(19) Octrooicentrum Nederland

(11) **2017545**

(12) B1 **OCTROOI**

(21) Aanvraagnummer: **2017545**

(22) Aanvraag ingediend: **28/09/2016**

(51) Int. Cl.:
A01N 43/90 (2016.01) **A23L 3/3463** (2016.01) **A61K 31/7048** (2016.01) **A61P 31/10** (2016.01) **A61K 47/10** (2017.01) **A01P 3/00** (2017.01) **A01N 25/02** (2017.01)

(41) Aanvraag ingeschreven:
06/04/2018

(43) Aanvraag gepubliceerd:
-

(47) Octrooi verleend:
06/04/2018

(45) Octrooischrift uitgegeven:
09/04/2018

(73) Octrooihouder(s):
Eucaryo Beheer B.V. te Nijmegen.

(72) Uitvinder(s):
Wilhelmus Antonius Maria van Lith te Nijmegen.

(74) Gemachtigde:
drs. J. Renkema te Geleen.

(54) **COMPOSITION COMPRISING A BIOACTIVE MOLECULE**

(57) The invention relates to a process for preparing a composition comprising a bioactive molecule comprising the steps of

a. Dissolving a bioactive molecule having at least one acid group with a base at pH=>11 to obtain a solution A

b. Neutralizing the solution A with an acid to obtain the composition comprising a bioactive molecule having a pH between 6 and 9,

wherein PEG is added before the neutralizing step in case either the base and/or the acid is an inorganic compound, and to a composition comprising a high concentration of natamycin.

COMPOSITION COMPRISING A BIOACTIVE MOLECULE

The invention relates to a method for preparing a water containing composition comprising a bioactive molecule comprising an acid functionality, to a composition obtainable with such method, to a composition comprising the bioactive molecule and to the use of such compositions.

Bioactive molecules having an acid functionality are known in the art. Such molecules in general have poor solubility in water, but show activity against fungi, bacteria, may have anticancer activity or may be a plant hormone. Some of these compounds may have a ring structure, comprising a number of conjugated double bonds, hydroxyl groups and a mycosamine group; examples of such compounds are natamycin, amphotericin B, Nystatin and Filipin. Examples of plant hormones are Giberrellic acid (GA3, GA4, GA7); other compounds are Auxin (indole-3-acetic acid), Indole-3-butyric acid - Abcissic acid (S-ABA) and betulinic acid. All components have in common that they have at least one carboxylic acid group and show bioactivity, and low solubility in water, for example less than 100 mg/l water.

One particular example of a bioactive molecule is Natamycin, also known as pimaricin, which is an effective anti-fungal agent. It exerts a wide range of *in vitro* activity against molds and yeast, particularly of the Candida, Aspergillus, Penicillium, Cephalosporium and Fusarium species. After its isolation in 1955, it has found widespread application in the preservation of foods, in particular bakery products, meat products, beverages and dairy products (in particular cheese). Natamycin appears to possess a broader spectrum of activity than many other fungicides allowed for food application.

Despite the potential of natamycin for combatting fungi, its use in clinical medicine is still limited. This is mainly due to the low solubility of natamycin in various solvents, both aqueous and organic, that are compatible for human administration. For example, the solubility in water is 30–50 mg L^{-1}, and in ethanol it is 0.04–1.2 g L^{-1}; only the dissolved fraction has antifungal activity. In conventional natamycin-based pharmaceutical formulations, such as creams for topical treatments of skin infections, the natamycin is present in a predominantly insoluble crystalline form, leading to a low bioavailability of active natamycin. Attempts to increase the amount of dissolved natamycin in topical formulations often gave a small increase of dissolved natamycin and an antifungal activity that was still insufficient for many applications. Other attempts indeed resulted in significantly higher amounts of dissolved natamycin, but such formulations had the disadvantage that the antifungal activity had not

increased accordingly (or had even decreased), or that the presence of undesired solubilizers was required. An incidental successful medical application of natamycin is in the treatment of corneal fungal infections, and the prevention of such infections in contact-lens users. However, these formulations are suspensions wherein the amount of soluble and biologically active natamycin is very small.

Thus, there is a need for pharmaceutical formulations having higher amounts of dissolved natamycin, without compromising the antifungal activity and/or without components that are undesired from a pharmaceutical point of view.

Further, when taken orally, little or no natamycin is absorbed from the gastrointestinal tract, making it inappropriate for treating systemic infections. Attempts to solubilize natamycin in vehicles that are safe for parenteral administration in humans have not yet been successful. Thus, there is also a need for effective natamycin formulations that are suitable for parenteral application.

The low solubility of natamycin also poses problems for its application as a food preservative. Although application of natamycin in crystalline form may in some cases be effective for that purpose, there is actually also a need for natamycin compositions comprising more finely divided forms of solid natamycin or natamycin that is completely dissolved. For example, the conventional application of natamycin as an aqueous suspension of crystals is undesired in view of the clogging of spray nozzles and the formation of a heterogeneous distribution when the suspension is applied on a surface (of *e.g.* cheese). Furthermore, more finely divided forms of natamycin or completely dissolved natamycin may allow the application of a lower dosage of natamycin for attaining the same antifungal effect.

A particular application of natamycin is the protection of agricultural products (in particular plant propagation material such as seed and flower bulbs) against fungi. The effectivity of natamycin however appears to be limited on such products, possibly because solid natamycin has limited access to fungi that reside in crevices or below the surface of the agricultural product. Natamycin that is either completely dissolved or that is present as smaller particles may therefore be more effective. On the other hand, existing fungicides such as thiram can be phytotoxic when applied in concentrations that are effective for combatting fungi. Also azole-fungicides generate resistance in targeted fungi, for example in Aspergillus fumigatus through 3 separate mutations since the early 90's. Natamycin is not prone to cause

resistance development due to its mode of action directly on the ergosterol of the fungal cell membrane.
Thus, the low solubility of natamycin is an obstacle that needs to be overcome to attain a higher effectivity in several applications. Another advantage of a high concentration of dissolved natamycin is that natamycin stock solutions can be prepared.

It is therefore an objective of the present invention to provide natamycin that is in a form that is capable to effectively combat fungi. In particular, it is aimed to provide a composition of natamycin that has a higher concentration of natamycin than known compositions, and which composition is stable over a prolonged period of time.
The composition comprising natamycin at a high concentration can also be used as stock solutions to prepare compositions having lower concentration of natamycin, for example by diluting with water.

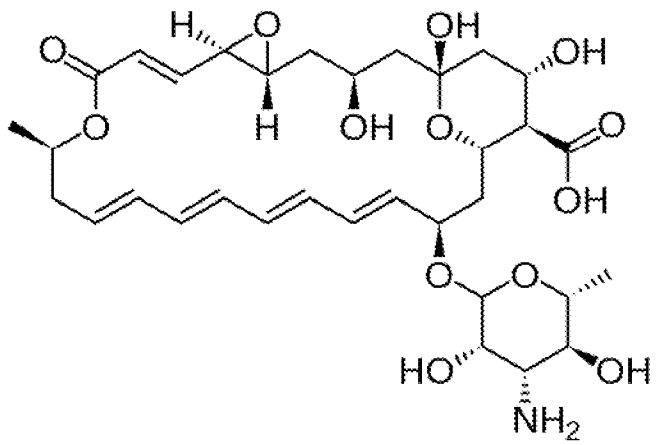
It is in particular an objective to provide an effective formulation of a bioactive molecule, in particular natamycin, that is suitable for medical application, for the preservation of foods and/or agricultural products against fungi.

It has now surprisingly been found that one or more of these objectives can be reached by a process for preparing a composition comprising a bioactive molecule comprising the steps of

a. Dissolving a bioactive molecule having at least one acid group with a base at pH=>11 to obtain a solution A
b. Neutralizing the solution A with an acid to obtain the composition comprising the bioactive molecule having a pH between 6 and 9,
wherein PEG is added before the neutralizing step in case either the base and/or the acid is an inorganic compound.

The bioactive molecule can be present as the pure chemical component known from literature, or it can be present as an adduct with one of the components added to the process or formed during the process of preparing the composition according to the invention. In particular the bioactive molecule can be present as the reaction product of the pure component with the base (inorganic or organic base) which can give an ion pair having an increased solubility in an aqueous environment.
The term bioactive molecule here and hereinafter has to be understood to comprise both the pure bioactive molecule and any adduct which can be formed upon reaction with one of the components of the composition.

As an example natamycin may be present as the pure chemical molecule according to formula (I), or as an adduct with other components, like the base, acid and/or the optional PEG component.



formula (I), natamycin.

Without being wanted to be bound by any theory, it is believed that these adducts can be reversible and may have a higher solubility than the neutral natamycin and provide bioactive natamycin upon dilution or application to a biological system.

The term natamycin here and hereinafter has to be understood to comprise both the pure natamycin and any adduct which can be formed upon reaction with one of the components of the composition.

The bioactive molecule is preferably chosen from the group consisting of natamycin, amphotericin B, nystatin, filipin, giberrellic acid (GA3, GA4, GA7), auxin (indole-3-acetic acid), indole-3-butyric acid - abcissic acid (S-ABA) and betulinic acid. More preferably the bioactive molecule is chosen from natamycin and amphotericin B, most preferably bioactive molecule is natamycin.

In general, the amount of bioactive molecule ranges between 0.1-20 wt% , relative to the total weight of the composition, more preferably between 1-18 wt%, 3-12 or most preferably between 4 and 10 wt%. Preferably the amount of water ranges between 5 and 45 wt%, and/or the weight ratio between bioactive molecule and water is between 1: 0.5 and 1: 12. Preferably the composition contains PEG, preferably between 30 and 80 wt% PEG having a number average molecular weight between 200 and 30000, or between 250 and 10000.

