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NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

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(57) Abstract

The invention relates to a process for concentrating epothilones in culture media, a process for the production of epothilones, a process for separating epothilones A and B and a strain obtained by mutagenesis for the production of epothilones, as well as aspects related thereto. Crystal forms of epothilone B are also described.

FERMENTATIVE PREPARATION PROCESS FOR CYTOSTATICS AND CRYSTAL FORMS THEREOF

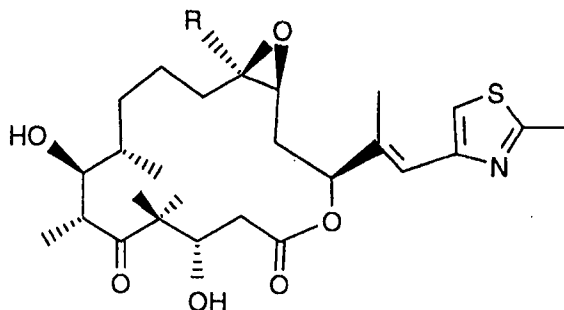
The invention relates to a new biotechnological preparation process that can be used on an industrial scale for the production of epothilones, especially a process for concentrating these compounds in the culture medium, as well as a new strain for the fermentative preparation of these compounds. The invention also relates to new crystal forms of epothilones, especially epothilone B, obtainable by the new processes, their usage in the production of pharmaceutical preparations, new pharmaceutical preparations comprising these new crystal forms and/or the use of these compounds in the treatment of proliferative diseases such as tumours, or in the production of pharmaceutical formulations which are suitable for this treatment.

Background to the invention:

Of the existing cytotoxic active ingredients for treating tumours, Taxol® (Paclitaxel; Bristol-Myers Squibb), a microtubuli-stabilising agent, plays an important role and has remarkable commercial success. However, Taxol has a number of disadvantages. In particular, its very poor solubility in water is a problem. It therefore became necessary to administer Taxol® in a formulation with Cremophor EL® (polyoxyethylated castor oil; BASF, Ludwigshafen, Germany). Cremophor EL® has severe side effects; for example it causes allergies which in at least one case have led even to the death of a patient.

Although the Taxan class of microtubuli-stabilising anti-cancer agents has been commended as "perhaps the most important addition to the pharmaceutical armoury against cancer in the last decade" (see Rowinsky E.K., Ann. rev. Med. 48, 353-374 (1997)), and despite the commercial success of Taxol®, these compounds still do not appear to represent a really great breakthrough in the chemotherapy of cancer. Treatment with Taxol® is linked with a series of significant side effects, and a few primary classes of compact tumours, namely colon and prostate tumours, respond to this compound only to a small extent (see Rowinsky E.K., *inter alia*). In addition, the efficacy of Taxol can be impaired and even completely neutralised by acquired resistance mechanisms, especially those based on the overexpression of phosphoproteins, which act as efflux pumps for active ingredients, such as "Multidrug Resistance" due to overexpression of the multidrug transport glycoprotein "P-glycoprotein".

Epothilones A and B represent a new class of microtubuli-stabilising cytotoxic active ingredients (see Gerth, K. et al., J. Antibiot. 49, 560-3 (1966)) of the formulae:



wherein R signifies hydrogen (epothilone A) or methyl (epothilone B).

These compounds have the following advantages over Taxol®:

- a) They have better water-solubility and are thus more easily accessible for formulations.
- b) It has been reported that, in cell culture experiments, they are also active against the proliferation of cells, which, owing to the activity of the P-glycoprotein efflux pump making them "multidrug resistant", show resistance to treatment with other chemotherapy agents including Taxol® (see Bolag, D. M., et al., "Epothilones, a new class of microtubule-stabilizing agents with a Taxol-like mechanism of action", Cancer Research 55, 2325-33 (1995)). And
- c) it could be shown that they are still very effective *in vitro* against a Taxol®-resistant ovarian carcinoma cell line with modified β -tubulin (see Kowalski, R. J., et al., J. Biol. Chem. 272(4), 2534-2541 (1997)).

Pharmaceutical application of the epothilones, for example for tumour treatment, is possible in an analogous manner to that described for Taxol, see for example US 5.641.803; US 5.496.804; US 5.565.478).

In order to be able to use the epothilones on a larger scale for pharmaceutical purposes, however, it is necessary to obtain appropriate amounts of these compounds.

