein the reverse phase chromatography elution is carried out in isocratic or gradient manner.
13. The method of purification as claimed in claim 12, wherein the gradient elution is achieved by changing the content of organic solvent, pH and/or buffer molarity during elution.
14. The method of purification as claimed in claim 6, wherein the water-immiscible solvent is selected from ethyl acetate, propyl acetate, butyl acetate, amyl acetate, chloroform, dichloromethane, methyl-t-butyl ether, diethyl ether, butanol, hexane, heptane, cyclohexane or mixture thereof.
15. The method of purification as claimed in claim 6, wherein the ion exchange chromatography is anion exchange chromatography or cation exchange chromatography.
16. The method of purification as claimed in claim 15, wherein the ion exchange chromatography is anion exchange chromatography.
17. The method of purification as claimed in claim 6, wherein the ion exchange chromatography is performed on resin selected from the group comprising diethyl aminoethyl, quaternary aminoethyl, quaternary ammonium, polyethyleneimine and quaternized polyethyleneimine type resins.

18. The method of purification as claimed in claim 6, wherein the ion exchange chromatography elution is carried out using a salt solution.
19. The method of purification as claimed in claim 18, wherein the salt is a halogenide.
- 5 20. The method of purification as claimed in 18, wherein the salt is an alkali or alkaline earth halogenide.
21. The method of purification as claimed in claim 6, wherein the evaporation is done using a thin or wiped film evaporator, falling film evaporator, forced circulation evaporator, short tube evaporator or long tube evaporator.
- 10 22. The method of purification as claimed in claim 5 or 6, wherein the ultrafiltration or nanofiltration is performed at pH 2.5 to 8.

**Fig. 1**

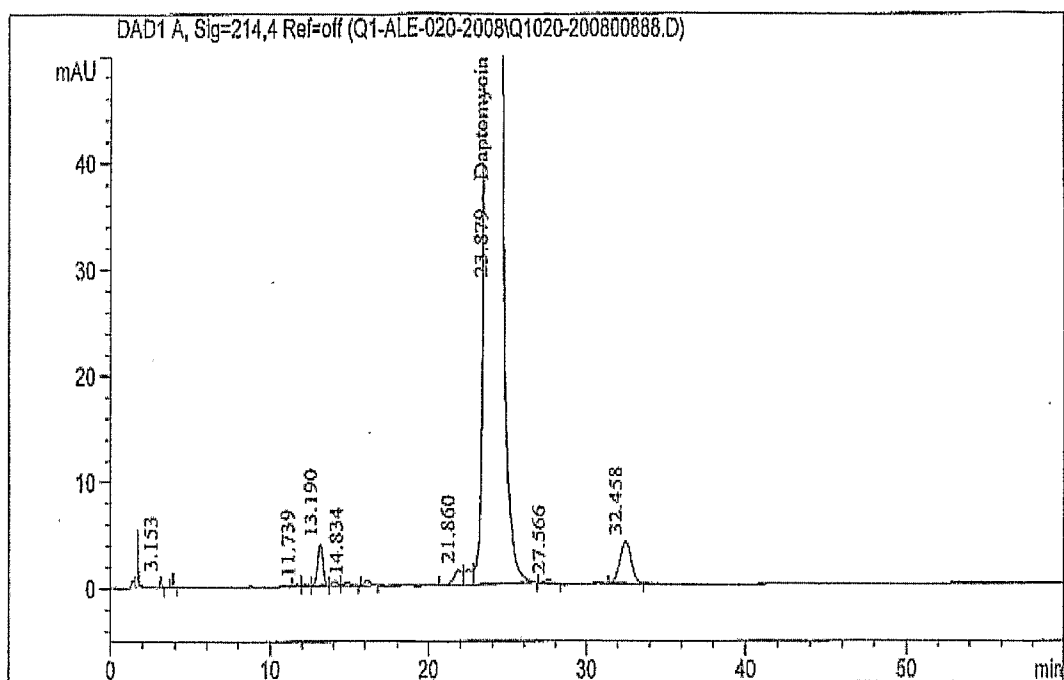


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2009/000265

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 1/20 (2006.01)

