

PATENT SPECIFICATION

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(54) A NEW POLYENIC ANTIBIOTIC /

(71) We, SPA SOCIETA PRODOTTI ANTIBIOTICI, S.p.A., of 8 Via Biella, Milan, Italy, a corporation organised under the laws of Italy, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention is concerned with a new polyenic antibiotic agent, the structural properties of which differ from other antibiotics of this class and which has a very high anti-fungal activity, especially against *Candida albicans* and also has an anti-*Trichomonas* activity.

The new antibiotic agent, which has our internal code number SPA—8—160, is hereinafter referred to by the USAN name, partricin methyl ester.

The present invention is also concerned with a chemical method of preparation of partricin methyl ester from a new antibiotic substance which we have called partricin and which is the subject of our British Patent Application No. 52270/70 (Serial No. 1,357,538).

Furthermore, the present invention is concerned with a method of isolation and purification of partricin methyl ester and its therapeutic utilisation, it having the above-mentioned activities but a much lower degree of toxicity than known polyenic compounds normally used therapeutically.

The polyenic antibiotics are a large class of compounds which are widely produced as metabolites by numerous micro-organisms, such as *Actinomycetes*.

Due to their highly unsaturated structure, with double conjugated bonds, as demonstrated by their characteristic ultra-violet spectrum, they can be classified as tetra-, penta-, hexa- and heptaenes.

These compounds are often extremely interesting for their high degree of activity toward a variety of fungi and yeasts and, therefore, not withstanding generally having only a slight or no activity against bacteria, they are widely used therapeutically. The fact

that the sensitive micro-organisms do not easily form strains which are resistant to these polyenic antibiotics and that, on the other hand, there are no satisfactory agents for the cure of infections caused by fungi and yeasts, either in the sulphonamide group of compounds or among the penicillins and tetracyclines, has provided a further interest in these products.

However, the widespread use of these polyenic antibiotics has been hindered by their toxicity which, by parenteral administration, is manifested by headache, nausea, vomiting and by contemporaneous increase of blood levels of urea nitrogen or of non-proteinic nitrogen and by the appearance of renal damage and haemolytic anaemia.

The new polyenic antibiotic agent partricin methyl ester provided by the present invention has a powerful anti-fungal activity, especially against *Candida albicans*, together with an activity against *Trichomonas* and, at the same time, has a low degree of toxicity.

The new antibiotic can be prepared from the new polyenic substance partricin (which we also refer to by our code number SPA—S—132) by treating a solution of partricin in an appropriate organic solvent with only a slight excess of diazomethane, in order to prevent the possible formation of products with major degrees of reaction, then leaving the reaction mixture to stand for a few hours or at least until the evolution of nitrogen has ceased, this evolution being an indication that reaction is taking place, and then precipitating the reaction product by the addition of an appropriate solvent.

It is particularly advantageous to carry out the reaction in the presence of basic substances, preferably ammonia or organic bases, such as triethylamine, in catalytic to stoichiometric quantities referred to the partricin. Although the mechanism of action is not completely known, the presence of these substances seems to be able to prevent or limit the formation of certain secondary reaction products otherwise detectable by thin layer chromatography, thus yielding a pro-

duct with high purity and microbiological activity.

The method and the reagent used lead us, according to the known procedures of synthesis reported in literature, to attribute to the material obtained the structure of a methyl ester of the antibiotic partricin; the physico-chemical and analytical data confirm this. The conditions of reaction, if suitably varied, give the above-mentioned product with unchanged characteristics of purity and yield, which is generally almost quantitative.

The solvents preferably employed for the reaction are dimethyl sulphoxide, dimethyl formamide, dimethyl acetamide, formamide, ethyl cellosolve, pyridine and numerous other organic solvents which have the ability to dissolve partricin.

The diazomethane used, which is a poisonous yellow gas and potentially explosive, is generally employed in ethereal solution but there can also be used the above-mentioned solvents, as well as others, such as alcohols and benzene; the presence of an excess of diazomethane is controlled by methods known for similar procedures and reported in literature. The reaction is usually completed in about 1-15 hours and is preferably carried out at a temperature of 0-40°C., generally at ambient temperature.

