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(54) Title: SULPHATE DERIVATIVE OF GALACTAN EXTRACTED FROM KLEBSIELLA

(57) Abstract

Sulphate derivative of galactan extracted from Klebsiella mainly composed of sulphate neutral oses in a proportion of 20 to 90% of hydroxyls. Preparation process, use as medicament of this new derivative.

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(56) Documents cited:

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The present invention relates to a new sulphate derivative of the galactan extracted from Klebsiella, its preparation process, its use as medicaments and the pharmaceutical compositions containing it.

The French Patent FR 2,574,429 describes acylglycan extracted from Klebsiella composed of approximately 80% neutral oses, 20% lipids, less than 2% proteins, having a molecular weight of approximately 12,500 and containing a chain formation of galactoses linked in position 1-3. They are endowed with anti-allergic and immunomodulating properties.

The French Patent Application No. 89.09305 filed on 11th July 1989, and the European Patent Application No. 904019874 describe galactan of low molecular weight extracted from Klebsiella, its preparation process, its use as medicaments, notably immuno-stimulating medicaments.

The present invention relates to a sulphate derivative of galactan extracted from Klebsiella, prepared from the galactan obtained previously.

Also a subject of the present invention is a new sulphate derivative of galactan extracted from Klebsiella, characterized in that it is mainly composed of sulphate neutral oses in a proportion of 20 to 90% of hydroxyls.

The composition in neutral oses is determined by gas phase chromatography after methanolysis according to the method of ZANETTA J. Chromato. (1972) 69, p. 291 or the method of KAMERLING (Biochem J. (1975), 151, p. 491).

More particularly a subject of the invention is a sulphate derivative of galactan characterized in that it is mainly composed of sulphate neutral oses in a proportion of 40 to 80% of hydroxyls.

Among the products, which are a subject of the invention, there is preferably retained a sulphate derivative of galactan characterized in that it is mainly composed of sulphate

neutral oses in a proportion of 50 to 70% of hydroxyls.

According to the invention, the sulphate derivative of galactan is characterized in that it is composed of a linear chain formation of galactose in position 1-3 and has a molecular weight of between 5,000 and 12,000 and preferably close to 7,000.

These polysaccharides are free from lipids and proteins.

The galactose composition is determined by gas phase chromatography, after reduction and methanolysis.

The lipids are determined by gas phase chromatography after methanolysis.

The proteins are determined by the method of LOWRY (J.B.C (1951), 193, p. 265-273).

The sequence of the chain formation of the galactoses is determined by standard methods of methylation, analysis by gas phase chromatography coupled with mass spectrometry, periodical oxidation, NMR of H^1 and C^{13}. The results show that the galactan is formed of a linear chain formation of galactose residues connected by 50% of alpha (1-3) bonds and 50% of beta (1-3) bonds, with a repetition pattern composed of 8 galactopyranose residues and 1 galactofuranose residue.

The sulphate derivative of galactan, which is a subject of the invention, can be prepared from various species of Klebsiella. Quite particularly there is retained the sulphate derivative of galactan, characterized in that it comes from Klebsiella pneumoniae, and notably from the strain deposited at the Pasteur Institute in Paris or the Collection Nationale de Culture de Microorganismes (CNCM) under the numbers 52145 and 1-163 and at the National Culture Type Collection under the No. 5055.

Also a subject of the present invention is a preparation process for the new sulphate derivative of galactan extracted from Klebsiella as defined above which consists of treating an acylglycan extracted from Klebsiella, mainly composed of approximately 80% neutral oses, 20% lipids, less than 2% proteins and having a molecular weight of approximately 12,500, by gentle acid hydrolysis, by high performance chromatography on an anion exchanger, recovering the unretained

fraction, subjecting to filtration on a gel, recovering the fraction containing the galactan of molecular weight comprised between 4,000 and 10,000, which process is characterized in that the fraction of galactan thus obtained is sulphated, then purified by dialysis and the sulphate derivative of galactan thus obtained is isolated.

The acylglycan extracted from Klebsiella used at the start can be obtained from a hydrosoluble bacterial extracted from Klebsiella by heating, followed by chromatographic fractionation. Such preparations have already been described in the French Patent Application FR 2,574,429 and the German Patent DE 3,543,267.

