

56509J.19

5 The invention relates to a polypeptide, hereinafter referred to as epidermin, having an antibiotic activity, a process for preparing it by means of a new strain of Staphylococcus epidermidis resistant to this substance, the new resistant strain, antibiotic preparation forms containing the active substance epidermin, and the use of the active substance for combating infectious diseases.

10 It is known from EP-A-0,027,710 that a particular strain of Staphylococcus epidermidis, namely Staphylococcus epidermidis NCIB 11536 (deposited at the National Collection of Industrial Bacteria, Aberdeen) produces a low-molecular weight antibiotic polypeptide with a broad activity on gram-positive pathogens, which  
15 lyses bacterial cells.

It is also known that the strain Staphylococcus epidermidis MF 205 (S.M. Taylor et al., Int. J. Mass Spectrom. and Ion Physics 48, 161-164, 1983) also produces an oligopeptide effective against gram-positive  
20 bacteria. It is not clear from this publication whether this strain is identical to the above-mentioned strain NCIB 11536.

It has now been found that a resistant mutant of Staphylococcus epidermidis, which was deposited on  
25 26.10.1984 at the "German Collection of Microorganisms" under the Number DSM 3095 and which is related to the above-mentioned strain NCIB 11536, produces

the similar polypeptide epidermin which has an antibiotic effect, preferably on gram-positive bacteria, according to a modified method of preparation and working up, and when epidermin is compared  
5 with the products of *Staphylococcus epidermidis* NCIB 11536 and MF 205, inter alia, it shows considerable differences in the composition of amino acids:

Comparison of the constituents:

10

|    |                   | Products of <i>Staph. epidermidis</i> |            |
|----|-------------------|---------------------------------------|------------|
|    |                   | MF 205                                | NCIB 11536 |
|    | Epidermin         |                                       |            |
|    | Asn               | 1                                     | -          |
|    | Asx               | -                                     | 2          |
| 15 | Glx               | -                                     | 3          |
|    | Pro               | 1                                     | 1          |
|    | Gly               | 2                                     | 2          |
|    | Ala               | 2                                     | 2          |
|    | Ile               | 2                                     | 1          |
| 20 | Phe               | 2                                     | 2          |
|    | Lys               | 2                                     | 1          |
|    | Lan               | 2                                     | -          |
|    | $\beta$ -Me-Lan   | 1                                     | -          |
|    | Dhb               | 1                                     | -          |
| 25 | Tyr               | 1                                     | 1          |
|    | S-(2-Aminovinyl)- |                                       |            |
|    | D-cysteine        | 1                                     | -          |
|    | X                 | -                                     | 1-2        |

30

Dhb corresponds to  $\alpha, \beta$ -dehydroaminobutyric acid,  
Asx and Glx indicate Asn/Asp and Gln/Glu, respectively.

After total hydrolysis of epidermin using  
35 6N hydrochloric acid (18 hours at 110°C) quantitative amino acid analysis by ion exchange chromatography (standard programme using the Biotronik LC 6000 E

apparatus) gave the following  $\alpha$ -amino acid composition:

Asp (1.00), Pro (0.95), Gly (2.09), Ala (2.03),  
Ile (2.03), Tyr (0.30), Phe (2.02) and Lys (2.00)  
5 (cf. Fig. 1). By adding thioglycolic acid the  
tyrosine content of the total hydrolysate was increased  
to the substantially stoichiometric value of 0.93.

The configuration of the protein amino acids  
10 was determined by gas chromatography of the n-propyl  
esters of pentafluoropropionylamino acid from the  
total hydrolysate. Separation was effected on  
the chiral stationary phase L-valine-tert.butylamide-  
polysiloxane (Chirasil-Val). By comparison with  
15 a test mixture of amino acids of known configuration  
the constituents listed above could be categorised  
as having the L configuration (cf. Fig. 2).