The most appropriate solvents for the precipitation of the reaction product are diethyl ether, benzene and water added in excess, as well as numerous others which have a low solvent capacity for the product. The product thus obtained, i.e. partricin methyl ester, can then be purified, if necessary, by the use of appropriate mixtures of solvents. Alternatively, for analytical or preparative purposes, the purification can be carried out by column chromatography on silica gel, employing different mixtures of solvents as eluents, for example, a mixture of pyridine and petroleum ether in the ratio 8:2 by volume.

With regard to the characterisation of the new compound, the polyenic structure of partricin remains unchanged, as demonstrated by the characteristic ultra-violet spectrum, so that it can be classified as a heptaene; it differs from partricin by not containing free carboxy groups and by having a "methyl ester" radical, as can be seen from the infra-red spectrum (see Fig. 1 of the accompanying drawings) (recorded in suspension in "Nujol"). ("Nujol" is a Registered Trade Mark), which has an absorbance band at 1715 cm⁻¹ due to the stretching vibration of C=O of the ester, this being absent in the starting substance, and in the N.M.R. spectrum recorded in solution of dimethyl sulphoxide where at 3.25 ppm the characteristic peak of a methyl radical in an "ester" group is present.

Fig. 2 of the accompanying drawings shews

the ultra-violet absorption spectrum of partricin methyl ester.

Similar to what has been reported in literature for other polyenic antibiotics, it should possess a lactonic macrocyclic nucleus, an amino group and some hydroxyl groups.

It is also characterised by the fact that it only contains the elements carbon, hydrogen, nitrogen and oxygen in the following approximate amounts by weight:

carbon:	63.3%
hydrogen:	7.8%
nitrogen:	3.2%
oxygen:	24.8%

determined on a sample free of moisture and ash; by the fact that, in ethanol solution at concentrations of about 0.01 mg/ml, it exhibits the following absorbance peaks under ultra-violet light (spectrum attached; Fig. 2):

maximum	minimum
401 mμ	391 mμ
378 mμ	367 mμ
359 mμ	348 mμ
340 mμ	

and finally, by the fact that, recording the infra-red spectrum in suspension in Nujol (spectrum attached; Fig. 1), it exhibits infra-red absorption maxima at the following frequencies: 3360, 1715, 1640, 1595, 1175, 1060, 1000, 848 and 763 cm⁻¹.

A further means for characterising the new substance and for distinguishing it from similar polyenic compounds, including the already mentioned partricin, is provided by thin layer chromatography, carrying out the chromatography on silica gel strips and using, as eluent, a mixture of butanol-ethanol-acetone-25% ammonium hydroxide (2:5:1:3), it has an R_f value of about 0.8. The spots are easily identified by exposure to ultra-violet light.

Particularly interesting is the behaviour of the product with regard to light; when measuring the ultra-violet absorption of a diluted solution left exposed for a long time to light, the whole spectrum is observed to shift to wavelengths about 4 mμ higher, thus modifying the intensity of the absorption peaks. This light-induced modification of the UV spectrum indicates that partricin methyl ester is not originally found in the "all-trans" form, but there is probably one or more "cis" double bonds which, as in known heptaenes, easily undergo a light stereoisomerisation towards the "trans" form.

The antibiotic partricin methyl ester is a yellow to dark yellow, crystalline solid which is very sparingly soluble in water and aqueous alkali (absence of acid functions) and in ether, petroleum ether, benzene and other non-polar solvents, moderately soluble in

other solvents, such as acetone and alcohols, while it is very soluble in dimethyl sulphoxide, pyridine, 2-ethoxyethanol, formamide, dimethyl formamide and dimethyl acetamide.

5 With some of these solvents, such as methyl-formamide and pyridine for example, the substance seems to form molecular complexes which give it a high solubility in many aqueous and organic solvent systems.