- separation of the epothilones by reversed-phase chromatography after being taken up in a suitable solvent, and elution with a mixture of nitrile/water (compulsory), preferably characterised in that the chromatography is carried out on a column of material, especially a RP-18 material, which is charged with hydrocarbon chains, such as hydrocarbon chains containing 18 carbon atoms, and an eluant comprising a nitrile, especially a lower alkyl-nitrile, in particular acetonitrile, is used, in particular a mixture of nitrile/water is used, especially a mixture of acetonitrile/water, preferably in a ratio of nitrile to water of about 1:99 to 99:1, primarily between 1:9 and 9:1, e.g. between 2:8 and 7:3, e.g. 3:7 or 4:6.
- single or multiple extraction of the residue (especially after evaporation) in a two-phase system consisting of water and a solvent immiscible with water, preferably an ester, in particular a lower alkyl lower alkanoate, such as ethyl acetate or isopropyl acetate;
- adsorption chromatography, in particular by adding to a column of silica gel and eluting with an appropriate solvent or solvent mixture, especially a mixture of ester/hydrocarbon, for example lower alkyl alkanoate / C₄-C₁₀-alkane, especially ethyl or isopropyl acetate / n-hexane, in which the ratio between the ester and hydrocarbon is preferably in the range 99:1 to 1:99, preferably 10:1 to 1:10, for example 4:1;
- dissolving the residue, which may be obtained after concentration, in an appropriate solvent such as an alcohol, e.g. methanol;
- mixing with activated carbon and removal thereof;
- recrystallisation, e.g. from appropriate solvents or solvent mixtures, for example consisting of esters, ester/hydrocarbon mixtures or alcohols, especially ethyl or isopropyl acetate : toluene 1:10 to 10:1, preferably 2:3 (epothilone A) or methanol or ethyl acetate (epothilone B);

whereby between each step being employed, the resulting solutions or suspensions are concentrated if necessary, and/or liquid and solid components are separated from one another, in particular by filtering or centrifuging solutions/suspensions. The more precise definitions mentioned below can be preferably used in the above individual steps.

Working up and purification are preferably carried out as follows: After harvest, a culture is separated into the liquid phase (centrifugate) and solid phase (cells) by means of centrifugation (tubular centrifuge or separator). The main part of the epothilones are found in the centrifugate, which is then directly mixed with a polystyrene resin, such as Amberlite XAD-16 [Rohm & Haas Germany GmbH, Frankfurt] or Diaion HP-20 [Resindion S.R.L.,

The invention relates in particular to the individual process steps named in the examples or any combination thereof, the culture media named therein, crystal forms and the strain described therein.

The invention also relates to new crystal forms of epothilone B, especially a crystal form of epothilone B described as modification B and in particular described as modification A.

The crystal forms can be distinguished in particular by their X-ray diagrams. X-ray diagrams taken with a diffractometer and using Cu-K α_1 -radiation are preferably used to characterise solids of organic compounds. X-ray diffraction diagrams are used particularly successfully to determine the crystal modification of a substance. To characterise the existing crystal modification A and crystal modification B of epothilone B, the measurements are made at an angle range (2θ) of 2° and 35° with samples of substance that are kept at room temperature.

The X-ray diffraction diagram thus determined (reflection lines and intensities of the most important lines) from crystal modification A (modification A) of epothilone B is characterised by the following table.

2θ	Intensity
7.7	very strong
10.6	weak
13.6	average
14.4	average
15.5	average
16.4	weak
16.8	weak
17.1	weak
17.3	weak
17.7	weak
18.5	weak
20.7	strong
21.2	strong

21.9	weak
22.4	weak
23.3	strong
25.9	average
31.2	weak
32.0	average

The invention also relates in particular to a new crystal form of epothilones B, which is characterised by a melting point of more than 120 °C, especially between 120 °C and 128°C, in particular 124-125 °C. Surprisingly, this value is considerably higher than the values previously described in literature. The invention relates especially to a crystal form of epothilone B, which is characterised by the X-ray diffraction diagram of the crystal form A and a melting point of above 120 °C, especially between 120 °C and 128 °C, for example between 124 °C and 125 °C.

The X-ray diffraction diagram thus determined (reflection lines and intensities of the most important lines) from crystal modification B (modification B) of epothilone B is characterised by the following table.

2θ	Intensity
6.9	very strong
8.0	weak
8.3	average
10.8	strong
11.5	average
12.4	weak
13.1	strong
15.5	weak
16.2	weak
16.7	average
18.1	average
18.6	average
20.4	weak

20.9	strong
21.3	weak
21.5	very weak
22.5	average
24.2	weak
25.1	average

The new crystal forms are especially stable, in particular crystal form A is to be regarded as the one which is more thermodynamically stable, and they are therefore suitable as active ingredients for solid forms of administration, for storing in solid form or as intermediates (with particularly good storability) in the preparation of solid or liquid forms of administration.

The invention also relates to the use of the new crystal forms, especially crystal form B, but primarily crystal form A (all referred to hereinafter as active ingredient) in the production of pharmaceutical preparations, new pharmaceutical preparations which contain these new crystal forms, and/or their use in the treatment of proliferative diseases, such as tumours. In the following, where pharmaceutical preparations or compositions which comprise or contain the active ingredient are mentioned, in the case of liquid compositions or compositions which no longer contain the crystal form as such, this is always understood to mean also the pharmaceutical preparations obtainable using the crystal forms (for example infusion solutions obtained using crystal forms A or B of epothilone B), even if they no longer contain the respective crystal form (for example because they exist in solution).