In case the bioactive molecule is natamycin, the ranges typically are between 0.1-20 wt%, more preferably between 1-18 wt%, 3-12 or most preferably between 4 and 10 wt%, as described above. In case bioactive molecule is amphotericin-B, the max concentration may be lower. The typical concentration of amphotericin-B range between 0.1-10 wt% or 0.2-5 wt%.

All weight percentages are defined relative to the total of the composition, unless specified otherwise.

Water is present in the process according to the invention. The amount of water can vary within wide ranges. The amount of water may range between 5 and 80 wt%, preferably between 5 and 45 wt%. Preferably a composition is obtained having a high content of natamycin, and a limited amount of water. In a preferred embodiment, the weight ratio between natamycin and water is between 1: 0,5 and 1: 12, to ensure a composition having a high amount of natamycin. Preferably the weight ratio between natamycin and water is between 1:0.55 and 1:10, or between 1:0.6 and 1:9.
This composition is concentrated and can be diluted for further use.

In the first step of the process, deprotonation of the natamycin takes place by reaction of natamycin with a base. The base can be an inorganic base, like for example KOH or NaOH, an organic base, or mixtures thereof.
The pH of the resultant solution is above pH 11, preferably above pH 13.
Preferably an organic base is used to prepare an adduct of natamycin which is dissolved in water. Examples of organic bases are choline hydroxide, choline chloride, choline bitartrate, choline monohydrogen-tartrate, choline-dihydrogen-citrate, tetramethylammonium hydroxide, tetraethylammonium hydroxide, tetrabutylammonium hydroxide and bPEI (branched polyethyleneimine).
Preferably the organic base is choline hydroxide.
Choline hydroxide preferably is applied as a solution in water, for example as a mixture containing 45 wt% choline hydroxide and 55 wt% water, or for example as a mixture of 25 wt% choline hydroxide in 75 wt% water. This mixture can be used as a solvent mixture for the natamycin, such that the total composition comprises between 25 and 40 wt% choline hydroxide.

The time needed to react natamycin with choline hydroxide should be sufficient for providing a clear solution A. The time typically ranges between 1 minute and 1 hour, or between 2 minutes and 30 minutes.

Choline hydroxide can also be added in more than 1 step, for example in 2 consecutive steps.

When an organic base is used, the base can be used to dissolve the natamycin, but the base can also act as a solvent for natamycin.

PEG can also be added as a cosolvent for natamycin after addition of base to the natamycin. In such cases the amount of choline hydroxide can range from 4 – 30 wt%, for example between 10 and 25 wt% relative to the total of the composition.

Preferably the PEG is a Poly Ethylene Glycol, which can have a number average molecular weight between 200 and 30000. In one embodiment PEG is applied having a lower molecular weight (for example between 200 and 1000, more preferably between 220 and 800 or between 250 and 600). In such cases PEG can be used as a liquid and compositions having excellent properties are obtained.
In another embodiment PEG having a molecular weight between 1000 and 10000 is applied. In such cases (especially at higher molecular weights, for example of at least from 5000, 6000 or 8000 to 10000) additional water may be needed to dissolve the high molecular weight PEG. The inventors found that in such cases natamycin may be present as dispersed crystals which can easily dissolve in the composition, rendering a composition having a quasi constant dissolved natamycin concentration: the composition contains dissolved natamycin and crystalline natamycin. When dissolved natamycin is taken from the composition, crystalline natamycin may be dissolved to generate the semi-constant natamycin concentration.
Compositions having lower molecular weight PEG (preferably between 200 and 1000) tend to be more stable compared to compositions having higher molecular weight PEG.

The weight ratio between natamycin and choline hydroxide may range between 1: 0.4 and between 1:100, preferably between 1:0.45 and 1:20, or between 1:0.5 and 1:8. The lower ratios (for example 1:0.5-1:3) are preferably applied when also PEG is present as solvent, the higher ratios (for example 1:3-1:100) are applied in the absence of PEG or when a limited amount of PEG is present (for example between 0.1 and 15 wt% PEG, relative to the total weight of the composition).

The weight ratio choline hydroxide and water needed to dissolve natamycin is at least 1.5, in order to have enough liquid to dissolve the natamycin. When the amount (weight) of water and choline hydroxide is less than 1.5 times the weight of natamycin, a paste or very high viscous substance is obtained which is less suitable for making the composition according to the invention.

In case the first step is carried out with an inorganic base (like for example KOH), than a PEG polymer should be added after formation of the ionic complex between natamycin and the

inorganic base. The PEG assists in forming a stable complex, especially after step b of the process of the present invention.

If added, the amount of PEG may range between 0.5 and 90 wt%, preferably between 20 and 87 wt%, or between 30 and 80 wt%.
Preferably the PEG is added at elevated temperature, preferably between 30 and 60 Cº. It is also possible to heat the PEG before addition of the mixture of natamycin and base component to a temperature between 40 and 85 ºC, to arrive at a solution A having a temperature between 30 and 60 ºC. A temperature of solution A below 30 ºC may lead to a composition having an enhanced viscosity, or even a paste like consistency. A temperature above 80 ºC may lead to excessive polymerization of PEG under the influence of KOH, which should preferably be avoided.

In the second step the reaction mixture is neutralized to a pH between 6 and 9 (preferably a pH between 7 and 8) by adding an acid component. The acid component can be an inorganic acid, like for example a solution of HCl, sulfuric acid and other strong acids, or the acid component can be an organic acid. Preferably the acid component used in step b is an organic acid.

Examples of organic acids are formic acid, acetic acid, propionic acid, sorbic acid, benzoic acid, lactic acid, malic acid, butanoic acid, hexanoic acid, hydroxybutanedioic acid, citric acid, fumaric acid, tartaric acid, ascorbic acid, octanoic acid and nicotinic acid. Preferably propionic acid is used for the neutralization of the solution A, when PEG is added to the composition.
In the absence of PEG, or when only low amounts (for example between 0.1 and 15 wt%) of PEG are applied, preferably the organic acid is chosen from the group consisting of propionic acid, fumaric acid, malic acid, tartaric acid and lactic acid.
The amount of acid is determined by the amount of base added to the bioactive molecule, in order to arrive at a solution having a pH between 6 and 9, preferably between 6.5 and 8.5, most preferably between 7 and 8.

In case the acid is an inorganic acid, PEG should be added before the neutralisation step, as explained above. Neutralization step B preferably is performed within a time of 1-60 minutes after addition of PEG, more preferably between 2 and 30 minutes after the addition of PEG, or between 3 and 15 minutes after the addition of PEG.
The start of the neutralization of the reaction mixture should not be too late, in order to minimize possible side reactions between KOH and PEG, and/or side reactions between KOH and natamycin.

The amount of acid is determined by measuring the pH of the resulting composition comprising the bioactive molecule. In general the molar amount of the acid will be close to the molar amount of base.

In one embodiment, the invention relates to a process for preparing composition comprising natamycin comprising the steps of

a) Dissolving 0.1-20 wt% of natamycin with an organic base dissolved in water at a pH of at least 13 to obtain a solution A,
b) Adding 30-90 wt% polyethylene glycol having a molecular weight between 200 and 800 daltons, to obtain a solution A',
c) Neutralizing the solution A' with an aqueous solution of an inorganic acid to obtain the composition comprising natamycin having a pH ranging from 6 to 9, preferably between 7 and 8,
wherein the organic base is chosen from the group consisting of choline hydroxide, tetramethyl ammonium hydroxide, tetra ethyl ammonium hydroxide.

In a preferred embodiment, the invention relates to a process for preparing composition comprising natamycin comprising the steps of

a) Dissolving 0.1-20 wt% of natamycin with an 5-35 wt% of an organic base dissolved in water at a pH of at least 13 to obtain a solution A,
b) Neutralizing the solution A with an organic acid to obtain the composition comprising natamycin having a pH ranging from 6 to 9, preferably between 6.5 and 8.5 or between 7 and 8,

wherein the organic base is chosen from the group consisting of choline hydroxide, tetramethyl ammonium hydroxide, tetra ethyl ammonium hydroxide and wherein the organic acid is chosen from propionic acid, citric acid, fumaric acid, malic acid, lactic acid, hexanoic acid or tartaric acid.

Preferably the organic base is choline hydroxide. Preferably the organic acid is propionic acid. Optionally 0.5-90 wt% of PEG may be added to solution A or A' or after the neutralization step. Preferably the PEG has a molecular weight between 200 and 800. The composition preferably contains between 5 and 45 wt% of water.

During step a) of the process according to the invention, generally an orange/brown colored solution is obtained. During the neutralization step the color becomes lighter when the pH drops. The color change is an indication for achieving a composition having a pH close to neutral. The actual pH value of the aqueous composition can be determined with known means, like for example pH indicator paper.

In a preferred embodiment, the invention relates to a process for preparing a composition comprising natamycin comprising the steps of

a) Dissolving 0.1-20 wt% of natamycin with 5-35 wt% of choline hydroxide dissolved in water at a pH of at least 13 to obtain a solution A,
b) Neutralizing the solution A with propionic acid to obtain the composition comprising natamycin having a pH ranging from 6 to 9, preferably between 7 and 8.

Optionally 0.5-90 wt% of PEG may be added to solution A or after the neutralizing step. Preferably the PEG has a molecular weight between 200 and 800. The composition preferably contains between 5 and 45 wt% of water.

In a preferred embodiment the invention relates to a process for preparing a composition comprising natamycin comprising the steps of

a) Dissolving 4-10 wt% of natamycin with 5-30 wt% of choline hydroxide dissolved in water at a pH of at least 13 to obtain a solution A,
b) Adding an aqueous choline hydroxide solution which is neutralized with propionic acid to obtain solution A',
c) Neutralizing the solution A' with propionic acid to obtain composition comprising natamycin having a pH ranging from 6 to 9, preferably between 7 and 8,

wherein the neutralized choline hydroxide solution is prepared from a choline hydroxide solution having between 15 and 50 wt% choline hydroxide in water, preferably from 25-45 wt% choline hydroxide in water.