10 With regard to microbiological activity, partricin methyl ester has little or no action against Gram-positive and Gram-negative bacteria but it is very active against numerous pathogenic fungi, in particular against

15 *Candida albicans*: the minimum inhibiting concentrations, determined on 10 strains of *Candida*, averaged 0.3—0.6 mcg/ml. The substance has shown to be nearly as active against *Trichomonas vaginalis*, where the minimum inhibiting concentration is about 0.6 mcg/ml.

In contradistinction to similar polyenic antibiotics, the toxicity of the new antibiotic agent is very low; the LD₅₀ (minimum dose necessary for killing half the animals being tested) is over 2000 mg/kg. in mice after oral administration.

Similar toxicity results are given by the new product in other animal species, for example rats: these doses are exceptionally high when compared with all the previously known polyenic antibiotics.

Local application of partricin methyl ester to normal and scarified rabbit skin, as well as to the conjunctival mucosa and to the corneal epithelium, show it to be perfectly tolerated.

The minimum haemolytic concentrations on rabbit red cells are about 40—80 mcg/ml after 1 hour, which is among the highest values reported for substances with polyenic structures and especially with heptaenic structures.

The numerous microbiological and pharmacological characteristics above described, make the antibiotic partricin methyl ester extremely interesting therapeutically for combatting many diseases caused by fungal infections in humans, animals and plants. Particularly interesting is the topical application thereof in the form of a tincture or ointment for dermatological use and of suppositories or inserts for vaginal use in fungal infections or in infections due to *Trichomonas*; in the case of plants, the substance can be sprayed on or mixed with fertilisers.

The observation of a low absorption of the new antibiotic through the intestinal wall and its high activity on *Candida albicans* make it particularly useful for combatting intestinal infections by oral administrations, which infections are especially frequent in humans after long-term antibiotic treatments with, for example, the tetracyclines and chloramphenicol, with antibacterial activity.

A particularly advantageous form of partricin methyl ester for clinical use is in association with one or more of the above-mentioned wide-spectrum antibiotics to prevent the appearance of fungal infections in the intestinal tract after the usual oral administrations.

In addition, it has been observed that the use of certain molecular complexes of partricin methyl ester, for example with dimethyl-acetamide, can improve the absorption of the compound, probably due to the increased capacity of solubilisation and diffusion. Therefore, the compound can also be of great interest in combatting numerous general infections by oral or parenteral administration.

For all uses, the new substance is admixed with an appropriate quantity of a pharmaceutically acceptable, solid or liquid carrier or diluent. If desired, at least one known wide-spectrum antibiotic can also be admixed therewith. Examples of pharmaceutical forms of administration include tablets, effervescent tablets, powders, granules, capsules and suspensions in oil or in other forms particularly adapted for oral administration.

The new antibiotic agent can also be administered by the parenteral route, i.e. by injection, using liquid, sterile vehicles. The formulations can include sterile solutions in, for example, dimethyl acetamide or ethyl cellosolve, or suspensions in water or injectable oils.

The following Examples are given for the purpose of illustrating the present invention:

Example 1

35 g. of partricin are dissolved in 280 ml. dimethyl sulphoxide and 250 ml. of 2% ethereal solution of diazomethane, obtained according to the standard methods reported in literature, are added dropwise. The addition is made while stirring slowly and at ambient temperature, taking the precautions necessitated by the poisonous and dangerous nature of diazomethane. A vigorous reaction immediately takes place, with the evolution of nitrogen, which gradually slows down and ceases when the quantity of diazomethane added is in stoichiometric excess.

The mixture thus obtained is left to stand overnight away from light, whereafter a sufficient quantity of ether is added to precipitate the reaction product. This material is collected by filtration and washed first by covering with ether and then digesting it with ether. The substance thus obtained, partricin methyl ester, is dried to constant weight under vacuum and at ambient temperature, to give an almost quantitative yield. Small quantities of residual solvent which may be present can be easily removed by washing with water.

