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(54) Title: PHARMACEUTICAL PREPARATION BASED ON RHAMNOLIPID AGAINST DERMATOLOGICAL DISEASES, E.G. PAPILLOMA VIRUS INFECTIONS		
(I)		
(57) Abstract The invention relates to a pharmaceutical preparation comprising as an active ingredient at least one rhamnolipid or a pharmaceutically acceptable salt thereof, and a carrier and/or diluent, preferably comprising a rhamnolipid of general formula (I), wherein R₁ = H, alpha-L-rhamnopyranosyl; R₂ = H, (a); R₃ = (C₅-C₂₀)-saturated, -mono- of poly-unsaturated alkyl; R₄ = (C₅-C₂₀)-saturated, -mono- of poly-unsaturated alkyl. The preparation is used for the treatment of dermatological diseases, e.g. Papilloma virus infections.		

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PHARMACEUTICAL PREPARATION BASED ON RHAMNOLIPID AGAINST
DERMATOLOGICAL DISEASES. E. G. PAPILLOMA VIRUS INFECTIONS

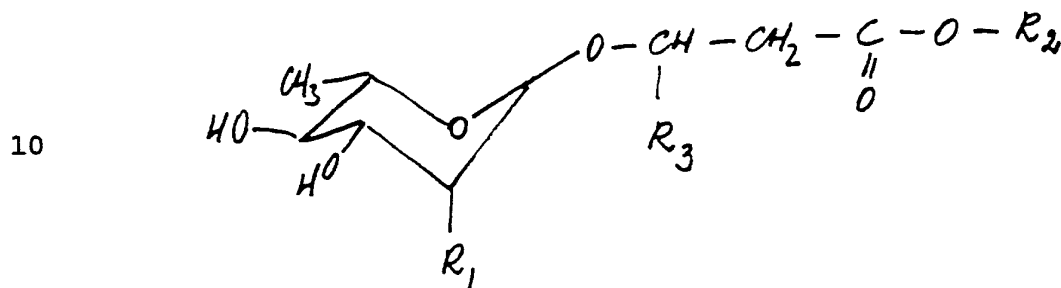
The present invention relates to a pharmaceutical preparation comprising as an active ingredient at least one rhamnolipid. In particular, the present invention relates to such a pharmaceutical preparation for treatment of
5 dermatological diseases, viral infections, and for cosmetic purposes.

Among bio-surfactants are glycolipids. Due to different combinations of carbohydrates and lipids, together with structural different bonds and different ionic states, there
10 are variety of glycolipids having mutually a strongly different hydrophilic/lipophilic balance. It is known that various strains of *Pseudomonas*, such as *Pseudomonas* *aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas oleovorans* are capable of extra cellular
15 secretion of rhamnolipids, when growing on soluble and insoluble carbon sources.

The present invention relates to pharmaceutical preparations comprising these rhamnolipids. Based on an extensive research, it has been found that these rhamnolipid
20 based pharmaceutical preparations exhibit a curing action on dermatological diseases, such as *Acnae vulgaris*, *Pytiriasis versicolor*, *Erythrasma*, *Piedra alba*, *Dermatitis allergica contactu*, *Neurodermitis circumscripta multilocularis*, *Neurodermitis diffusa*, *Neurodermitis erythrodermica*,
25 *Neurodermitis circumscripta chronica unilocularis*, *Neurodermitis verrucosa*, *Neurodermitis infantum*, *Prurigo chronica*, *Rosacea*, *Dermatitis rosacei formis steroidica*, *Psoriasis vulgaris*, *Lychen ruber planus*, *Strophulus infantum*, *Urticaria allergica*, *Dermatitis nummularis eczematoides*,
30 *Erythema exsudativum multiforme*, *Epydermolysis bullosa hereditaria dystrophica recessiva*, *Dermatitis seborrhoica*, *Erythema nodosum*, *Pemfigus vulgaris*, *Dermatitis herpetiformis*

Duhring, Lupus erythematosus² systemic, Herpes simplex infections, Papilloma virus infections, Ichtyosis vulgaris, Erythrodermia ichtyosiformis, Keratodermatitis palmoplantaris, Epidermolysis bullosa hereditaria simplex and the like.

5 Preferably, the rhamnolipid is a rhamnolipid of the general formula



Very active rhamnolipids are obtained when the
15 rhamnolipid is a di-rhamnolipid.

When the substituent R_2 is hydrogen, the rhamnolipid comprises only one lipid group. When the substituent R_2 is formed by the group $\text{CH} - \text{CH}_2 - \text{COOH}$,

R_4

20 the rhamnolipid molecule comprises two lipid units mutually connected by an ester bond.