In addition to these protein amino acids  
there are the following non-protein amino acids:  
20 lanthionine (2),  $\beta$ -methyllanthionine (1) and  $\alpha,\beta$ -  
dehydroaminobutyric acid (1); lanthionine has the  
meso configuration whilst  $\beta$ -methyllanthionine has  
the 2S,3S,6R configuration. In addition, epidermin  
contains an S-(2-aminovinyl)-D-cysteine component.

25 The new compound epidermin can be characterised  
by the following data:

1. Nature: colourless powder
2. Solubility: very readily soluble in mixtures  
30 of water/glacial acetic acid or methanol/glacial  
acetic acid, soluble in lower alcohols, insoluble  
in chloroform, acetone, diethyl ether, petroleum  
ether.
3. Colour reaction on silica gel plates: Ninhydrin,  
35 chlorine/TDM [TDM=4,4'-bis-(dimethylamino)-  
diphenylmethane], orcin/sulphuric acid, anisaldehyde/  
sulphuric acid. Non-destructive detection

in UV light at 254 nm and by spraying with water.

4. Thin layer chromatography: ready-made silica gel plates 60 F<sub>254</sub> (Merck) were used.
5. System A: chloroform/methanol/  
17% ammonia (2/2/1),  $R_F = 0.73$   
System B: chloroform/methanol/  
17% ammonia (70/35/10)  $R_F = 0.30$   
System C: n-butanol/glacial acetic acid/  
10 water (4/1/1)  $R_F = 0.05$
5. HPLC: see Fig. 7.
6. Stability: stable from pH 2 to pH 7; a sharp drop in activity occurs at higher pH values.
7. Molecular mass: in the region of 2160. May  
15 be contained as anions, e.g. chloride, acetate or phosphate, depending on the method of isolation used.
8. Ultra-violet absorption spectrum: in aqueous solution, long-wave maximum at 267 nm (Fig. 3).
- 20 9. Infra-red absorption spectrum: IR spectrum of a sample in a potassium bromide tablet (Fig. 4).
10. Nuclear magnetic resonance spectra  
25 <sup>1</sup>H-NMR spectrum: Fig. 5, <sup>13</sup>C-NMR spectrum: Fig. 6.

Suitable fragments for sequencing epidermin were obtained by tryptic cleavage. Reaction with  
30 trypsin rapidly resulted in a loss of the antibiotic activity. Macroscopically, a jelly-like white precipitate was observed on tryptic cleavage and this precipitate could be removed by centrifugation. The supernatant was lyophilised and subjected to  
35 gel chromatography on Sephadex G 25 (dextran gel made by Pharmacia) in 1% acetic acid.

The chemically uniform fraction (hereinafter

referred to as P1), which can be stained with ninhydrin, chlorine/TDM and water, proved to be the N-terminal fragment of the tryptic cleavage of epidermin (see below).

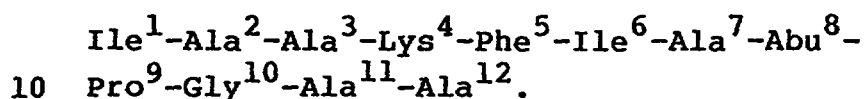
5       The extremely hydrophobic, jelly-like precipitate consisted of several chemical components which were formed during tryptic cleavage of the C-terminal fragment of the total molecule. A chemically uniform product (hereinafter referred to as P2) was obtained  
10 by dissolving the precipitate in dimethylformamide with subsequent gel chromatography on Sephadex LH-20 (dextran gel produced by hydroxypropylation of Sephadex G-25, Pharmacia) with dimethylformamide as eluant. P2 could be detected with water, chlorine/TDM  
15 and under UV light. The ninhydrin reaction on P2 proved negative.

Amino acid analysis of the fragment P1 gave the following composition: Pro (1), Lan (1),  $\beta$ -Me-Lan (1), Gly (1), Ala (2), Ile (2), Phe (1),  
20 Lys (2). Since isoleucine was determined to be the n-terminal amino acid by dansylation of the total molecule, and since the fragment P2 does not contain isoleucine, the fragment P1 must be the N-terminal cleavage product of the total molecule.  
25 The C-terminal amino acid of fragment P1 must be lysine, on account of the tryptic cleavage.