In a preferred embodiment, the invention relates to a process for preparing a composition comprising natamycin comprising the steps of

a) Dissolving 4-10 wt% of natamycin with 5-35 wt% of choline hydroxide dissolved in water at a pH of at least 13 to obtain a solution A,
b) Neutralizing the solution A with propionic acid to obtain composition comprising natamycin having a pH ranging from 6 to 9, preferably between 7 and 8.

Optionally 0.5-30 wt% of PEG may be added to solution A or after the neutralizing step. Preferably the PEG has a molecular weight between 1000 and 10000 and is dissolved in an equal amount of water before adding to the process. The composition preferably contains between 5 and 45 wt% of water.

In an embodiment, the invention relates to a process for preparing a composition comprising natamycin comprising the steps of

a) Dissolving 0.1-20 wt% of natamycin with 0.5-2.5 wt% of potassium hydroxide dissolved in water at a pH of at least 13 to obtain a solution A,
b) Adding 30-80 wt% polyethylene glycol having a molecular weight between 200 and 800 daltons, to obtain a solution B,
c) Neutralizing the solution B with propionic acid to obtain the composition comprising natamycin having a pH ranging from 6 to 9, preferably between 7 and 8.

The composition preferably contains between 5 and 45 wt% of water.

When an inorganic base is used to dissolve natamycin, it is possible to add less strong organic bases to make a stable composition. An example of such bases are amines and imines, like for example bPEI, which is a branched polyethyleneimine.

The invention also relates to a composition comprising the reaction product of 0.1-20 wt% of natamycin, 5-30 wt% of choline hydroxide and an organic acid, wherein the organic acid is selected from propionic acid, fumaric acid, malic acid, tartaric acid and lactic acid. The composition may further contain 5-90 wt% water, between 0-85 wt% PEG and additives. Preferably the composition comprises the reaction product between 4-10 wt% natamycin, between 5 and 10 wt% choline hydroxide, 50-85 wt% PEG having a molecular weight between 200 and 800 daltons,and the organic acid, wherein the composition comprises between 5-45 wt% water, preferably 5-20 wt% water.
The composition preferably comprises 1-18 wt% natamycin, or 3-12 wt% or 4-10 wt%, relative to the total composition.
The weight ratio between natamycine and water is preferably between 1:0.5 and 1:12.
The weight ratio between natamycine and choline hydroxide is preferably between 1:0.4 and 1:100. Preferably the composition comprises between 5 and 45 wt% water.

The composition of the invention may contain additives, in particular additives for improving the look and feel of the product once it contains the composition. In the case of seed, for example, it may comprise anti-sticking agents, agents that improve the flowability of the seed and/or agents that improve the optical appearance of the seed such as pigments and shining agents.
Accordingly, the invention further relates to a method for treating an agricultural product or a food product, comprising applying a composition of the invention on an agricultural product or a food product, respectively.
The invention further relates to an agricultural product or food product obtainable by such method.

The invention further relates to an agricultural product or food product comprising a composition of the invention.

A composition of the invention may also find application in the medical field. Therefore, the invention further relates to a composition of the invention for medical use. The invention in particular relates to a composition of the invention for the treatment of a fungal infection. The composition may for example be used as a cream or potage to treat topical infections, for example of the skin or mucus, such as in the genital area. The action of the composition may also be enteral or parenteral. Preferably, the auxiliary compound in the composition comprises a pharmaceutically acceptable polymer, e.g. PEG.

Materials and Methods

Use is made of the following experimental procedures

1) Preparing the natamycin compositions

The compositions comprising natamycin were prepared as 100 g formulations. Exemplary is the preparation of a formulation comprising 5 wt% of natamycin (50,000 ppm). The natamycin is supplied by Shandong Freda Biotechnology Co., Ltd.. The natamycin used is of >90% purity, therefore 5.5 grams of this natamycin is used for the preparation of 100 g formulations. All other materials, except thiram, are supplied by Acros Organics. Water used is tap water.

During the preparation, natamycin is dissolved at pH 13-14 by adding a alkaline solution of an anorganic base such as KOH (7 wt% - 25 wt%) of or an organic base such as choline hydroxide (25 wt% - 45 wt%). Heated PEG-200, supplied by Acros Organics or an additional amount of organic base can be added. Thereafter neutralization to pH 6.5-8 or other pH is performed with an organic acid such as propionic acid. Organic acids are liquid (e.g. propionic acid 98% and lactic acid aqueous solution 85%) or in powder/crystalline form (fumaric acid, malic acid, tartaric acid). Solid organic acids require a longer mixing and neutralization time than liquid organic acids.

A 150 mL glass vessel is used for the preparation steps. A magnetic stirrer is used for mixing materials whenever the viscosity is low enough. A spoon is used for mixing higher viscosity mixtures, especially when mixing natamycin powder with the first amount of alkaline solution. Adding too much alkaline solution in 1 step can create lumps of natamycin, which are difficult to dissolve. A glass vessel is used for heating PEG-200 in a magnetron upon which the temperature is measured with an infra-red thermometer. The pH is measured using pH indicator paper. Before neutralization the pH is often found to be above pH 13. During neutralization the color of the mixture is becoming lighter below pH 10, the color is especially lighter in the target

pH range. Also the viscosity of the mixture is reducing at the same time the color is lightening. The final solution is mostly clear and off red-brown in color.
Upon 12x dilution with 11 parts of water mixed with 1 part of natamycin solution the dilution can show crystallization after a period of several hours to 24 hours. The dilution ratio of 12x is chosen as this dilution rate appears to show relatively more crystallization than other dilution ratio's. It is also a ratio that maybe used to produce solutions from stock solutions under practical conditions.

Example 1.

Different compositions according to the present invention have been prepared with PEG 200 and PEG 6000 as polymers, choline hydroxide as base and propionic acid as neutralizing compound. Composition shave been prepared with 5 to 20 wt% natamycin , which all show to be stable.
The results are shown in table 1.

In each experiment a correction has been made for the purity of natamycin. For example if a composition is prepared having 5 wt% natamycin, and the purity of natamycin is 90%, the amount of natamycin (90%) is 5/0.9 = 5.55 g/100 g of composition. In this example >90% purity natamycin, supplied by Shandong Freda Biotechnology Co., Ltd., is used. The amount of natamycin is weighed in a glass vessel of 150 ml. The suitable amount of choline hydroxide 45 wt% aqueous solution, supplied by Acros Organics, is added in 1 or 2 steps to the natamycin. In the first step not more than 1.5x the amount of natamycin is added to make a smooth mixture. If water is used this is added after the amount of choline hydroxide has been mixed with the natamycin. The water facilitates obtaining a light brown solution. When no water is used the result of the dissolving step maybe a mixture with a paste constitution. PEG-200 is heated in a glass vessel in a magnetron to the described first temperature. The temperature is measured after mixing by an infra-red thermometer. The heated PEG-200 is added to the mixture of natamycin, choline hydroxide solution and optionally water. A spoon and then magnetic stirrer is used to process this mixture into a uniform solution. The temperature is measure again and indicated as the second temperature value. The color of the solution is dark reddish brown with a pH higher than 13. The warm mixture is then neutralized to pH 6.5-8 by adding with a syringe the indicated amount of liquid propionic acid of 98% purity. During the neutralization the color of the solution is becoming lighter below pH 10. The pH is measured again using pH indicator paper. The propionic acid neutralizing agent is added after the addition of PEG
At higher concentrations of natamycin the PEG needs to be of higher temperature for sufficient temperature increase of the mixture to obtain clear solutions including PEG.

Higher molecular weight PEG's such as PEG-6000 or PEG-1500 can be used after dissolving in approximately equal amount of water and heating. The viscosity of the final solution after storage during several days using higher molecular weight PEG will be relatively higher.
The final solution is reddish-brown in color, from low viscosity to higher viscosity depending on the concentration of natamycin and forming stable solutions.
Upon 12x dilution with 11 parts of water mixed with 1 part of natamycin solution the dilution can show crystallization after a period of several hours to 24 hours.

	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9
Natamycine 90% purity (Wt%)	5,6	7,4	8,3	5,6	8,3	11,1	16,7	22,2	5,6
Polymer	PEG200	PEG200	PEG200	PEG200	PEG200	PEG200	PEG200	PEG200	PEG6000
Final conc. Polymer (Wt%)	78,0	78,4	78,0	30,0	75,7	72,1	63,8	48,6	20,0
Temperature before mixing	57'C → 44'C	63'C → 37'C	83'C → 47'C	20'C → 39'C	63'C → 42'C	83'C → 45'C	83'C → 45'C	83'C → 42'C	67'C → 40'C
base	choline OH	choline OH	choline OH	choline OH	choline OH	choline OH	choline OH	choline OH	choline OH
amount base (Wt%)	3,35	4,3	4,95	22,5	4,9	6,3	7,4	10,8	19,2
water (Wt%)	11,6	7,7	6,05	27,5	9	7,7	9,1	13,2	43,4
Neutralizing agent	propionic acid	propionic acid	propionic acid	propionic acid	propionic acid	propionic acid	propionic acid	propionic acid	propionic acid
Amount neutralizing agent (Wt%)	1,4	2,2	2,65	14,4	2,1	2,8	3	5,2	11,8
ratio (w/w) Natam/polymer	1/15,6	1/15,8	1/15,6	1/6	1/10,1	1/7,2	1/4,2	1/2,45	1/4
clear or turbid	clear	slightly milky	slightly milky	clear	clear	clear	clear	turbid	clear
Color	reddish brown	reddish brown	reddish brown	dark reddish brown	dark reddish brown	dark reddish brown	dark reddish brown	dark reddish brown	light brown
Viscosity (indication)	low	low	low	low	medium	medium	high	very high	low
Stability (visual)	●●●●●	●●●●	●●●●	●●●●●	●●●	●●●●●	●●●●●	●●●●	●●●●●
Crystals or other structures in 12x dilution with water after 24 hours (visual)	●●●●●			●●●●●					●●●●●

Tabel 1 examples according to the invention.