The hydrosoluble bacterial extracted from Klebsiella has been prepared according to processes described in Patents FR 2,490,496 and EP 49182.

In the preferred conditions for implementing the process which is a subject of invention:

- The gentle acid hydrolysis of the acylglycan is carried out in a 1% solution of acetic acid by heating to 100°C for 90 minutes; the precipitate formed, which contains the lipid fraction, is eliminated by centrifuging, for example for 30 minutes at 2,000 G. The supernatant which contains the galactan is collected and can be lyophilized.
- The galactan is then separated from the supernatant by chromatographic fractionation. The fraction composed of neutral oses is collected.
- The fractionation can be carried out by chromatography on anion exchangers, preferably by high performance chromatography, for example on Magnum 9SAX Whatman. High performance chromatography on anion exchangers consists of separating the galactan by elution with water. The fraction of interest, composed of neutral oses, is located by spectrophotometric detection at 200 nm and at 492 nm after coloration with the phenol-sulphuric acid test, according to the method of DUBOIS (Anal. Chem. (1956) 28, p. 350).
- Said fraction, constituted of neutral oses, is subjected to filtration on a gel which allows the recovery of the fraction containing the galactan of molecular weight comprised between

4,000 and 10,000. The filtration on gel is carried out on commercially available supports, for example on Sephadex or Biogel agarose; Biogel A-1.5M is preferably used.

- The sulphation of the galactan fraction is carried out by means of a sulphonic acid such as chlorosulphonic acid in an amine such as pyridine or by means of a sulphonate such as piperidine N-sulphonate by heating the reaction medium.

The dialysis is carried out against distilled water.

Also a subject of the invention is the sulphate derivative of galactan such as that obtained during the implementation of the process described above.

The sulphate derivative of galactan, which is a subject of present invention, possesses very useful pharmacological properties; notably it is endowed with remarkable immunostimulating properties as well as having a good tolerance. Notably it possesses the property of stimulating the production of Il_1 and TNF at the level of the macrophages and above all of stimulating the generation of free radicals (polynuclear). It also allows the synergy of the effect of the GM-CSF (Granulomonocyte-Colony Stimulating Factor).

Also a remarkable anti-elastase activity is noted, both against bovine pancreatic elastase, and against human leucocytary elastase. Also these products show an anti- coagulant activity.

These properties are illustrated further on in the experimental part.

These properties justify the use of the sulphate derivative of galactan, as defined above, as medicaments.

These medicaments find their use, for example, in the treatment or prevention, in man, of immuno-depressions, infectious illnesses caused by bacteria or viruses, and especially by the AIDS virus, in the treatment of illness due to parasites, toxic infections, in the treatment of post- hospital and post-surgical infections and allergies of all origins. These medicaments can also be used very advantageously in the treatment of bone marrow transplants and post-chemotherapy medullary aplasias.

The usual dose, variable according to the product used,

the subject treated and the affection in question, can be, for example, 0.5 to 5 mg per day by oral route.

Also these medicaments find their use, for example, in pneumology in the treatment of emphysema, pneumonia, bronchitis, pulmonary disorders caused by nicotiniom or atmospheric pollution, in cardiology in the treatment of arthritis, as well as, for example, in dermatology in the treatment of psoriasis, burns, buloses and in the ageing of the skin, in gastro-enterology in the treatment of acute pancreatitis and in a general manner in the treatment of all affections implicating the malfunctioning of the elastase as well as in the treatment of thrombo-embolic illness.

The usual dose, variable according to the product used, the subject treated and the affection in question can be, for example, from 1 to 300 mg per day by intravenous route in man.

Also a subject of the invention is the pharmaceutical compositions which contain the sulphate derivative of galactan extracted from Klebsiella, as defined above, as active ingredient.

As medicaments, the sulphate derivative of galactan extracted from Klebsiella, as defined above, can be incorporated in pharmaceutical compositions intended for digestive, parenteral or local route.