If necessary, the substance can be purified by treating it with suitable mixtures of sol-

vents, such as dimethyl sulphoxide-ether, dimethyl sulphoxide-acetone-ether, dimethyl acetamide-water or the like.

Example 2

10 g. of partricin are dissolved in 100 ml. of dimethylacetamide and 70 ml. of 2% ethereal solution of diazomethane are added dropwise at the temperature of 26°C. The reaction mixture is kept for 6 hours, with slow stirring, at the same temperature and then a sufficient quantity of ether is added to precipitate the reaction product. The precipitate is filtered, washed thoroughly with ether and dried under vacuum to give an almost quantitative yield of partricin methyl ester.

Example 3

50 g. of partricin are dissolved in 500 ml. of dimethylsulphoxide and then 3 ml. of concentrated ammonium hydroxide are added, with stirring, i.e. enough to bring the pH of the solution to 9—9.2, this being measured after dilution of a sample to 1% by adding a mixture of dimethylsulphoxide-water (1:1).

350 ml. of an approximately 2% ethereal solution of diazomethane are then added to the reaction mixture, which is slowly stirred for 6 hours at ambient temperature, after which time, excess ether is added to precipitate the reaction product, which is isolated from the solvent mixture and treated with 250 ml. of acetone, with stirring, and then with excess ether. The crystalline product in suspension thus obtained is filtered and washed with ether to give the desired partricin methyl ester.

Example 4

To a solution of 50 g. of partricin in 500 ml. of dimethylsulphoxide, is added, with stirring, an almost stoichiometric quantity of concentrated ammonium hydroxide, i.e. enough to bring the pH of the solution to about 11, measuring this value after dilution of a sample to 1% by adding a mixture of dimethylsulphoxide-water (1:1). 350 ml. of an approximately 2% ethereal solution of diazomethane are then rapidly added dropwise, whereafter the reaction mixture is further worked up as described in Example 3 to give partricin methyl ester.

Example 5

A solution of 100 g. of partricin in 1000 ml. of dimethylsulphoxide is prepared and 6 ml. of concentrated ammonium hydroxide and 700 ml. of 2% ethereal solution of diazomethane are added, with stirring, at ambient temperature. The reaction mixture is kept at ambient temperature, with slow stirring, for 8 hours and then most of the ether and excess diazomethane are distilled off under reduced pressure, whereafter excess water is added to precipitate the reaction product. The precipitate is separated by filtration, washed

thoroughly with water and dried under vacuum. The product obtained is slurried with 95% aqueous acetone to extract any impurities and fractions of more toxic matter and then the washed solid is filtered and dried to give high yields of partricin methyl ester in the form of a dark yellow crystalline solid.

Example 6

20 g. of partricin are dissolved in 160 ml. of dimethylsulphoxide and then 7 ml. of triethylamine and 140 ml. of 2.3% ethereal solution of diazomethane are added. The mixture is stirred for 6 hours at ambient temperature and then worked up as described in Example 3 to give the desired partricin methyl ester.

Example 7

To a solution of 20 g. of partricin in 250 ml. of pyridine is rapidly added dropwise excess 2.5% ethereal solution of diazomethane. The mixture is left to stand overnight at about 25°C. and then the reaction product is isolated and purified in the manner described in Example 1.

Example 8

12 g. of partricin methyl ester, obtained by one of the procedures described in the above Examples, are dissolved in 60 ml. of pyridine and the solution obtained is percolated through a chromatographic column consisting of 400 g. of silica gel (0.05—0.20 mm.) in a mixture of pyridine-petroleum ether (8:2).

The same solvent mixture is used in the subsequent elution. During the chromatography, 100 ml. fractions of eluate are collected and pooled after having been individually tested for purity by means of thin layer chromatography (Kieselgel F₂₅₄ plate; solvent system: butanol - ethanol - acetone - concentrated ammonium hydroxide, 2:5:1:3). To the solution containing the pure product is added excess ether to precipitate the solute. The precipitate is collected by filtration and washed thoroughly with ether to give almost pure partricin methyl ester, in the form of a microcrystalline, yellow powder. For analytical purposes, it can be further purified, if desired, with mixtures of solvents, such as dimethylsulphoxide ether, dimethylsulphoxide - water or dimethylacetamide - water.