The substituents R_3 and R_4 may be selected from straight or branched $(\text{C}_5 - \text{C}_{20})$ -saturated mono or poly-unsaturated alkyl groups. Preferred are unbranched, saturated
25 alkyl groups having the general formula $(\text{CH}_2)_x - \text{CH}_3$, wherein $x = 4 - 20$. More preferably $x = 4$ or 6 for the alkyl groups.

A very active pharmaceutical preparation is obtained when it comprises the rhamnolipid is (alpha-L-rhamnopyranosyl-(1,2) alpha-L-rhamnopyranosyl)-3-
30 hydroxydecanoyl-3-hydroxydecanoic acid.

Based on a non-limitative example, the isolation of rhamnolipid and the use in a pharmaceutical preparation according to the present invention will be described. Herein
the following methods will be used.

35

1. Isolation and characterization

From oil well drilling mud bacterial strains have been isolated, which strains can synthesize rhamnolipids both on a soluble carbon source (glucose) as on a insoluble carbon

source (glycerol, gas oil). These³ isolated bacterial strains have been characterized as Pseudomonas strains (BBL Minitek, Numerical Determination and Identification System, Becton, Dickinson and Company).

5

2. Concentration and purification

The rhamnolipids present in the medium due to extra cellular secretion have been concentrated and purified using different procedures.

10

a) acid precipitation

The pH of the supernatant was adjusted to pH 1.5-2.0 using hydrochloric acid, then it was evaporated to 1/10 of the initial volume and left over the night at 4°C. Pellet
15 (0.5-2.0 g/l) was centrifuged at 17.300 x g for 30 minutes at 4°C and subsequently extracted using CH₂Cl₂, filtrated, evaporated, resuspended in water and precipitated again.

b) foam fractionation

20 Compressed air was introduced into the supernatant and foam formed was passed into a separate container, or was introduced in acidified water (pH 1.5-2.0).

c) chromatography

25 Supernatant was applied on a preparative column (Amberlit XAD-8 or XAD-2 resin, Rohm & Haas). The column was equilibrated using water, and after absorption rinsed with water. The active compounds were eluted using a lower alkyl alcohol, such as ethanol and methanol. The solvent used was
30 evaporated under vacuum and the concentrate was acidified and precipitated as described above.

3. Chemical detection and characterization

Thin layer chromatography was performed analytically
35 using silica gel 60F 254 plates (Merck), and performed preparatively using Kemika plates. For an optimal separation the following solvent mixtures may be used
CHCl₃-MeOH-acetic acid-water (25:15:4:2; 12:15:4:2; 25:25:4:2)
CHCl₃-MeOH-acetic acid (80:15:5)

Hexane-isopropyl ether-acetic acid (15:10:1)
CHCl₃-MeOH-NH₄OH (25%)-water (65:25:4:2)
propanol-NH₄OH (25%) (4:1).

5 EXAMPLE

Pseudomonas aeruginosa was cultured at a temperature of 32°C. The following culture media were very suitable for rhamnolipid production.

a) 5 g glucose, 5 g peptone, 2 g yeast extract,
10 5 g NaCl, 0,5 g KH₂PO₄, 2 g MgSO₄.H₂O, 3 g KNO₃, 1 ml Gottlieb solution and 1 liter water.

b) 20 g glucose, 10 g yeast extract, 20 g CaCO₃ and 1 liter water.

Glucose may be replaced by glycerol, whereby a two
15 phase system is formed. As a carbon source glycerol provides a better yield in comparison to glucose, but a more complicated process is required. The change in surface tension of the culture media to 28-31 mN/m was measured using a White's ring tensiometer. A decrease of the surface tension
20 is a good indication of the yield at the end of the fermentation. The rhamnolipid concentration was measured spectrophotometrically using anthrone reagents. After separation of the biomass by centrifugation, the supernatant was chromatographed on silica gel 60 F 254 plates and on
25 Kemika plates.

The presence of glycolipids was proved using the following reagents, alpha naphthol for lipids, diphenylamine for glycolipids, and beta naphthol thymol and anthrone for carbohydrates and oligosaccharides. Using these reagents, it
30 is proved that the compounds are of the glycolipid type. Using alphacyclodextrin, reagent and dye for fatty acids, it is proved that the lipophilic/hydrophilic part of the molecule consists of fatty acid chains having an even number of carbon atoms. Negative results were obtained using
35 reagents for unsaturated lipids. Using alkali KMnO₄ and ammoniacal AgNO₃, the presence of glycosidic bonds has been proved. Using alkali hydrolysis ester bonds between fatty acids are split, and using acidic hydrolysis, the

5

O-glucosidic bond between a sugar group and the OH group of the lipid part of the molecule is split.