Stability (visual)		Crystals or other structures in 12x dilution with water after 24 hours (visual)	
●●●●●	Clear	●●●●●	Clear
●●●●	Slightly milky	●●●●	Slight cloud of crystals
●●●	Milky, later some sediment	●●●	Cloud of crystals
●●	Sediment	●●	Layer of crystals
●	Large amount of sediment	●	Thick layer of crystals

All compositions have a high amount of natamycine, are stable and do not crystallize.

Example 2

Example 2 shows compositions comparable to the compositions in example 1, but prepared in a different order: neutralization with propionic acid is performed before the addition of PEG. The results are summarized in table 2.

	2,1	2,2	2,3	2,4	2.5	2.6
Natamycine 90% purity (Wt%)	5,60	5,60	5,60	5,60	11,10	5,60
Polymer	PEG200	PEG200	PEG200	PEG200	PEG200	PEG300
Final conc. Polymer (Wt%)	48,7	48,7	69,4	29,4	44,2	47,9
Temperature before mixing	87'C -> 48'C	20'C -> 32'C	57'C -> 47'C	60'C -> 40'C	97'C -> 52'C	60'C -> 42'C
base	choline OH	choline OH	choline OH	choline OH	choline OH	choline OH
amount base (Wt%)	16,2	16,2	9	22,5	16,2	16,3
water (Wt%)	20	20	11	27,5	20	20,1
Neutralizing agent	propionic acid	propionic acid	propionic acid	propionic acid	propionic acid	propionic acid
Amount neutralizing agent (Wt%)	9,5	9,5	5	15	8,5	10,1
ratio (w/w) Natam/polymer	1/9,8	1/9,8	1/13,8	1/6	1/9	1/9,6
clear or turbid	clear	clear	clear	clear	clear	clear
Color	dark reddish brown	dark reddish brown	dark reddish brown	dark reddish brown	dark reddish brown	dark reddish brown
Viscosity (indication)	low	low	low	low	low	low
Stability (visual)	●●●●●	●●●●●	●●●	●●●●●	●●●●●	●●●●●
Crystals or other structures in 12x dilution with water after 24 hours (visual)			●●	●●●●●		

Table 2

Example 3.

Experiments are performed with different organic acids, for neutralizing the natamycin solutions. Results are shown in table 3.

The natamycin amount used is calculated from its purity. In this example >90% purity natamycin, supplied by Shandong Freda Biotechnology Co., Ltd., is used. The amount of natamycin for most objects is 5.5 grams, weighed in a glass vessel of 150 ml. The suitable amount of choline hydroxide 45% aqueous solution is added in 1 or 2 steps to the natamycin. In the first step not more than 1.5x the amount of natamycin is added to make a smooth mixture. After this addition, the natamycin – choline hydroxide mixtures are further diluted with additional choline hydroxide (45 wt% in water).

In a final step, all compositions are neutralized with organic acids under stirring with a magnetic stirrer. The final pH is confirmed using a pH indicator paper. The reaction temperature during neutralization ranged between 35'C – 47'C.

The solutions are visually observed for color, clarity/turbidity and viscosity. Solutions are diluted 12x with water by mixing 11 parts of water with 1 part of solution. Crystal formation in this dilution is visually checked after 6 hours and 24 hours.

	3.1	3.2	3.3	3.4	3.5	3.6	3.7
Natamycine 90% purity (Wt%)	5,6	5,6	5,6	5,6	5,6	5,6	5,6
Polymer	none	none	none	none	none	none	none
Final conc. Polymer (Wt%)	0	0	0	0	0	0	0
Temperature before mixing	20'C → 42'C	20'C → 39'C	20'C → 39'C	20'C → 45'C	20'C → 39'C	20'C → 39'C	20'C → 37'C
base	choline OH	choline OH	choline OH	choline OH	choline OH	choline OH	choline OH
amount base (Wt%)	33,5	32,4	34,5	33,6	30,6	29,1	33,3
Water (Wt%)	40,7	39,5	41,9	40,8	37	35,3	40,1
Neutralizing agent	propionic acid	citric acid	fumaric acid	malic acid	lactic acid 85%	hexanoic acid	tartaric acid
Amount neutralizing agent (Wt%)	20,2	22,5	18	20	26,8	30	21
ratio (w/w) Natam/polymer	1/0	1/0	1/0	1/0	1/0	1/0	1/0
clear or turbid	clear	clear	clear	clear	clear	clear	clear
Color	light brown color	light brown color	light brown color	light brown color	light brown color	light brown color	light brown color
Viscosity	low	low	low	low	low	low	low
Stability	stable	stable; pH 6.4; dark brown after storage	stable	stable	stable	stable	stable; after 10 days still not very dark
Crystal or other structures formation in 12x dilution with water	after 24 hours: ●●●●●	after 6 hours: ●●	after 24 hours: ●●●●	after 6 hours: ●●●●	after 24 hours: ●●●●	after 2 hours: ●	after 24 hours: ●●●●●
Bio-activity Antifungal	●●●●●	●●●●	●●●●●	●●●●	●●●●●	●●●●●	●●●●●
Plant growth	●●●●●	●●●●	●●●●●	●●●●	●●●●	●	●●●●●

Table 3

Bio-activity Antifungal		Plant growth	
●●●●●	No fungi growth	●●●●●	Healthy growth
●●●●	Little, slow fungi growth	●●●●	Some abnormal growth
●●●	Fungi growth	●●●	Abnormal growth
●●	Fungi growth covering some seeds	●●	Many abnormal growth
●	Heavy fungi growth covering all seeds	●	Almost no growth

The compositions of example 3 have been tested as dilutions for its bioactivity (antifungal) and support of plant growth.

The bio-activity of compositions on fungi is determined at various dilutions on the growth and survival of Aspergillus niger spores. The growth of the Aspergilus niger culture is measured by OD (optical density) by OD600.

Method

Aspergillus niger spores and the medium are added to a 96-wells plate and incubated to germinate the spores. After germination of spores the formulation is added in triplo in various dilutions together with additional medium and an amount of luciferine. The well plate is incubated in a plate-reader that measures every 5 minutes both the OD as well as the emitted luminescence.

The antifungal activity and plant growth results of the different examples have been visually inspected. High antifungal activity is rated as 5 dots, while poor antifungal activity is indicated with a single dot.

Excellent plant growth stimulation is indicated with 5 dots, while poor plant growth is indicated with a single dot.

Example 4

Compositions have been prepared with different organic bases. Results are shown in table 4.

The natamycin amount used is calculated from its purity. In this example >90% purity natamycin, supplied by Shandong Freda Biotechnology Co., Ltd., is used. For most objects the amount of 5.5 grams natamycin of 90% purity is weighed in a glass vessel of 150 ml. The suitable amounts of choline hydroxide 45% aqueous solution is added in 1 or 2 steps to the natamycin.

In the first step not more than 1.5x the amount of natamycin is added to make a smooth mixture.

25 wt% aqueous solutions of tetra ethyl ammonium hydroxide and tetra methyl ammonium hydroxide were supplied by Acros Organics. These is added in 2-3 steps to the natamycin and mixed in with a spoon.

PEG-200 is heated in a glass vessel in a magnetron to the described first temperature. The temperature is measured after mixing by an infra-red thermometer. The heated PEG-200 is added to the mixture of natamycin, tetra ethyl ammonium hydroxide solution. A spoon and then magnetic stirrer is used to process this mixture into a uniform solution.

All objects are finally neutralized with organic acids under stirring with a magnetic stirrer. The final pH is confirmed using a pH indicator paper. The final temperature of all objects is in a range of 30'C – 50'C.

The solutions are visually observed for color, clarity and viscosity. Solutions are diluted 12x with water by mixing 11 parts of water with 1 part of solution. Crystal formation in this dilution is visually checked after 24 hours.

	1.1	1.2	4.1	4.2	4.3	5.0
Natamycine 90% purity (Wt%)	5,60	7,40	5,60	7,40	7,40	5,60
Polymer	PEG200	PEG200	PEG200	PEG200	PEG200	PEG200
Final conc. Polymer (Wt%)	78	78	78	65	65	61
Temperature before mixing	57'C → 44'C	63'C → 37'C	57'C	57'C	57'C	70'C → 50'C
base	choline OH	choline OH	TEAOH	TEAOH	TMAOH	KOH + bPEI
amount base (Wt%)	3,4	4,3	3,8	6,3	6,3	1,6 + 8
water (Wt%)	11,6	8,1	11,3	18,6	18,8	14,8
Neutralizing agent	propionic	propionic	propionic	lactic 85%	propionic	propionic
Amount neutralizing agent (Wt%)	1,4	2,2	1,25	2,75	2,0	9,0
ratio (w/w) Natam/polymer	1/15,6	1/15,6	1/15,6	1/13	1/13	1/12,2
clear or turbid	clear	slightly milky	clear	clear	clear	clear
Color	reddish brown	reddish brown	light brown color	dark red-brown color	dark red-brown color	dark red-brown color
Viscosity (visual)	low	low	low	low	low	low
Stability (visual)	•••••	•••••	•••••	•••••	•••••	•••••
Crystal formation in 12x dilution with water after 24 hours (visual)	•••••		••		•••••	•••••

Table 4 examples with different organic bases including tetraethylammoniumhydroxide and tetramethylammoniumhydroxide.