The corresponding pharmaceutical compositions can be, for example, solid or liquid and be presented in the pharmaceutical forms commonly used in human medicine, such as for example, plain or sugar-coated tablets, capsules, granules, solutions, syrups, suppositories, lyophilized or non-lyophilized injectable preparations, pessaries, creams, ointments, lotions, drops, collyria, aerosols; they are prepared according to usual methods. The active ingredient or ingredients can be incorporated with the excipients usually employed in these pharmaceutical compositions, such as talc, gum arabic, lactose, starch, magnesium stearate, cocoa butter, aqueous or non-aqueous vehicles, fatty substances of animal or vegetable origin, paraffin derivatives, glycols, various wetting, dispersing or emulsifying agent, preservatives.

There will now be given non-limiting examples of the

implementation of the invention.

EXAMPLE 1 : Sulphate derivative of the galactan extracted from Klebsiella.

Chlorosulphonic acid (0.4 cm^3) is added to pyridine cooled to 0°C in ice. After returning to ambient temperature, the galactan extracted from Klebsiella pneumoniae (25 mg) in suspension in pyridine (24 cm^3) is added. The mixture is heated for 3 hours at 80°C, then cooled to ambient temperature, diluted with distilled water (30 cm^3) and finally treated with a 2.5M solution of soda (5 cm^3). The obtained solution is concentrated under vacuum, dialyzed with distilled water for 3 days, frozen and lyophilized.

Thus the desired sulphate derivative of galactan extracted from Klebsiella is obtained.

In the product obtained the hydroxyls are sulphated in a proportion of 60%. The molecular weight is close to 7,000.

Preparation of the galactan extracted from Klebsiella pneumoniae.

334 mg of acylglycans of Klebsiella (obtained as indicated in Example 1 of the French Application No. 2,574,429 from the strain deposited at the Pasteur Institute under the No. I-163) is solubilized in 33.4 cm^3 of a 1% acetic acid solution and the mixture is heated to 100°C for 90 minutes. After cooling, the precipitate containing the lipid fraction is separated out by centrifuging at 2000 g for 30 minutes. The supernatant is lyophilized and 200 mg of residue is isolated which is redissolved in 4 cm^3 of water. Chromatography takes place, using 1 cm^3 fractions, on a high performance anion exchanger chromatography Whatman Magnum 9 SAX (9.4 mm *50cm) column, eluting with water for 30 minutes, at a flow rate of 2 cm^3/minute with detection at 200 nm. The fraction containing the neutral oses, located at 492 nm, is isolated, after the phenol-sulphuric test. 55 mg of the neutral fraction is isolated by lyophilization.

- <u>isolation of the galactan</u>

The previous lyophilisate is dissolved in a water-acetic acid-pyridine (973-7-20) buffer, then it is subjected to filtration on gel by chromatography on a Biogel A.1.5M

(2 cm x 1.5m) column, balanced with the same buffer. The fraction detected in the phenol-sulphuric test is collected. After sterilising by filtration on a 0.22 micron membrane and lyophilizing, 22 mg of the expected galactan is obtained.

EXAMPLE 2: Sulphate derivative of galactan extracted from Klebsiella

Chlorosulphonic acid (4 cm^3) is added to pyridine cooled to 0°C in ice. After returning to ambient temperature, the galactan extracted from Klebsiella pneumoniae (prepared as indicated in the preparation given in Example 1) (85 mg) in suspension in pyridine (24 cm^3) is added. The mixture is heated for 2 hours at 80°C, then cooled to ambient temperature and diluted with distilled water (30 cm^3). The pH of the reaction mixture is brought to 6 by the addition of a 2.5M solution of soda. The solution obtained is dialyzed with distilled water, then frozen and lyophilized.

Thus 32 mg of sulphate derivative of galactan extracted from Klebsiella pneumoniae is obtained.

EXAMPLE 3 :

Tablets corresponding to the following formula were prepared:

- Product of Example 1 ..... 1 mg -
Excipient s.q. for a tablet completed at ..... 100 mg
(detail of excipient: lactose, starch, talc, magnesium stearate).

EXAMPLE 4:

Tablets corresponding to the following formula were prepared:

- Product of Example 2 ..... 1 mg
- Excipient s.q. for a tablet completed at ..... 100 mg
(detail of excipient: lactose, starch, talc, magnesium stearate).