10 Volumina of the supernatant comprising rhamnolipids was passed over an amberlit XAD-8 or XAD-2 column (Rohm & Haas). The column was washed with water. The rhamnolipids were eluted using 100% MeOH. The fractions comprising rhamnolipids were evaporated and subsequently added to pure water. 1 N HCl was added to precipitate the rhamnolipids. Precipitated rhamnolipids were centrifuged at 3000 rpm for 10 minutes. The rhamnolipids now precipitated were washed using pure water and subsequently centrifuged at 3000 rpm for 10 minutes. Using 1/10 N NaOH the pH of the precipitated lipids were adjusted to 7.2. After lyophilization 10 g of the lyophilized preparation was dissolved in 50 ml propanol and applied to a silica column (Waters HPLC, volume 500 ml) equilibrated with hexane. Using 5 l propanol impurities were eluted. The rhamnolipids were eluted using a solvent mixture comprising propanol-25% NH₄OH (4:1). The active fractions were evaporated and dissolved in water, again precipitated in 1 N HCl, centrifuged at 3000 rpm, and the precipitants were adjusted to pH 7.2 using 0.1 N NaOH, and lyophilized.

The pure rhamnolipids obtained were used for preparing pharmaceutical preparations according to the invention. These preparations may comprise solutions, ointment, cremes, gels, fluids, powders, pills and the like.

Dependent on the disease to be treated, such as dermatological diseases, the seriousness of the disease, the age of the patient, and the like, pharmaceutical preparations may comprise 0.05 to 10 % by weight active rhamnolipid. Preferably, the rhamnolipid concentration is 0.1 to 2.0 % by weight, more preferably 0.1 to 1.0% by weight. The under limit is determined by the activity of the present rhamnolipid and the upper limit is predominantly determined economically.

35

Formulation example

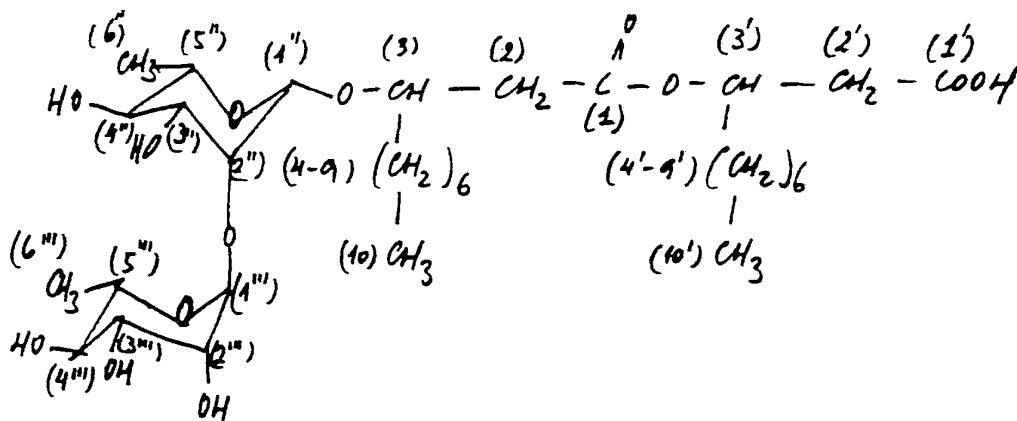
A ointment base was enriched using 1.0% by weight rhamnolipid. The rhamnolipid was (alpha-L-rhamnopyranosyl-

(1,2) alpha-L-rhamnopyranosyl)-3-hydroxydecanooyl-3-hydroxydecanoic acid, and was identified using

- i) ^1H and ^{13}C -NMR data; and
- ii) mass spectrophotometric data.

5

10



7

¹³C-NMR (75 MHz; DMSO-d₆)

	<u>C</u>	<u>ppm</u>	<u>C</u>	<u>ppm</u>	<u>C</u>	<u>ppm</u>
5	1"	97,7	1	173,3	1'	170,6
	2"	77,3	2	40,8	2'	40,2
	3"	70,4 ^a	3	72,2 ^a	3'	71,4 ^a
	4"	73,2	4	33,7	4'	32,5
	5"	68,9	5	24,8	5'	24,1
10	6"	17,9	6 }	28,8-29,2	6' }	28,8-29,2
	1'"	102,2	7 }		7' }	
	2'"	70,4 ^a	8	31,4	8'	31,4
	3'"	70,8 ^a	9	22,2	9'	22,2
	4'"	72,4	10	14,0	10'	14,0
15	5'"	68,8				
	6'"	17,8				

a: assignments may be reversed

20

¹H-NMR (300 MHz, DMSO-d₆)

sugar part

	<u>H</u>	<u>ppm</u>	
25	1" ; 1'"	4,8; 4,9	2x singlet (2H)
	2" ; 2'"		
	↓	3,20 --> 4,10	multiplet (8H)
	5" ; 5'"		
	6" ; 6'"	1,21	doublet, J=5,9 Hz (6H)