C07K 1/16 (2006.01)

A61K 38/12 (2006.01)

C12P 21/04 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, Medline, CAPLUS (keywords - daptomycin, LY 146302, cubicin, purity, purify, amorphous, chromatography, column, reverse phase)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6696412 B1 (KELLEHER et al.) 24 February 2004 See abstract, column 9 line 10 – column 12 line 5 and column 14, lines 12-64	1-3, 5-9, 14-16, and 17-22
X	US 2003/0045678 A1 (KEITH et al.) 6 March 2003 See Figure 6	4
X	US 4874843 A (BAKER) 17 October 1989 See abstract, column 2, line 40 – column 3, line 68 and column 4, lines 1-3	5 and 10-13

☒ Further documents are listed in the continuation of Box C
 ☒ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 17 September 2009	Date of mailing of the international search report 29 SEP 2009
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer MARYKA GAUDIO AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6225 6128

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN2009/000265

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US Re. 32333 (HAMILL et al.) 20 January 1987 See examples 1-6	5-22

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

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

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

[See Supplemental Box]

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

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box

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

Continuation of Box No: III

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

In assessing whether there is more than one invention claimed, I have given consideration to those features which can be considered to potentially distinguish the claimed combination of features from the prior art. Where different claims have different distinguishing features they define different inventions.

This International Searching Authority has found that there are different inventions as follows:

- Claims 1-3 are directed to amorphous daptomycin having at least 96% purity. It is considered that amorphous daptomycin having at least 96% purity comprises a first distinguishing feature.
- Claim 4 is directed to amorphous daptomycin having a particular XRD pattern. It is considered that amorphous daptomycin having this XRD pattern comprises a second distinguishing feature.
- Claims 5-22 are directed to a method of purification of daptomycin comprising the steps of:
 - a. filtration of a fermentation broth,
 - b. optional concentration of the filtrate from step (a) by hydrophobic interaction chromatography, ultrafiltration or nanofiltration,
 - c. purification of daptomycin by loading the filtrate from step (a) or the concentrate from step (b) on reverse resin followed by elution,
 - d. recovery of purified daptomycin from the elute of the reverse phase chromatography and
 - e. depyrogenation and lyophilization of the recovered daptomycin to give highly pure formulated product.

It is considered that this method comprises a third distinguishing feature.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

The only feature common to all of the claims is daptomycin. However daptomycin is well known in the art. This means that the common feature can not constitute a special technical feature within the meaning of PCT Rule 13.2, second sentence, since it makes no contribution over the prior art.

Because the common feature does not satisfy the requirement for being a special technical feature it follows that it cannot provide the necessary technical relationship between the identified inventions. Therefore the claims do not satisfy the requirement of unity of invention *a posteriori*.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/IN2009/000265

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

Patent Document Cited in Search Report		Patent Family Member			
US 6696412		AU 30978/01	BR 0107731	CA 2398726	
		CN 1404487	CN 101240013	CZ 20022830	
		EP 1252179	EP 1586580	HU 0203969	
		IS 6470	KR 20070044079	MX PA02007132	
		NO 20023476	NZ 520324	PL 356898	
		RU 2002122114	US 2005009747	US 2007191280	
		WO 0153330	ZA 200205763		
US 2003045678		AU 2008207496	CA 2432096	CA 2432187	
		CN 1592753	CN 1982330	EP 1343811	
		EP 1383794	EP 1908770	JP 2008214348	
		KR 20080036661	NZ 554405	US 2002111311	
		US 2003045484	US 2006014674	US 2009197799	
		WO 02056829	WO 02059145	WO 02096936	
US 4874843		AU 17493/88	BG 47349	EP 0294990	
		HU 47154	IL 86601	JP 1047388	
		NZ 224873	US 5912226	ZA 8803887	
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.					
END OF ANNEX					