To clarify the structure of P1 further, the C-terminal amino acid lysine was enzymatically removed by means of carboxypeptidase B (Boehringer  
30 Mannheim). The resulting fragment P12 could be isolated in pure form by gel chromatography on Sephadex G-25 (1% acetic acid). P12 consists of the following amino acids: Pro (1), Lan (1),  $\beta$ -Me-Lan (1), Gly (1), Ala (2), Ile (2), Phe (1),  
35 Lys (1).

The sulphur bridges of the thioether amino acids lanthionine and  $\beta$ -methyllanthionine prevent

sequence analysis according to the Edman procedure. By reacting P12 with Raney-Ni W2, a sulphur-free dodecapeptide was obtained. meso-Lan was converted into D- and L-alanine and  $\beta$ -methyllanthionine was converted into D-aminobutyric acid and L-alanine. The dodecapeptide sequence was clarified by Edman degradation and FAB spectrometry:



Various investigations for the arrangement of the sulphur bridges in the fragment P12 yielded the following structure:

15



20 The C-terminal fragment P2 obtained by tryptic cleavage was able to be characterised as follows: Asn (1), Lan (1), Gly (1), Phe (1), Tyr (1),  $\alpha$ -ketobutyric acid and S-(2-aminovinyl)-D-cysteine.

The  $\alpha$ -ketobutyric acid originates from the dehydroaminobutyric acid which directly follows lysine<sup>13</sup> in the sequence of the total molecule. Tryptic cleavage liberates the amino group of the dehydroaminobutyric acid; since dehydroamino acids with a free amino group are unstable, they are converted, inter alia, into  $\alpha$ -keto acids.

The tryptic fragment P2 is blocked at the N-terminal end with the  $\alpha$ -ketobutyric acid (ninhydrin reaction on P2 proves negative). In addition to the amino acids lanthionine, glycine, phenylalanine, tyrosine and aspartic acid, which are identified by total hydrolysis and amino acid analysis, P2 has a further component which is destroyed by acid

total hydrolysis. This was able to be characterised by means of its signals in the  $^{13}\text{C}$ -nuclear resonance spectra of epidermin (Fig. 6) and of the fragment P2 (Figure 8).

5 An indication of the chemical nature of this amino acid was obtained by reacting P2 with Raney-Ni W2, from which two main products were formed. Both products isolated by preparative HPLC showed, after total hydrolysis, an additional D-alanine  
10 group in the amino acid chromatogram which was produced by this reaction.

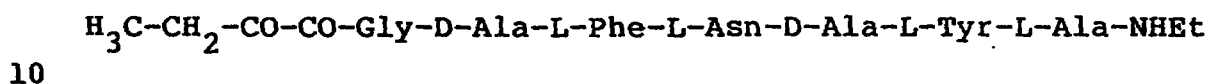
The structure of this component could be clarified by heterogeneous hydrogenation of the native antibiotic. The four reaction products  
15 H1, H2, H3 and H4 were purified by semi-preparative HPLC, totally hydrolysed and subjected to gas chromatography on a chiral stationary phase (Fig. 15). Compared with the chromatogram in Fig. 2, an additional peak occurred in H1 and H3 which was identified  
20 by its mass spectrum as being S-(2-aminoethyl)-D-cysteine. In epidermin, this amino acid was present as the acid-unstable component S-(2-aminovinyl)-D-cysteine.

The two peptides P21 and P22 formed from  
25 the C-terminal fragment P2 by treatment with Raney nickel W2 were able to be characterised as follows by amino acid analysis and gas chromatography on Chirasil-Val:

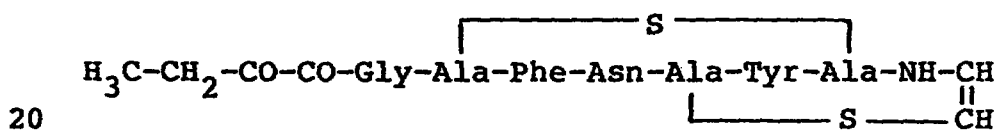
| 30 | Reaction products       | P 21    | P 22             |
|----|-------------------------|---------|------------------|
|    | Amino acid analysis and | 2 D-Ala | 1 D-Ala          |
|    | gas chromatography on   | 1 L-Ala | <u>meso</u> -Lan |
|    | Chirasil-Val            | 1 L-Phe | 1 L-Phe          |
|    |                         | 1 L-Tyr | 1 L-Tyr          |
| 35 |                         | 1 L-Asp | 1 L-Asp          |
|    |                         | 1 Gly   | 1 Gly            |

In addition, both products were blocked at the C-terminal by ethylamine. During desulphurisation with simultaneous hydrogenation S-(2-aminovinyl)-D-cysteine decomposed to form D-alanine and ethylamine.

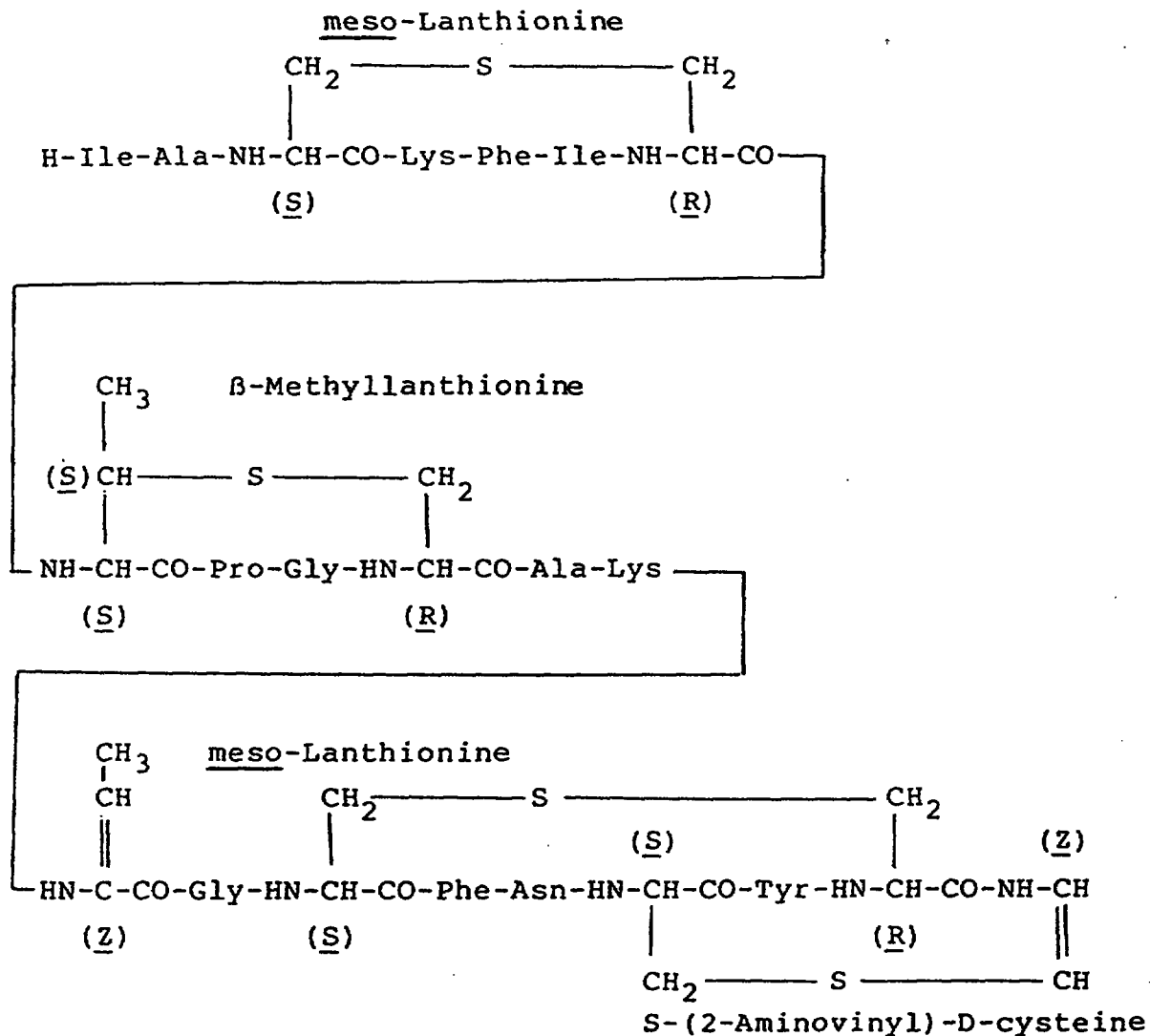
5 Sequencing of the bridge-free heptapeptide P21 (partial hydrolysis; 0.1 N hydrochloric acid, 93°C, 16 hours) yielded the following structure:



The arrangement of the meso-lanthionine bridge present in P22 could be reconciled, by partial hydrolysis and various chemical investigations on enzymatic fragments of P21 and P22, only with  
15 the following structure of the fragment P2 derived from tryptic cleavage:



Formal substitution of the 2-ketobutyric acid, which blocks the P2 fragment at the N-terminal, by the amino acid dehydrobutyrine which is present  
25 in the native antibiotic, and subsequent fragment condensation by the tryptic peptides P1 and P2, resulted directly in the following sequence for the heterodet tetracyclic peptide antibiotic epidermin:



The halves of the individual thioetheramino acids closer to the N-terminus are of D configuration (corresponding to the S configuration in the R,S nomenclature).

The invention thus relates to a uniform and therefore pure product known as epidermin, and to processes for preparing, isolating and purifying it.

The producer, Staphylococcus epidermidis DSM 3095, is cultured aerobically at 37°C in a complex medium with the composition 2 to 4% meat

extract, 1 to 3% malt extract and 0.25 to 1%  $\text{CaCO}_3$  or 0.25 to 0.5%  $\text{Ca(OH)}_2$ . (The percentages given are always percent by weight throughout, unless otherwise specified). The maximum antibiotic activity  
5 is achieved after 18-23 hours.

Attempts to concentrate the antibiotic were made, for example, on culture filtrate from 10 litre fermenters, and the effectiveness of individual steps was checked by the plate diffusion test.  
10 The active component could be concentrated by extraction of the culture filtrate, freed from cells and lime, with n-butanol. Extraction with n-butanol, however, was only possible at the natural end-pH of 8.0 of the culture filtrate. Further purification  
15 could be achieved by separating off the lipid contaminants by ether precipitation. For this, the butanol extract was evaporated, and the residue was dissolved in methanol and stirred into five times the quantity of cold diethyl ether. The activity was left behind  
20 completely in the precipitate (Figure 9).

Another particularly suitable method of concentration is adsorption of the centrifuged culture filtrate on Amberlite XAD-8 or related types of polymer based on acrylic esters (Serva). The attachment  
25 of epidermin is effected not by simple adsorption but by the cation-exchanging activity of free acrylic acid groupings in the resin. This is confirmed by the fact that the active component can only be released from the resin by elution with methanol/conc.  
30 HCl (99:1). The strongly acidic eluate has to be neutralised with ammonia before being concentrated by evaporation in vacuo. After this working up by adsorption on Amberlite XAD-8 there is no need for subsequent reprecipitation from methanol/ether.  
35 Isolation of the epidermin by adsorption may also be carried out directly in the culture broth during the cultivation of the microorganism.

Stability tests and chromatographic investigations were carried out on the lyophilised butanol extract. Incubation of the extract with aqueous solutions of different pH values resulted in a sharp decrease in activity from pH 10 onwards, whereas the antibiotic is stable in the range from pH 2 to 7.

Thin layer chromatography of the evaporated butanol extract showed a plurality of compounds which can be stained with various spray reagents. Bioautograms carried out in parallel yielded important indications as to the nature of the new active substance. In acidic and virtually all neutral systems the antibiotic remained on the baseline in thin layer chromatography on silica gel 60, whereas chromatography with alkaline eluants gave  $R_F$  values of between 0.3 and 0.75. The combination of bioautography with thin layer chromatography in alkaline systems showed a correlation with a compound which could be stained with ninhydrin.