The following Examples illustrate pharmaceutical compositions according to the present invention:—

Example 9

Vaginal suppositories

Each vaginal suppository contains:

partricin methyl ester	0.025 g.
dimethyl acetamide	0.065 g.
polyethylene glycol 1540 mono-stearate	1.200 g.
cetyl alcohol	0.610 g.

Example 10

Vaginal suppositories

Each vaginal suppository contains:

5	partricin methyl ester	0.025 g.
	dimethyl sulphoxide	0.075 g.
	polyethylene glycol 1540 mono-stearate	1.200 g.
	cetyl alcohol	0.600 g.

Example 11

Vaginal suppositories

Each vaginal suppository contains:

10	partricin methyl ester	0.025 g.
	dimethyl acetamide	0.065 g.
15	polyethylene glycol 1540 mono-stearate	1.200 g.
	polyethylene glycol 4000	0.610 g.

Example 12

Vaginal suppositories

Each vaginal suppository contains:

20	partricin methyl ester	0.025 g.
	fatty acid triglycerides	1.775 g.

Example 13

Ointment

Composition:

25	partricin methyl ester	3 g.
	dimethyl acetamide	5 g.
	lanolin	20 g.
	cetyl alcohol	32 g.
	oleyl alcohol	7.5 g.
30	sorbitan trioleate	7.5 g.
	polyethylene glycol 1540 mono-stearate	25 g.

Example 14

Ointment

Composition:

35	partricin methyl ester	3 g.
	dimethyl sulphoxide	6 g.
	lanolin	20 g.
	cetyl alcohol	31 g.
40	sorbitan trioleate	10 g.
	polyethylene glycol 1540 mono-stearate	30 g.

Example 15

Ointment

Composition:

45	partricin methyl ester	3 g.
	dimethyl sulphoxide	6 g.
	lanolin	20 g.
	cetyl alcohol	31 g.
50	oleyl alcohol	10 g.
	polyethylene glycol 1540 mono-stearate	30 g.

Example 16

Ointment

Composition:

partricin methyl ester	3 g.
petroleum jelly	70 g.
white oil	27 g.

WHAT WE CLAIM IS:—

1. Partricin methyl ester, which has ultra-violet absorption maxima at 401, 378, 359 and 340 $m\mu$ and ultra-violet absorption minima at 391, 367 and 348 $m\mu$; has infra-red absorption maxima at 3360, 1715, 1640, 1595, 1175, 1060, 1000, 848 and 763 cm^{-1} , has an Rf value of about 0.8 on silica gel using butanol - ethanol - acetone - 25% ammonium hydroxide (1:5:1:3) and is a yellow to dark yellow crystalline solid which is very sparingly soluble in water and aqueous alkali and in ether, petroleum ether and benzene and is very soluble in dimethyl sulphoxide, dimethyl formamide, dimethyl acetamide, pyridine and 2 - ethoxyethanol. 60
2. Process for the preparation of partricin methyl ester, wherein partricin is reacted in an organic solvent with a slight excess of diazomethane. 65
3. Process according to claim 2, wherein the reaction is carried out at a temperature of 0—40°C. 70
4. Process according to claim 3, wherein the reaction is carried out at ambient temperature. 75
5. Process according to any of claims 2—4, wherein the partricin methyl ester formed is precipitated out from the reaction mixture by the addition of a solvent which has a low solvent capacity for said methyl ester. 80
6. Process according to claim 1 for the preparation of partricin methyl ester, substantially as hereinbefore described and exemplified. 85
7. Partricin methyl ester, whenever prepared by the process according to any of claims 2—6. 90
8. Pharmaceutical compositions, comprising partricin methyl ester in admixture with a solid or liquid pharmaceutical diluent or carrier. 95
9. Pharmaceutical compositions according to claim 8, wherein at least one known wide-spectrum antibiotic is also present. 100

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Agents for the Applicants.