Example 5

Table 5.1 shows examples of compositions prepared with an inorganic base (KOH) instead of the preferred organic base. Also two comparative exaperiments have been added: comparative experiment A describes an experiment, where no base is added, but only PEG was added to the natamycin: no soluble composition could be obtained.

Comparative experiment B shows the effect of dissolving natamycin with KOH, and subsequently neutralizing with HCl, but in the absence of PEG. No suitable composition could be obtained having a high dissolved natamycin concentration.

The natamycin amount used is calculated from its purity. In this example >90% purity natamycin, supplied by Shandong Freda Biotechnology Co., Ltd., is used. For these experiments the amount of 5.5 grams natamycin of 90% purity is weighed in a glass vessel of 150 ml. The suitable amounts of water or potassium hydroxide aqueous solution is added in 1 or 2 steps to the natamycin. In the first step not more than 1.5x the amount of natamycin is added to make a smooth mixture.

PEG-200 is heated in a glass vessel in a magnetron to the described first temperature. The temperature is measured after mixing by an infra-red thermometer. The heated PEG-200 is added to the mixture of natamycin, potassium hydroxide solution or water. A spoon and then magnetic stirrer is used to process this mixture into a uniform solution or suspension in case of comparative experiment A.

All experiments with alkaline solution are finally neutralized with HCL 10% solution or propionic acid to pH 6.5-8 under stirring with a magnetic stirrer. The final pH is confirmed using a pH indicator paper. The final temperature of all experiments can be high when putting for a second time in the magnetron.

The bio-activity of compositions on fungi is determined at various dilutions on the growth and survival of Aspergillus niger spores. The growth of the Aspergilus niger culture is measured by OD (optical density) by OD600.

The bioactivity of compositions on oomycetes is determined at 10x dilution ratio's used with a culture of Phytophtora infestans on plates by judging the growth and survival of the P. infestans by visual inspection by a skilled microbiologist.

In the absence of use of an alkali solution the composition is a suspension which is unstable and forms a deposit of crystals at the bottem. The bio-activity against fungi is average and the bioactivity against oomycetes is low. Example 5.3 shows the a good bio-activity against fungi and a fairly good bioactivity against oomycetes. A high temperature combined with a high concentration of alkali solution for example 5.3 and 5.4 reduces the bioactivity against fungi to a very low level.

Table 5.2 shows examples of compositions prepared with KOH and in addition bPEI (branched polyethylene-imine) molecular weight 600 supplied by Polysciences Europe GmbH.

The bPEI is introduced in the process after dissolving natamycine with KOH solution. A 50% bPEI acquous solution is mixed with the KOH-natamycine mixture. Thereupon the process is continued by adding heated PEG and finally the neutralization step.

	A	B	5.1	5.2	5.3	5.4	5.5
Natamycine 90% purity	5,6	5,6	5,6	5,6	5,6	5,6	5,6
Polymer	PEG200	None	PEG6000	PEG200	PEG200	PEG200	PEG6000
Final conc. Polymer (Wt%)	80	0	35	81	79	89	34
Temperature before mixing	84'C → 55'C		86'C → 48'C	80'C → 51'C	79'C → 53'C	110'C → 100'C	50'C → 35'C
type of base	none	KOH	KOH	KOH	KOH	KOH	KOH
base (Wt%)	0	1,05	1,05	1,25	3,1	1,3	1,05
Water (Wt%)	14,4	88,4	53	11,2	9,4	3,7	54
Neutralizing agent	none	HCL 10% sol.	HCL 10% sol.	propionic acid	propionic acid	propionic acid	HCl 10% sol
Amount neutralizing agent (Wt%)	0	5	5	1	2,5	1	5
ratio (w/w) Natam/polymer	1/16	1/0	1/7	1/16,2	1/16	1/17,8	1/7
clear or turbid	turbid	turbid	turbid	dark	black	black	turbid
Color	white	white	brown-yellow	dark red brown	very dark reddish brown	very dark reddish brown	brown yellow
Viscosity	Low viscocity	Very high viscosity	Medium viscosity	Low viscosity	Medium viscosity	Medium viscosity	high
Stability	Not stable. Settling in layer of crystals.	Paste.	Not stable. Sediment and higher viscosity.	A little sediment after storage	Becoming more viscous. Stable.	Stable	
Bioactivity on fungi	●●●		●●●●●	●●●●●	●●	●●	●●
Bioactivity on oomycetes	●		●	●●●●	●●●●●	●●●●	

Table 5.1 Use of KOH as base.

	5.6	5.7	5.8	5.9	5.10
Natamycine 90% purity (Wt%)	5,60	5,60	5,60	5,60	5,60
Polymer	PEG200	PEG200	PEG200	PEG200	PEG200
Final conc. Polymer (Wt%)	61	67	70	74	75
Temperature before mixing	70'C → 50'C	72'C → 50'C	72'C → 50'C	72'C → 50'C	75'C → 55'C
base	KOH + bPEI	KOH + bPEI	KOH + bPEI	KOH + bPEI	KOH + bPEI
amount base (Wt%)	1,6 + 8	1,6 + 6	1,6 + 4	1,6 + 2	1,6 + 1
water (Wt%)	15	15	15	15	15
Neutralizing agent	propionic	propionic	propionic	propionic	propionic
Amount neutralizing agent (Wt%)	9,0	5,0	3,5	2,5	2,1
ratio (w/w) Natam/polymer	1/12,2	1/13,4	1/14	1/14,8	1/15
clear or turbid	clear	clear	clear	clear	clear
Color	dark red-brown color	dark red-brown color	dark red-brown color	dark red-brown color	dark red-brown color
Viscosity (visual)	low	low	low	low	low
Stability (visual)	•••••	•••••	•••••	•••••	•••••
Crystal formation in 12x dilution with water after 24 hours (visual)	•••••	•••••	•••••	•••••	••••

Table 5.2 Use of KOH and bPEI as bases.

Bioactivity on fungi spores		Bioactivity on oomycetes spores	
•••••	Complete control at >1500x dilution	•••••	Complete control at 150x dilution
••••	Complete control at 1500x dilution	••••	Complete control at 150x dilution for limited
•••	Complete control at <1500x dilution	•••	Reduction of oomycetes growth
••	Reduction of fungi growth	••	Limited reduction of oomycetes growth
•	No reduction of growth	•	No reduction of growth

Example 6

In example 6 three different bioactive molecule have been dissolved with the method according to the present invention.

Betulinic acid - 98% is supplied by Xi'an Lyphar Biotech Co.,LTD;

Amphotericin-B - 86% is supplied by XI'AN HEALTH BIOCHEMICAL TECHNOLOGY CO.,LTD ;

Gibberellic acid GA3 - 90% is supplied by BIOSYNTH AG.

Compositions have been prepared by taking the bioactive component, adding the base with water to dissolve the bioactive compound, followed by adding warm PEG. After stirring to make a clear solution, the neutralizing agent has been added to prepare the solution of the bioactive.

Results are summarized in table 6.

	6.1	6.2	6.3
Bioactive	Betulinic acid	Amphotericin-B	Gibberellic acid GA3
Final conc bioactive (Wt%)	2,0	2,5	10,0
Polymer	PEG200	PEG200	PEG200
Final conc. Polymer (Wt%)	92	84	56
Temperature before mixing	100'C → 75'C	100'C → 75'C	60'C → 40'C
base	choline OH	KOH	KOH
amount base (Wt%)	1,1	0,7	2
Water (Wt%)	4,1	9,8	23
Neutralizing agent	propionic acid	sulphuric acid 10% sol.	HCl 10% sol.
Amount neutralizing agent (Wt%)	0,5	3,5	9
ratio (w/w) Bioactive/polymer	1/46	1/33,4	1/5,6
clear or turbid	clear	clear	clear
Color	yellowish	very dark, reddish	very slight yellow
Viscosity	Low viscocity	Low viscosity	Low viscosity
Stability	Stable.	Stable	Stable

Table 6.

Example 7

Treating seed with a natamycin composition

The seeds were treated with the natamycin composition were typically prepared as a 400 ppm mixture, i.e. a mixture comprising 0.4 g of natamycin per kg of seed. For example, when a composition comprising 5 wt% of natamycin was used (50,000 ppm), 0.16 g of the composition was first diluted to a weight of 2.0 g (4,000 ppm). Then, this diluted mixture was applied to 20 g of seed. The water evaporated during 8 hours in the air at room temperature, yielding a portion of seeds comprising 400 ppm of natamycin.

Comparative experiments were carried out with thiram, using a 42-S formulation obtained from Bayer Cropscience in the USA containing 42% thiram. This solution was diluted by adding water to 0.16 g of the thiram solution until it reached the weight of 2 g. Then, this diluted mixture was applied to 20 g of seed. A coating around the seeds was obtained by allowing the solvent to evaporate during 8 hours in the air at room temperature, yielding a portion of seeds comprising

3.36 g of thiram per kg of seed (3360 ppm). A second formulation on seed was prepared comprising 0.840 g of thiram per kg of seed (840 ppm).
The seeds were then placed on germination paper (paper obtained from Allpaper BV, type T10 D 140*200, 550 g/m-2) and the growth of fungus was monitored as the seeds germinated, typically during 7–30 days at room temperature. In comparative experiments, untreated infected seeds or thiram-treated infected seeds were used. The results of this essay are as effectivities of the growth inhibition of fungus compositions, represented on a scale of 1–5 (1 = no effect; 2 = small effect; 3 = standard effect; 4 = good effect; 5 = excellent effect). The effect of the composition on the growth of the plant is represented on a scale of 1-5 (1 = strong negative effect; 2 = fairly strong negative effect; 3 = small negative effect; 4 = no effect; 5 = positive effect).