The result of these preliminary tests was that the antibiotic is a strongly basic peptide which could not be further purified by column chromatography on silica gel 60 since it could not be eluted from the column with acidic or neutral systems.

Chromatography of the Amberlite XAD eluate or of the butanol extract, freed from lipids, on Sephadex LH-20 with methanol/acetic acid (95:5) separated a large number of small peptides, amino acids and salts from the medium away from the antibiotic (Fig. 9).

In the subsequent multiplicative countercurrent distribution following the procedure developed by Craig, it was possible to draw on the experience gained during extraction of the antibiotic from the culture filtrate. In a first liquid-liquid distribution with the system n-butanol/ethyl acetate/0.1 N acetic acid (3:1:3) the antibiotic remained on

the baseline. In the second Craig distribution with the neutral system 2-butanol/0.05 N ammonium acetate (1:1) the antibiotic was at the mid-point of the apparatus. Ammonium acetate could be removed again by

5 lyophilisation under a high vacuum.

After freeze drying, epidermin was obtained as a powder which was uniform in all the thin layer systems used.

Whereas purification in accordance with the  
10 procedure of European Patent Application 0,027,710 was carried out by freezing/thawing extraction, evaporation of the extracting agents, ultrafiltration, precipitation with ammonium sulphate, ion exchange chromatography and gel filtration on Sephadex G-50 or G-25 or G-15 or on  
15 Biogel P2, the purification according to the invention yields a uniform pure product by adsorption of the centrifuged culture filtrate on Amberlite XAD-8 followed by gel chromatography on Sephadex LH-20 and subsequent two-fold countercurrent distribution according to Craig.  
20 Thus, the methods of isolation and purification according to the above-mentioned European patent application and according to the invention are totally different.

Other differences from the known process according  
25 to the above-mentioned EP-A-0,027,710 consist in the choice of the complex nutrient solution. Whereas the known nutrient solution Brain Heart Infusion (37 g BHI/1) gave only a very small yield of antibiotic, a nutrient solution according to the invention consisting  
30 of 2 to 4% meat extract, 1 to 3% sugar or sugar alcohols, such as malt extract, maltose, galactose, lactose, mannitol, glucose or glycerol, and 0.25 to 1% calcium carbonate and/or 0.25 to 0.5% calcium hydroxide, gave very good results. The best production was  
35 achieved with a nutrient solution having the following composition: 3% meat extract, 2% malt extract and 0.37%

calcium hydroxide. Of all the C sources, maltose gave the best production after malt extract. Glucose should only be used in conjunction with other C sources. A combination of lactose and maltose, or galactose and maltose, also gave good results. All the other conventional C sources showed no or very little production. The addition of all 20 amino acids as an N source in concentrations of 2 g/l each gave the same results as the meat extract. Casamino acid (Difco) is a suitable source of N only if tryptophan (1-2 mm) and vitamins are added. Suitable vitamins include (preferred concentrations being shown in brackets): biotin (0.006 mg/l), nicotinic acid (2.3 mg/l), thiamine (1.0 mg/l), pyridoxine.HCl (12.0 mg/l), calcium pantothenate (1.2 mg/l).

Fermentation is carried out with good ventilation at temperatures of between 34 and 37°C. The best production pattern is obtained if the pH is between 6.0 and 7.0 before fermentation. In the absence of carbonates or hydroxides of divalent cations such as calcium carbonate or calcium hydroxide, no production takes place. After the addition of calcium carbonate, for example, the pH value shows a characteristic pattern, falling into the acid range, in which case no production occurs. When the pH value subsequently rises into the slightly alkaline range production begins. Instead of calcium carbonate it is also possible to use magnesium carbonate, whilst calcium hydroxide gives better results than calcium carbonate. With 50 mM calcium hydroxide, production can be increased compared with that obtained with 25 mM calcium carbonate. When the sources of C (sugars) are utilised by the strain, organic acids are formed which are complexed by divalent cations and at the same time the medium is buffered.