EXAMPLE 5:

Aerosols were prepared delivering doses each of which contains:

- Product of Example 1 ..... 0.5 mg -
Emulsifying agent ..... 0.15 mg -
Propellant ..... 50.00 mg

EXAMPLE 6:

A cream corresponding to the following formula was prepared:

- Product of Example 2 ..... 1 mg

Excipient: 2-octyl-dodecanol alcohol, ketostearylic alcohol, sodium sulphate, methyl and propyl parahydroxybenzoate, purified water ..... 10 mg.

Biochemical study

The activity of the sulphate derivative of galactan extracted from Klebsiella is determined by measurement of the inhibition of syncitia (Gruters et al Nature Vol. 330 p.74 - 77.(5, November 1987)) and by determination of the protective activity exercised vis-a-vis certain cell cultures.

A) Preparation and titration of virus

1° / Preparation of virus

- the supernatant of H 9 III_B cells (chronically infected with HIV), cultured for 48 hours starting with 10^5 cells/cm^3
- centrifuged supernatant, filtered at 0.45 micromole, aliquoted and stored at -80°C.

2° / Titration of virus

- reverse transcriptase (RT)

On the fresh supernatant 4.0×10^6 cpm/cm^3

After thawing 4.5×10^6 cpm/cm^3

- MT4/MTT test

technique: 100 microlitre dilutions of the virus in series in a microtitration plate

- addition of 100 microlitres of cellular suspension containing 5×10^4 MT4 cells
- culture 7 days at 37°C, 5% CO_2
- addition of MTT (tetrazolium salt viability colouring agent)
- incubation 4 hours at 37°C.
- halting the reaction by the addition of a hydrochloric acid solution in isopropane
- reading of the optical density (OD) at 540 nm (after dissolution of crystals).

Results

The relationship of the optical density of the infected cells (viral cytotoxicity = weak OD) over the optical density of infected cells (viability and maximum OD) as a function of the dilution of the virus (average over 3 wells).

The more the cells are infected the more this relationship is weak.

B) Protective activity of the sulphate derivative of the galactan extracted from Klebsiella

1° / Inhibition of Syncitia

A co-culture of H 9 III cells (chronically infected 10^5 cells/cm^3) with SUP T1 cells (non-infected: $2X10^5$ cells /cm^3) is carried out.

There is syncitia formation which is counted with a microscope.

Each type of cell is pre-incubated or non-preincubated with the studied product, for one night at 37°C.

If there is no pre-incubation, the studied product is added to cells at the moment the culture is carried out.

When there is inhibition of Syncitia formation in the treated cells relative to the control cells, the studied product is protective.

The results obtained are shown hereafter:

TABLE 1

co-culture with the studied product without previous incubation

Product Example 1 in micrograms/cm^3	Percentage of inhibition Test no. 1	Test no. 2
1	0	10
10	42	17
100	100	100

TABLE 2

pre-incubation with the studied product for one night at 37°C before the co-culture

Product of Example 1 in micrograms/cm^3	Percentage of inhibition	
	H 9 III cells	Sup Ti cells
1	17	23
10	23	27
100	100	100

2°/ MT4/MTT Test

MT4 cells at 10^6 cells/cm^3 are used. Dilution to 1/2 takes place with or without the studied product at different concentrations. 100 microlitres of HIV virus at different dilutions and 100 microlitres of pre-treated cell suspension are introduced onto a microtitration plate.

Incubation takes place at 37°C for 7 days, the MTT is added and a reading is taken with a spectrophotometer.

The percentage inhibition of the infection of MT4 cells is determined.

The results obtained are shown hereafter:

TABLE 3

Product of Example 1 in micrograms/cm^3	Percentage of inhibition
1	74
10	99
100	84

3°/ H9 test

H9 cells are used which are treated with polybrene, washed, and taken up in 100 microlitres of medium, with or without the studied product at different concentrations.

After incubating for 2 hours at 37°C, 100 microlitres of HIV virus at 1/10 is added, the whole is kept for 1 hour at 37°C, then washed.