The effectivity of compositions of the invention were compared to to those of antifungal compositions comprising thiram. The results obtained with “plant seed essay #1” are visualized in Figures 1 and 2, each containing a photograph comprising two test areas (left and right) of Tartaros wheat seed (harvest of 2014) naturally infected with fungus (predominantly Fusarium). All areas record the situation after seven days of sowing. The left area of Figure 1 contains untreated seed, while the right area contains the natamycin composition of entry 5.5 of Table 5, which is present in a concentration of 400 ppm on the seed and has an antifungal effectivity of 4. The two areas in Figure 2 contain a formulation of thiram of different concentration. In the left area the concentration is 3360 ppm, in the right area it is 840 ppm. In both areas, fungus growth is inhibited, the extent of which correspond to an effectivity of 3 on the indicated scale of 1–5 (standard effectivity). However, this is to a smaller extent than the inhibition by the natamycin composition of Figure 1 (effectivity of 4). Moreover, it can clearly be seen that thiram has an phytotoxic effect as compared to natamycin, since the germination of the seeds in Figure 2 is substantially less developed. The effect is the largest with the highest concentration of thiram (left area of Figure 2). It can thus be concluded that natamycin compositions of the invention are more effective and less phytotoxic than known compositions such as thiram compositions.
It was further shown that natamycin compositions are highly stable when stored in the dark at room temperature, since no decrease in effectivity has been observed for compositions of more than one year old.

CONCLUSIES

1. Een werkwijze voor het bereiden van een samenstelling omvattende een bioactief molecuul omvattende de stappen voor:
het oplossen van een bioactief molecuul met ten minste een zure groep met een base bij pH =>11 om een oplossing A te verkrijgen,
het neutraliseren van de oplossing A met een zuur om de samenstelling omvattende een bioactief molecuul te verkrijgen met een pH tussen 6 en 9,
waarin PEG wordt toegevoegd voor de neutralisatiestap wanneer de base en/of het zuur een anorganische verbinding is,
waarin het bioactieve molecuul wordt gekozen uit de groep bevattende natamycine, amfotericine B, nystatine, filippine, gibberelline zuur (GA3, GA4, GA7), auxine (indool-3-azijnzuur, indool-3-boterzuur) – abscisinezuur (S-ABA) en betuline zuur en
waarin de base is gekozen uit de groep bevattende choline hydroxide, tetramethylammonium hydroxide, tetraethylammonium hydroxide, tetrabutylammonium hydroxide and bPEI (branched polyethyleneimine).

2. De werkwijze volgens conclusie 1, waarin het bioactieve molecuul natamycine is, en de base choline hydroxide is.

3. De werkwijze volgens een der conclusies 1-2, waarin het gewichts% van het bioactieve molecuul tussen 0,1-20 gew% is, met meer voorkeur tussen 1-18 gew%, 3-12 gew% of met de meeste voorkeur tussen 4 en 10 gew%.

4. De werkwijze volgens een der conclusies 2 of 3, waarin de gewichtsverhouding tussen natamycine en water tussen 1:0,5 en 1:12 is, bij voorkeur is de gewichtsverhouding tussen natamycine en water tussen 1:0,55 en 1:10, of tussen 1:0,6 en 1:9.

5. De werkwijze volgens conclusie 4, waarin de hoeveelheid organische base in het bereik van 4-30 gew% ligt, bijvoorbeeld tussen 10 en 25 gew% ten opzichte van de totale samenstelling.

6. De werkwijze volgens een der bovenstaande conclusies, waarin het bioactieve molecuul natamycine is en de organische base choline hydroxide is en waarin de gewichtsverhouding tussen natamycine en choline hydroxide in het bereik is tussen 1: 0,4 en 1:100, bij voorkeur tussen 1:0,45 en 1:20, of tussen 1:0,5 en 1:8.

7. De werkwijze volgens een der conclusies 1-6, waarin de organische zuren zijn gekozen uit de groep bevattende mierenzuur, azijnzuur, propionzuur, sorbinezuur, benzoëzuur, melkzuur, appelzuur, boterzuur, hexaanzuur, hydroxybutaandizuur, citroenzuur, fumaarzuur, wijnsteenzuur, ascorbinezuur, octaanzuur en nicotinezuur; bij voorkeur is het organische zuur propionzuur.

8. De werkwijze volgens een der bovenstaande conclusies, waarin 20-80 gew% PEG met een molecuulgewicht tussen 200 en 1000 daltons wordt toegevoegd.

9. Een samenstelling omvattende het reactieproduct van 0,1-20 gew% van natamycine, 5-30 gew% van choline hydroxide en een organische zuur, 5-90 gew% water en optioneel 0-70 wt% PEG, waarin het organische zuur wordt geselecteerd uit propionzuur, fumaarzuur, appelzuur, wijnsteenzuur en melkzuur.

10. De samenstelling volgens conclusie 9, waarin de samenstelling 1-18 gew% natamycine omvat, of 3-12 gew% of 4-10 gew%, ten opzichte van de totale samenstelling.

11. De samenstelling volgens een der conclusies 9-10, waarin de gewichtsverhouding tussen natamycine en water bij voorkeur tussen 1:0,5 en 1:12 is.

12. De samenstelling volgens een der conclusies 9-11, waarin de gewichtsverhouding tussen natamycine en choline hydroxide bij voorkeur tussen 1:0,4 en 1:100 is.

13. De samenstelling volgens een der conclusies 9-12, waarin de samenstelling 20-80 gew% PEG omvat met een molecuulgewicht tussen 200 en 1000 daltons.

14. Een werkwijze voor het behandelen van een agrarisch product of een voedsel product, omvattende het aanbrengen van een samenstelling volgens een der conclusies 9-13, of verkregen met de werkwijze volgens een der conclusies 1-8 op, respectievelijk, een agrarisch product of een voedsel product.

15. Een agrarisch product of voedsel product omvattende een samenstelling volgens een der conclusies 9-13, of verkregen met de werkwijze volgens een der conclusies 1-8.

16. Samenstelling volgens een der conclusies 9-13 of verkregen met de werkwijze volgens een der conclusies 1-8 voor gebruik als geneesmiddel.

17. Samenstelling volgens een der conclusies 9-13, of verkregen met de werkwijze volgens een der conclusies 1-8 voor de behandeling van een schimmelinfectie.