Example 10			Example 16		
Vaginal suppositories			Ointment		
Each vaginal suppository contains:			Composition:		
5	partricin methyl ester	0.025 g.	partricin methyl ester	3 g.	55
	dimethyl sulphoxide	0.075 g.	petroleum jelly	70 g.	
	polyethylene glycol 1540 mono-stearate	1.200 g.	white oil	27 g.	
	cetyl alcohol	0.600 g.			
Example 11			WHAT WE CLAIM IS:—		
Vaginal suppositories			1. Partricin methyl ester, which has ultra-violet absorption maxima at 401, 378, 359 and 340 m μ and ultra-violet absorption minima at 391, 367 and 348 m μ ; has infra-red absorption maxima at 3360, 1715, 1640, 1595, 1175, 1060, 1000, 848 and 763 cm ⁻¹ , has an Rf value of about 0.8 on silica gel using butanol - ethanol - acetone - 25% ammonium hydroxide (1:5:1:3) and is a yellow to dark yellow crystalline solid which is very sparingly soluble in water and aqueous alkali and in ether, petroleum ether and benzene and is very soluble in dimethyl sulphoxide, dimethyl formamide, dimethyl acetamide, pyridine and 2 - ethoxyethanol.		
10	Each vaginal suppository contains:		2. Process for the preparation of partricin methyl ester, wherein partricin is reacted in an organic solvent with a slight excess of diazomethane.		
	partricin methyl ester	0.025 g.	3. Process according to claim 2, wherein the reaction is carried out at a temperature of 0—40°C.		
	dimethyl acetamide	0.065 g.	4. Process according to claim 3, wherein the reaction is carried out at ambient temperature.		
15	polyethylene glycol 1540 mono-stearate	1.200 g.	5. Process according to any of claims 2—4, wherein the partricin methyl ester formed is precipitated out from the reaction mixture by the addition of a solvent which has a low solvent capacity for said methyl ester.		
	polyethylene glycol 4000	0.610 g.	6. Process according to claim 1 for the preparation of partricin methyl ester, substantially as hereinbefore described and exemplified.		
Example 12			7. Partricin methyl ester, whenever prepared by the process according to any of claims 2—6.		
Vaginal suppositories			8. Pharmaceutical compositions, comprising partricin methyl ester in admixture with a solid or liquid pharmaceutical diluent or carrier.		
20	Each vaginal suppository contains:		9. Pharmaceutical compositions according to claim 8, wherein at least one known wide-spectrum antibiotic is also present.		
	partricin methyl ester	0.025 g.			
	fatty acid triglycerides	1.775 g.			
Example 13			VENNER, SHIPLEY & CO.,		
Ointment			Chartered Patent Agents,		
Composition:			Rugby Chambers,		
25	partricin methyl ester	3 g.	2, Rugby Street,		
	dimethyl acetamide	5 g.	London, WC1N 3QU.		
	lanolin	20 g.	Agents for the Applicants.		
	cetyl alcohol	32 g.			
	oleyl alcohol	7.5 g.			
30	sorbitan trioleate	7.5 g.			
	polyethylene glycol 1540 mono-stearate	25 g.			
Example 14					
Ointment					
35	Composition:				
	partricin methyl ester	3 g.			
	dimethyl sulphoxide	6 g.			
	lanolin	20 g.			
	cetyl alcohol	31 g.			
40	sorbitan trioleate	10 g.			
	polyethylene glycol 1540 mono-stearate	30 g.			
Example 15					
Ointment					
45	Composition:				
	partricin methyl ester	3 g.			
	dimethyl sulphoxide	6 g.			
	lanolin	20 g.			
	cetyl alcohol	31 g.			
50	oleyl alcohol	10 g.			
	polyethylene glycol 1540 mono-stearate	30 g.			