The invention also relates especially to the use of a new crystal form of epothilone B, especially the crystal form B or in particular crystal form A, in the production of pharmaceutical preparations, characterised by mixing a new crystal form of epothilone B with one or more carriers.

The invention also relates to a method of treating warm-blooded animals suffering from a proliferative disease, characterised by administering a dose of epothilone B which is effective for treating said disease in one or the new crystal forms to a warm-blooded animal requiring such treatment, also including in particular the treatment with those preparations that are produced using one of the new crystal forms; and/or the use of a new crystal form of epothilone B in such a treatment.

A large range of container sizes may be considered. When selecting the size of the container, the factors to be taken into consideration are especially the solubility of epothilones in an aqueous medium, easy handling, and if appropriate, storage of the container. It is preferable to use containers which hold between ca. 200 and 1000 ml of infusion solution.

Owing to their good formulating properties, the new crystal forms of epothilone B according to the invention are especially suitable for the simple and reproducible production of the said infusion solutions. However, the new crystal forms are especially suitable for the production of pharmaceutical formulations which contain the active ingredient in solid form, for example oral formulations.

Pharmaceutical formulations for oral application may be obtained by combining the active ingredient with solid carriers, if desired by granulating the resultant mixture, and further processing the mixture, if desired or if necessary, after adding suitable adjuvants, into tablets, dragée cores or capsules. It is also possible to embed them in plastic substrates which enable the active ingredient to be diffused or released in measured quantities.

Suitable pharmaceutically employable carriers are especially fillers, such as lactose, saccharose, mannitol or sorbitol, cellulose preparations, and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, and binders, such as starches, for example maize, wheat, rice or potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropyl methyl cellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone, and/or, if desired, disintegrators, such as the above-mentioned starches, crosslinked vinylpyrrolidones, agar, alginic acid or a salt thereof, such as sodium alginate. Adjuvants are in particular flow-improving agents and lubricants, e.g. silicates, talcum, stearic acid or salts thereof, such as magnesium or calcium stearate and/or polyethylene glycol. Dragée cores are provided, if desired, with appropriate gastric-juice-resistant coatings, using *inter alia* concentrated sugar solutions, gum arabic, talcum, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, or coating solutions in suitable organic solvents, or in order to produce gastric-juice-resistant coatings, solutions of appropriate cellulose preparations, such as ethyl cellulose phthalate or hydroxypropyl methyl cellulose phthalate. Capsules are dry capsules consisting of gelatin or pectin, and if

required, a softener such as glycerol or sorbitol. The dry capsules may contain the active ingredient in the form of granules, for example with fillers, such as lactose, binders, such as starches, and/or lubricants, such as talc or magnesium stearate, and where appropriate stabilisers. In soft capsules, the active ingredient may be present in dissolved or preferably suspended form, whereby oily adjuvants such as fat oils, paraffin oil or liquid propylene glycols are added; stabilisers and/or antibacterial additives may also be added. Dyes or pigments can be added to the tablets or dragée coatings, for example to improve identification or to distinguish different dosages of active ingredient.

The usage in the treatment of a proliferative disease with one of the crystal forms B and in particular A preferably takes place whereby the crystal form (preferably as for the usage in the preparation of an infusion solution, as described above) is administered to a warm-blooded animal, especially a human, in a dose which can be determined at between 20 and 133%, preferably between 25 and 100%, of the Maximum Tolerated Dose (MTD) by standard methods, for example using a modified Fibonacchi series, in which the increases in dosages for successive amounts are 100%, 67%, 50% and 40% followed by 33% for all subsequent doses; and, if necessary, one or more further doses administered in the dosage range given above for the first dose, each dose after a period of time which allows sufficient recovery of the individual being treated after the preceding administration, in particular one week or more after the first administration, preferably 2 to 10 weeks, especially 3 to 6 weeks after each preceding administration. In general, this treatment scheme, in which a high dosage is administered once, twice or several times with sufficiently long intervals between the individual administrations for recovery to take place, is preferred over a more frequent treatment with lower doses, since hospitalisation is less frequent and for a shorter period and an improved anti-tumour effect can be expected. The dosage of epothilone B for humans is preferably between 0.1 and 50 mg/m², preferably between 0.2 and 10 mg/m².

The following Examples serve to illustrate the invention without limiting its scope.

Caution: When handling epothilones, appropriate protective measures must be taken, where necessary, in view of their high toxicity.

The 750 litre fermenter used in the following is a refined steel fermenter from the company Alpha AG, Nidau, Switzerland.