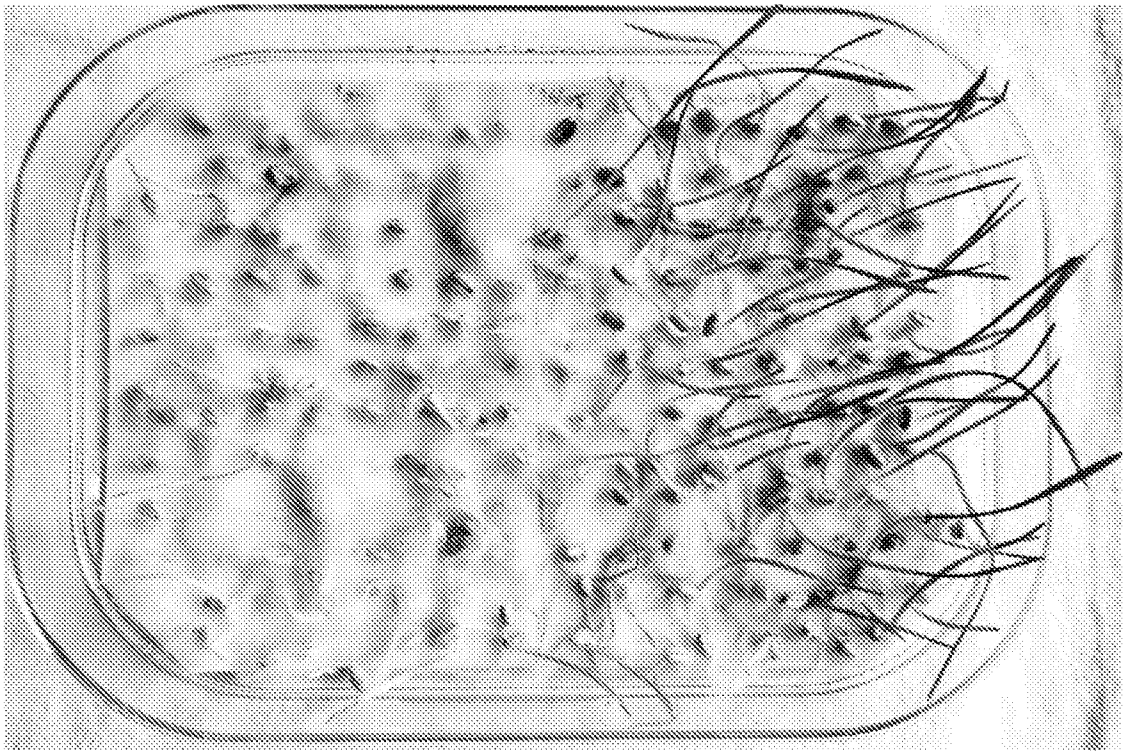


Figure 1

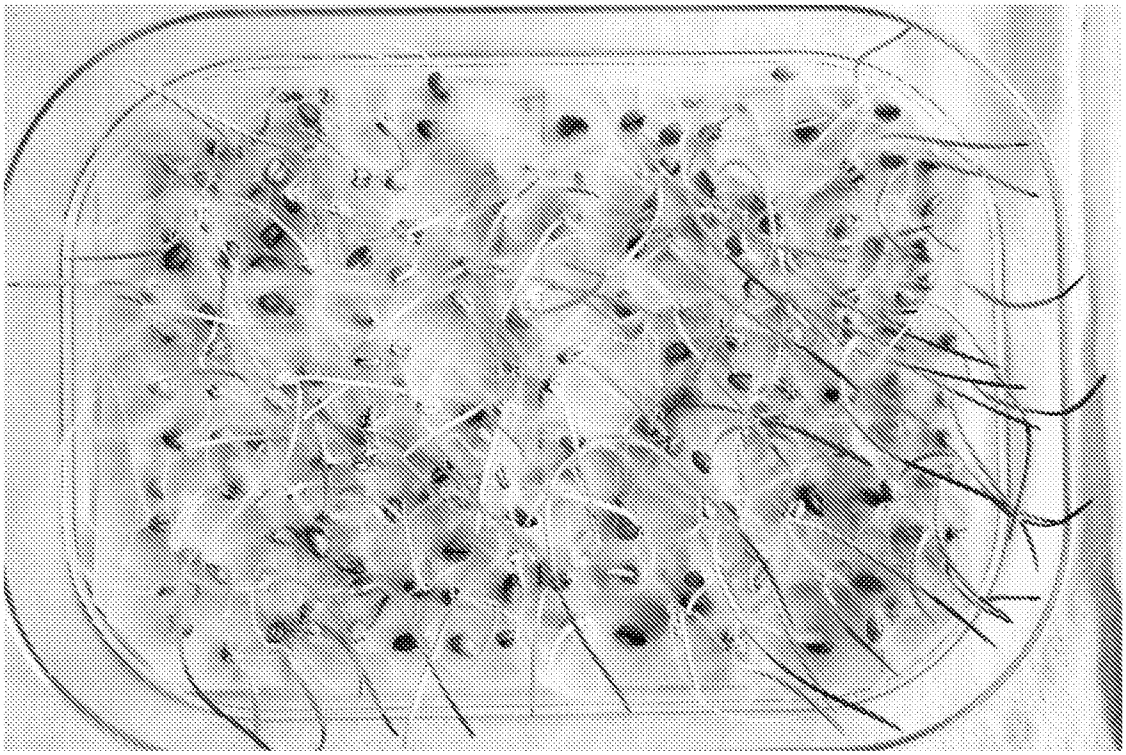


Figure 2

ABSTRACT

The invention relates to a process for preparing a composition comprising a bioactive molecule comprising the steps of

a. Dissolving a bioactive molecule having at least one acid group with a base at pH=>11 to obtain a solution A
b. Neutralizing the solution A with an acid to obtain the composition comprising a bioactive molecule having a pH between 6 and 9,

wherein PEG is added before the neutralizing step in case either the base and/or the acid is an inorganic compound, and to a composition comprising a high concentration of natamycin .

Rijksdienst voor Ondernemend Nederland

RAPPORT BETREFFENDE HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK

Octrooiaanvrage 2017545

Classificatie van het onderwerp[1]: A01N 43/90; A23L 3/3463; A61K 31/7048; A61P 31/10; A61K 47/10; ; A01N25/02; A01P3/00	Onderzochte gebieden van de techniek[2]: A61K, A01N, A23L
Computerbestanden: EPODOC, WPI, REGISTRY, CAS-ONLINE	Omvang van het onderzoek: Volledig
Datum van de onderzochte conclusies: 28 september 2016	Niet onderzochte conclusies: –

Van belang zijnde literatuur

Categorie[2]	Vermelding van literatuur met aanduiding, voor zover nodig, van speciaal van belang zijnde tekstgedeelten of figuren.	Van belang voor conclusie(s)
X	CN 102742581 A (SHANXI PROVINCE INST OF MICROBIOLOGY) 24 oktober 2012 & online machinevertaling EPO http://translationportal.epo.org/emtp/translate/?ACTION=description-retrieval&COUNTRY=CN&ENGINE=google&FORMAT=docdb&KIND=A&LOCALE=nl_NL&NUMBER=102742581&OPS=ops.epo.org/3.2&SRCLANG=zh&TRGLANG=en, opgehaald van het internet op 15 maart 2017 * WPI & EPODOC samenvatting; pagina 2 van de machinevertaling * – – –	1-10, 16-19
X	WO 2004/105491 A (DSM IP ASSETS BV) 9 december 2004 * het gehele document; in het bijzonder pagina's 4, 5, 9 * – – –	1-3, 6, 16, 17
X	US 2012/0196003 A (SNABE TORBEN) 2 augustus 2012 * het gehele document; in het bijzonder paragrafen [0071], [0089] * – – –	1
A	US 2015/0173365 A (VALENT BIOSCIENCES CORP) 25 juni 2015 * het gehele document * – – –	

1 Gedefinieerd volgens International Patent Classification (IPC).
2 Verklaring van de categorie-aanduiding: zie apart blad.

Schriftelijke Opinie

Octrooiaanvrage 2017545

Categorie	Vermelding van literatuur met aanduiding, voor zover nodig, van speciaal van belang zijnde tekstgedeelten of figuren.	Van belang voor conclusie(s)
A	T. R. M. Tan et al., "Characterization of a Polyethylene Glycol-Amphotericin B Conjugate Loaded with Free AMB for Improved Antifungal Efficacy", PLoS ONE, volume 11 (3), maart 2016, pagina's 1-18 * het gehele document * – – – – –	

Datum waarop het onderzoek werd voltooid: 15 maart 2017	De bevoegde ambtenaar: L. Bechger **Octrooicentrum Nederland,** **onderdeel van Rijksdienst voor Ondernemend Nederland**

Categorie van de vermelde literatuur:

X: op zichzelf van bijzonder belang zijnde stand van de techniek

Y: in samenhang met andere geciteerde literatuur van bijzonder belang zijnde stand van de techniek

A: niet tot de categorie X of Y behorende van belang zijnde stand van de techniek

O: verwijzend naar niet op schrift gestelde stand van de techniek

P: literatuur gepubliceerd tussen voorrangs- en indieningsdatum

T: niet tijdig gepubliceerde literatuur over theorie of principe ten grondslag liggend aan de uitvinding

E: octrooiliteratuur gepubliceerd op of na de indieningsdatum van de onderhavige aanvrage en waarvan de indieningsdatum of de voorrangsdatum ligt voor de indieningsdatum van de onderhavige aanvrage.

D: in de aanvrage genoemd

L: om andere redenen vermelde literatuur

&: lid van dezelfde octrooifamilie; corresponderende literatuur

Rijksdienst voor Ondernemend Nederland

AANHANGSEL

Behorende bij het Rapport betreffende het Onderzoek naar de Stand van de Techniek, Octrooiaanvrage 2017545

Het aanhangsel bevat een opgave van elders gepubliceerde octrooiaanvragen of octrooien (zogenaamde leden van dezelfde octrooifamilie), die overeenkomen met octrooigeschriften genoemd in het rapport. De opgave is samengesteld aan de hand van gegevens uit het computerbestand van het Europees Octrooibureau per 8 maart 2017. De juistheid en volledigheid van deze opgave wordt noch door het Europees Octrooibureau, noch door Octrooicentrum Nederland gegarandeerd; de gegevens worden verstrekt voor informatiedoeleinden.

In het rapport genoemd octrooigeschrift		Datum van publicatie	Overeenkomende octrooigeschriften		Datum van publicatie
WO 2004105491	A1	09-12-2004	US 2006241061	A1	26-10-2006
			EP 1641343	A1	05-04-2006
			CN 1798503	A	05-07-2006
			CA 2527120	A1	09-12-2004
US 2012196003	A1	02-08-2012	BR 112013018856	A2	09-08-2016
			US 2015216189	A1	06-08-2015
			NZ 611007	A	24-04-2015
			AU 2012210484	A1	26-06-2014
			CN 103491807	A	01-01-2014
			MX 2013008555	A	21-08-2013
			AR 084942	A1	10-07-2013
			WO 2012101256	A1	02-08-2012
US 2015173365	A1	25-06-2015	AU 2014364823	A1	16-06-2016
			EP 3082421	A1	26-10-2016
			WO 2015095312	A1	25-06-2015

Rijksdienst voor Ondernemend Nederland

SCHRIFTELIJKE OPINIE

Octrooiaanvrage 2017545

Indieningsdatum: 28 september 2016	Voorrangsdatum:
Classificatie van het onderwerp[1]: A01N 43/90; A23L 3/3463; A61K 31/7048; A61P 31/10; A61K 47/10; A01N25/02; A01P3/00	Aanvrager: Eucaryo Beheer B.V.

Deze schriftelijke opinie bevat een toelichting op de volgende onderdelen:

☒ Onderdeel I	Basis van de schriftelijke opinie
☐ Onderdeel II	Voorrang
☐ Onderdeel III	Vaststelling nieuwheid, inventiviteit en industriële toepasbaarheid niet mogelijk
☐ Onderdeel IV	De aanvraag heeft betrekking op meer dan één uitvinding
☒ Onderdeel V	Gemotiveerde verklaring ten aanzien van nieuwheid, inventiviteit en industriële toepasbaarheid
☐ Onderdeel VI	Andere geciteerde documenten
☐ Onderdeel VII	Overige gebreken
☒ Onderdeel VIII	Overige opmerkingen

De bevoegde ambtenaar:
L. Bechger

Octrooicentrum Nederland,
onderdeel van Rijksdienst voor Ondernemend Nederland

1 Gedefinieerd volgens International Patent Classification (IPC).

Schriftelijke Opinie

Octrooiaanvrage 2017545

Onderdeel I Basis van de schriftelijke opinie

Deze schriftelijke opinie is opgesteld op basis van de op 28 september 2016 ingediende conclusies.