Figure 10 shows the results in terms of the curve representing the number of living bacteria and the production of antibiotic expressed in mm of inhibitory area against *Micrococcus luteus* ATCC 9341, of the media according to the invention compared with Brain Heart Infusion Medium (Difco) according to the above-mentioned European patent.

The following increases in activity at the moment of maximum production were obtained in the plate diffusion test using a calibrated line.

The activity in Brain Heart Infusion nutrient solution was equated with 100%.

|                                                                 |      |
|-----------------------------------------------------------------|------|
| a) Brain Heart Infusion Agar                                    | 100% |
| b) 3% meat extract, 2% malt extract,<br>25 mM calcium carbonate | 200% |
| c) 3% meat extract, 2% malt extract,<br>50 mM calcium hydroxide | 320% |

These data do not constitute absolute production values. However, a comparison of the values achieved in the plate diffusion test clearly shows that a significant increase in yields is achieved by using the procedure according to the invention compared with the known procedure.

The strain used to produce epidermin is characterised as follows according to Schleifer & Kloos [Int. J. Syst. Bact. 25, 50-61 (1975)]:

|                        |                                                 |
|------------------------|-------------------------------------------------|
| Gram coloration        | : positive                                      |
| Cell size              | : 0.5-0.8 $\mu$ m diameter                      |
| Colony size            | : about 1 mm in diameter                        |
| Appearance of colonies | : smooth, glossy, slightly raised in the centre |
| Colour of colonies     | : greyish-white                                 |
| Cells in culture       | : often single cells or groups of two, seldom   |

|    |                         |                                                                                                                        |
|----|-------------------------|------------------------------------------------------------------------------------------------------------------------|
|    |                         | larger clumps                                                                                                          |
|    | Haemolysis              | : cultures spread on blood slides showed strong haemolysis                                                             |
| 5  | Anaerobic growth        | : the strain grows even under anaerobic conditions                                                                     |
|    | Lysozyme sensitivity    | : the cells are resistant to lysozyme in quantities of up to 2 mg/ml                                                   |
| 10 | Lysostaphin sensitivity | : the cells are sensitive to lysostaphin even in quantities of 20 µg/ml                                                |
|    | Epidermin resistance    | : demonstrated up to 1 mg/ml                                                                                           |
|    | Other resistances       | : in liquid culture the strain shows a marked resistance to Streptomycin (up to 1 mg/ml) and Spectinomycin (0.5 mg/ml) |
| 15 |                         |                                                                                                                        |
|    | Increased NaCl content  | : the strain still grows well even up to a sodium chloride content of 15% by weight.                                   |
| 20 |                         |                                                                                                                        |

Colonies or spread-out cultures are very adhesive.

25        The utilisation of various C sources with acid formation and other enzyme reactions was determined using the Api-Staph System (Biomérieux, Nürtingen).

         This system is based on a combination of  
20 biochemical reactions which can be traced back  
30 to the classification of KLOOS & SCHLEIFER [J.Clin. Microbiol. 1, 82-88 (1975)].