The cells are taken up in 1 cm^3 of medium with or without the studied product, and distributed in 2 wells, 500 microlitres per well and $2.X10^5$ cells by well.

On D + 4, 500 microlitres of culture medium with or without the studied product is added.

On D + 8, half of the supernatant is replaced with new medium.

On D + 11, a quantitative analysis by reverse transcriptase is carried out on the supernatant and an immunofluorescent determination is carried out on the cells. The results obtained are shown hereafter:

TABLE 4

	Reverse Transcriptase		Immunofluorescence	
	cpm/cm^3 x 10^4	% inhibition	% fluorescent cells	% inhibition
Non-infected cells	1.68		0	
Infected cells	291		51	
Product of Example 1 (concentration in micrograms/cm^3				
1	6.42	76	3	94
10	1.16	100	0	100
100	1.12	100	0	100

The results obtained in the different tests show that the studied product very significantly inhibits the formation of syncitia and that it protects the studied cells from infection caused by the HIV virus.

Study of anti-elastase activity.

The anti-elastase activity has been determined by spectrophotometric quantitative analysis vis-a-vis human leucocytary elastase (by a similar technique to that described by Boudier et al. Clin. Chim. Acta. 132, 309-315 (1983)).

Product of Example	Human leucocytary elastase
1	Ki : 1 x 10^{-8}M

CLAIMS

1. - A sulphate derivative of galactan extracted from Klebsiella, characterized in that it is mainly composed of sulphate neutral oses in a proportion of 20 to 90% of hydroxyls.

2. - Sulphate derivative of galactan according to claim 1, characterized in that it is mainly composed of sulphate neutral oses in a proportion of 40 to 80% of hydroxyls.

3. - Sulphate derivative of galactan according to claim 1 or 2, characterized in that it is mainly composed of sulphate neutral oses in a proportion of 50 to 70% of hydroxyls.

4. - Sulphate derivative of galactan according to any one of claims 1, 2 or 3, characterized in that it is composed of a linear chain formation of galactose in position 1-3 and has a molecular weight of between 5,000 and 12,000.

5. - Sulphate derivative of galactan according to any one of claims 1, 2, 3 or 4, characterized in that it comes from Klebsiella pneumoniae.

6. - Sulphate derivative of galactan according to claim 5, characterized in that it comes from the strain deposited at the Pasteur Institute in Paris or the Collection Nationale de Culture de Microorganismes (CNCM) under numbers 52145 and 1-163 and at the National Culture Type Collection under the No. 5055.

7. - Preparation process for the new sulphate derivative of galactan extracted from Klebsiella as defined in any one of claims 1 to 6 which consists of treating an acylglycan extracted from Klebsiella, mainly composed of approximately 80% neutral oses, 20% lipids, less than 2% proteins and having a molecular weight of approximately 12,500, by gentle acid hydrolysis, high performance chromatography on an anion exchanger, collecting the non-retained fraction, subjecting it to filtration on gel, collecting the fraction containing the galactan of molecular weight of between 4,000 and 10,000, which process is characterized in that the fraction of galactan thus obtained is sulphated, purified by dialysis and the sulphate derivative of galactan thus obtained is isolated.

8. - Process according to claim 7, characterized in that the sulphatation of the galactan fraction is carried out using a sulphonic acid in an amine or by means of a sulphonate operating by heating the reaction medium and the dialysis is carried out against distilled water.

9. - The sulphate derivative of galactan as obtained **by the process defined in any one of claims 7 or 8.**

10. Medicaments, characterized in that they are composed of sulphate derivatives of galactan as defined in any one of claims 1 to 6.

11. - Medicament, characterized in that it is composed of the sulphate derivative of galactan as defined in claim 9.

12. - Pharmaceutical compositions, characterized in that they contain, as active ingredient, at least one of the medicaments as defined in any one of claims 10 or 11.

13. A sulphate derivative of galactan according to claim 1 substantially as herein described with reference to any one of the illustrative examples.

14. A process according to claim 7 substantially as herein described with reference to any one of the illustrative examples.

15. A pharmaceutical composition according to claim 1 substantially as herein described with reference to any one of examples 3 to 6.

DATED THIS 20th DAY OF November 1990

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