30

lipid part

	<u>H</u>	<u>ppm</u>	
	2,2'	2,43, 2,53	2x doublet, J=6,0 Hz (4H)
	3,3'	{ 3,20 - 4,1	multiplet (2H)
35		5,24	
	4,4'	1,6	multiplet (4H)
	5,5'-> 9,9'	1,3	multiplet (16H)
	10,10'	0,97	triplet, J=6,3 Hz (6H)

massaspectra

8

m/z : 673 $[M + H + Na]^+$ m/z : 695 $[M + H + 2Na]^+$ m/z : 525 $[M - C_{10}H_{18}O_2 + 2Na]^+$ minus terminal lipid5 m/z : 379 $[M - C_{10}H_{18}O_2 - \text{rhamnose} + 2Na]^+$ minus
terminal lipid and rhamnose

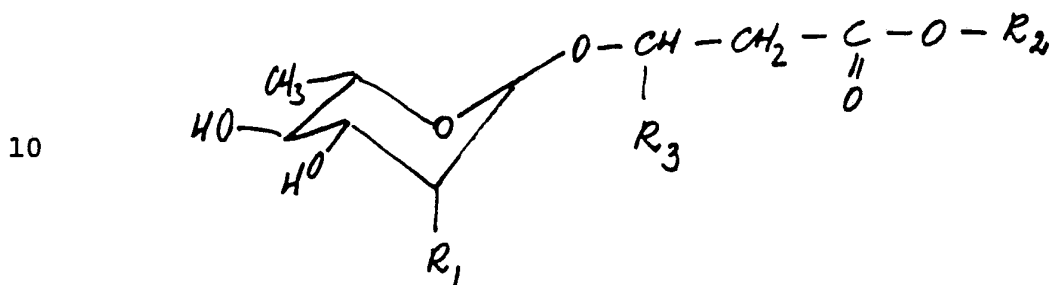
For human patient the following dermatological diseases were treated using the preparation according to the

10 formulation example: Psoriasis pustulosa, Neurodermitis chronica multilocularis, Lichen ruber planus and Acne papulopustulosa. The treatment of affected skin surfaces for a period of about two weeks resulted in a substantially total disappearance of the features of the dermatological diseases.

CLAIMS

1. Pharmaceutical preparation comprising as an active ingredient at least one rhamnolipid or a pharmaceutically acceptable salt thereof, and a carrier and/or diluent.

2. Preparation as claimed in claim 1, comprising a rhamnolipid of the general formula:



wherein

15 $R_1 = \text{H, } \alpha\text{-L-rhamnopyranosyl;}$

$R_2 = \text{H, } -\text{CH} - \text{CH}_2 - \text{COOH;}$

R_4

$R_3 = (\text{C}_5\text{-C}_{20})\text{-saturated, -mono- of poly-unsaturated alkyl;}$

20 $R_4 = (\text{C}_5\text{-C}_{20})\text{-saturated, -mono- of poly-unsaturated alkyl;}$

3. Preparation as claimed in claim 2, wherein

$R_3 = -(\text{CH}_2)_x - \text{CH}_3$, and $x = 4\text{-}20$.

25

4. Preparation as claimed in claim 3, wherein $x = 4$ or 6 .

5. Preparation as claimed in claim 2-4, wherein

30 $R_4 = -(\text{CH}_2)_x - \text{CH}_3$, and $x = 5\text{-}20$.

6. Preparation as claimed in claim 5, wherein $x = 4$ or 6 .

7. Preparation as claimed in claim 2-6, wherein
R₁ = alpha-L-rhamnopyranosyl.

8. Preparation as claimed in claim 2-7, wherein x = 6.

9. Preparation as claimed in claim 2-8, wherein
R₂ = - CH - CH₂ - COOH;



and y = 4-20.

10. Preparation as claimed in claim 9, wherein
y = 4 or 6.

11. Preparation as claimed in claim 1-10, wherein the
rhamnolipid is (alpha-L-rhamnopyranosyl-(1,2) alpha-L-
rhamnopyranosyl)-3-hydroxydecanooyl-3-hydroxydecanoic acid.

12. Preparation as claimed in claim 1-11, for treatment
of dermatological diseases.

13. Preparation as claimed in claim 12, for treatment
of Papilloma virus infections.

14. Preparation as claimed in claim 1-11, for use as a
cosmetic preparation.