Onderdeel V Gemotiveerde verklaring ten aanzien van nieuwheid, inventiviteit en industriële toepasbaarheid

1. Verklaring

Nieuwheid	Ja: Conclusie(s)	1-19
	Nee: Conclusie(s)	-
Inventiviteit	Ja: Conclusie(s)	11-15
	Nee: Conclusie(s)	1-10, 16-19
Industriële toepasbaarheid	Ja: Conclusie(s)	1-19
	Nee: Conclusie(s)	-

2. Literatuur en toelichting

In het rapport betreffende het onderzoek naar de stand van de techniek worden de volgende publicaties genoemd:

D1: CN 102742581 A (SHANXI PROVINCE INST OF MICROBIOLOGY) 24 oktober 2012
D2: WO 2004/105491 A (DSM IP ASSETS BV) 9 december 2004
D3: US 2012/0196003 A (SNABE TORBEN) 2 augustus 2012
D4: US 2015/0173365 A (VALENT BIOSCIENCES CORP) 25 juni 2015
D5: T. R. M. Tan et al., "Characterization of a Polyethylene Glycol-Amphotericin B Conjugate Loaded with FreeAMB for Improved Antifungal Efficacy", PLoS ONE, volume 11 (3), maart 2016, pagina's 1-18

Interpretatie van de conclusies

De term 'bioactief molecuul met ten minste een zure groep' in conclusie 1 is te breed omdat deze een oneindige groep van bioactieve moleculen kan inhouden en allerminst duidelijk is of de vermeende uitvinding betrekking heeft op deze oneindige groep van bioactieve moleculen. Derhalve is deze term geïnterpreteerd als zijnde de groep bioactieve moleculen gekozen uit de groep zoals opgesomd in conclusie 2, omdat ook in het licht van de beschrijving de uitvinding betrekking heeft op deze groep van bioactieve moleculen.

Nieuwheid en inventiviteit

Uit D1 is bekend een werkwijze voor het bereiden van een samenstelling omvattende een bioactief molecuul ("preparation of natamycin-hydroxypropyl-beta-cyclodextrin inclusion complex", zie EPODOC samenvatting) omvattende de stappen voor:
– het oplossen van een bioactief molecuul met ten minste een zure groep ("natamycine") met een base bij pH=>11 om een oplossing A te verkrijgen ("Step one: natamycin dissolved in alkaline

solution, pH value of 11.5-13", pagina 2, regel 2 van de machinevertaling),
– het neutraliseren van de oplossing A met een zuur om de samenstelling omvattende een bioactief molecuul ("natamycin") te verkrijgen met een pH tussen 6 en 9 ("After completion of the reaction, the acid solution in the reaction system was neutralized", pagina 2, regel 6 van de machinevertaling, of lees: "adding acid solution to neutralize into reaction system", WPI samenvatting),
– waarin hydroxypropyl-beta-cyclodextrine wordt toegevoegd voor de neutralisatiestap.
Voorbeeld van een geschikt (organisch) zuur is oxaalzuur, voorbeeld van een geschikte (anorganische) base is natriumhydroxide (pagina 2, regels 8-11).
De onderhavige aanvraag verschilt nu daarin van D1 dat er PEG in plaats van hydroxypropyl-beta-cyclodextrine wordt toegevoegd (omdat er een anorganische base wordt gebruikt). Conclusie 1 van de onderhavige aanvraag is derhalve nieuw.
Volgens de beschrijving van de onderhavige aanvraag is bij het gebruik van anorganische basen en/of zuren het toevoegen van PEG nodig om te assisteren in het vormen van een stabiel complex (zie pagina 7, regels 1-2) van het bioactieve molecuul in oplossing. Ook de rol van cyclodextrine in D1 is om een stabiel complex te vormen ("inclusion complex") van het bioactieve molecuul in oplossing. Nergens uit de aanvraag blijkt dat de samenstellingen met PEG stabieler zijn of effectiever zijn dan samenstellingen met andere complexvormende verbindingen zoals cyclodextrine. Nu het effect gelijk wordt gesteld moet het objectieve technische probleem waar de vakman zich voor ziet gesteld, worden geformuleerd als het verschaffen van een werkwijze voor het bereiden van een samenstelling omvattende een bioactief molecuul en een complexvormende verbinding (stabilisator) voor het behandelen van een agrarisch of voedselproduct of als geneesmiddel, als alternatief voor de werkwijze voor het bereiden van de samenstelling uit D1.
Aangezien het algemeen bekend is om voor (agriculturele) samenstellingen omvattende bioactieve moleculen PEG als stabilisator/oplosmiddel/emulgator te gebruiken (zie bv. D4 en D5) ligt het voor de gemiddelde vakman voor de hand samenstellingen omvattende een bioactief molecuul te bereiden volgens een werkwijze waarin PEG in plaats van cyclodextrine wordt toegevoegd.
Conclusie 1 is derhalve een niet-inventief alternatief, evenals de van deze conclusie afhankelijke conclusies 2, 3 en 9 (zie o.a. pagina 3, regels 1-2). De onafhankelijke conclusies 16, 17, 18 en 19 zijn ook bekend uit D1 (zie o.a. pagina 1, regels 21-24) en worden daarmee eveneens niet inventief bevonden.
De maatregelen van conclusies 4-8 en 10 zijn gebruikelijke maatregelen in het vakgebied en worden beschouwd als niet meer dan een van de verschillende mogelijkheden die de vakman afhankelijk van de omstandigheden zal selecteren zonder het uitoefenen van inventieve arbeid. Zo zal een hoeveelheid base in het bereik van 4-30 gew%, afgestemd zijn om een basische pH van => 11 te krijgen. Deze conclusies zijn derhalve evenmin inventief.

Ook ten opzichte van D2 als meest nabije stand van de techniek worden tenminste conclusies 1-3, 6, 16 en 17 niet inventief bevonden.
D2 openbaart een werkwijze voor het bereiden van een samenstelling omvattende een bioactief molecuul ("stable aqueous solution of natamycin fungicide") omvattende de stappen voor:
– het oplossen van een bioactief molecuul met ten minste een zure groep ("natamycine") met een

base bij pH=>11 om een oplossing A te verkrijgen ("an ethanolic aqueous solution having a pH of 12.8 was prepared"... "natamycin was added", voorbeeld 1, pagina 9),
– het neutraliseren van de oplossing A met een zuur om de samenstelling omvattende een bioactief molecuul ("natamycin") te verkrijgen met een pH tussen 6 en 9 ("a solution containing 57 ppm of natamycin and 0.2% of ethanol with a pH of 6.5 was obtained", voorbeeld 1).
Gegeven voorbeelden van geschikte (organische) zuren in D2 zijn citroenzuur en melkzuur, voorbeelden van geschikte basen natriumhydroxide (NaOH) en ammoniumhydroxide (NH_4OH).
De onderhavige aanvraag verschilt nu daarin van D2 dat een organische base wordt gebruikt. De in de onderhavige aanvraag gebruikte organische basen tetramethylammonium hydroxide of tetraethylammonium hydroxide zijn niet meer dan organische derivaten van ammonium hydroxide, waarvan algemeen bekend is dat zij evenzeer geschikt zijn voor toepassing in een werkwijze voor het bereiden van samenstellingen omvattende een bioactief molecuul, bijvoorbeeld voor gebruik in de behandeling van een agrarisch of voedselproduct. Het ligt voor een vakman dan ook voor de hand om, als alternatief voor de (anorganische) base ammoniumhydroxide zoals in D2, een (organische) base als tetramethylammonium hydroxide toe te passen.
Tenminste conclusies 1-3, 6, 16 en 17 zijn niet inventief.

De resterende conclusies 11, 12, 13, 14 en 15 hebben betrekking op een samenstelling omvattende het reactieproduct van de specifieke combinatie en in specifieke hoeveelheden van natamycine, choline hydroxide, een organisch zuur en water, o.a. bereid volgens de werkwijzestappen van conclusie 1. Volgens de beschrijving resulteert deze specifieke combinatie, en bereid volgens de stappen van werkwijzeconclusie 1, tot stabiele samenstellingen, met een goede bioactiviteit op schimmels zonder dat de plantgroei (zaadontkieming) nadelig wordt beïnvloed, vergeleken met andere (antischimmel) samenstellingen omvattende een bioactief molecuul (zie pagina 22, regels 27-31 van de beschrijving).
Deze specifieke kenmerken zijn niet bekend uit de in het rapport genoemde documenten en worden daarin evenmin gesuggereerd. De conclusies 11-15 voldoen daarom aan de eisen van nieuwheid en inventiviteit.

D3 is inventiviteitsbezwarend voor tenminste conclusie 1 volgens eenzelfde redenering als bij D1.

Onderdeel VIII Overige opmerkingen

De volgende opmerkingen met betrekking tot de duidelijkheid van de conclusies, beschrijving, en figuren, of met betrekking tot de vraag of de conclusies nawerkbaar zijn, worden gemaakt:

Zinsneden achter woorden als "bij voorkeur", "met meer voorkeur" of "met de meeste voorkeur" zijn niet beperkend en kunnen daarom worden weggedacht.

Conclusie 11 betreft een samenstelling omvattende een reactieproduct van o.a. natamycine, een organische base en een organisch zuur. Deze samenstelling is niet perse bereid volgens de werkwijze stappen van conclusie 1 en kan derhalve bereid zijn volgens meerdere routes,

bijvoorbeeld een waarbij het bioactieve molecuul wordt opgelost bij een zure pH (i.p.v. een basische), gevolgd door het neutraliseren van de oplossing. Omdat voor deze route geen enkele onderbouwing in de beschrijving is te vinden (sterker nog: een essentiële maatregel van de vermeende uitvinding lijkt te zijn het deprotonoreren van de zure groep in het bioactieve molecuul met een base (zie pagina 5, regels 13-15)), is deze conclusie 11 onnodig breed.