**Table 1:** Utilisation of C sources and other enzyme reactions

|    | C source or enzyme                                                                                                                                | Producer | Staph. epid.<br>Kloos &<br>Schleifer | Staph.epid.<br>Api - Staph |
|----|---------------------------------------------------------------------------------------------------------------------------------------------------|----------|--------------------------------------|----------------------------|
| 5  | Control                                                                                                                                           | -        | -                                    | -                          |
|    | D-Glucose                                                                                                                                         | +        | n.d.                                 | +                          |
|    | D-Fructose                                                                                                                                        | +        | +                                    | +                          |
| 10 | D-Mannose                                                                                                                                         | (+)      | (+)                                  | +                          |
|    | Maltose                                                                                                                                           | +        | +                                    | +                          |
|    | Lactose                                                                                                                                           | (+)      | (+)                                  | +                          |
|    | D-Trehalose                                                                                                                                       | -        | -                                    | -                          |
|    | D-Mannitol                                                                                                                                        | -        | -                                    | -                          |
| 15 | Xylitol                                                                                                                                           | -        | -                                    | -                          |
|    | D-Melibiose                                                                                                                                       | -        | n.d.                                 | -                          |
|    | Raffinose                                                                                                                                         | -        | n.d.                                 | -                          |
|    | D-Xylose                                                                                                                                          | -        | -                                    | -                          |
|    | Sucrose                                                                                                                                           | +        | +                                    | +                          |
| 20 | α-Methylglucoside                                                                                                                                 | -        | n.d.                                 | -                          |
|    | N-Acetylglucosamine                                                                                                                               | -        | n.d.                                 | ±                          |
|    | Nitrate reduction                                                                                                                                 | +        | +                                    | ±                          |
|    | Phosphatase                                                                                                                                       | +        | +                                    | +                          |
|    | Formation of acetyl-                                                                                                                              | +        | n.d.                                 | +                          |
| 25 | methylcarbinol<br>(Voges-Proskauer<br>reaction)                                                                                                   |          |                                      |                            |
|    | Arginine dehydrolase                                                                                                                              | -        | n.d.                                 | ±                          |
|    | Urease                                                                                                                                            | +        | n.d.                                 | +                          |
| 30 | <hr/> + positive reaction<br>- negative reaction<br>(+) clearly positive reaction does not occur until later<br>± variable result<br>n.d. no data |          |                                      |                            |
| 35 |                                                                                                                                                   |          |                                      |                            |

The strain *Staphylococcus epidermidis* DSM 3095 was inoculated on slanting tubes with a medium having the following composition:

|   |                             |     |                           |
|---|-----------------------------|-----|---------------------------|
|   | Peptone                     | 10  | g                         |
| 5 | Disodium hydrogen phosphate | 2   | g                         |
|   | Meat extract                | 8   | g                         |
|   | Glucose                     | 10  | g (autoclaved separately) |
|   | Common salt                 | 3   | g                         |
|   | pH                          | 7.2 |                           |

- 10 then incubated overnight at 37°C and subsequently frozen at -20°C. A fresh tube was thawed for each new test mixture since losses of activity were observed on lengthy storage.

- A more precise description of the preparation  
15 of the antibiotic epidermin will now be given:

Fermentation can be carried out in suitable shaking flasks and in order to prepare larger quantities of substance fermenters with a capacity of 200 litres or more may be used.

- 20 For flask tests, 500 ml Erlenmeyer flasks with a lateral inlet were used. The flasks were filled with 100 ml of nutrient solution and autoclaved for 20 minutes at 121°C. The inoculant used was 1% of a preliminary culture 4 hours old. Incubation  
25 was carried out at 37°C in a shaking machine rotating at 140 rpm.

- For fermentation on a 10 litre scale, fermenters with a useful capacity of 10 litres (Model MF-14 made by New Brunswick Scientific Co., New Brunswick,  
30 USA) were filled with 9.9 l of nutrient solution and autoclaved for 30 minutes at 134°C. After cooling, the nutrient solution was inoculated with 100 ml of a preliminary culture 4 hours old and fermented at 37°C, 0.6 vvm and at a speed of 240 rpm

for the blade stirrer. The relatively strong foaming is counteracted by repeatedly adding sterile polyol.

For fermentation on a 25 litre scale, a 25 litre fermenter (Type b 25 made by Braun/Melsungen, with a recirculating system) was filled with 25 l of nutrient solution with the addition of 3 ml of polyol and sterilised in situ at 121°C for 30 minutes. The inoculant used was 300 ml of a preliminary culture 4 hours old. The fermentation was carried out at 37°C, 0.6 vvm and 1000 rpm.

The course of fermentation on a 10 litre scale in a nutrient solution containing 30 g of meat extract, 20 g of malt extract and 5 g of calcium carbonate per litre is shown in Figure 11. Intensive growth combined with the utilisation of the C source provided, with formation of acid, can be detected as the pH value falls. With the start of alkalisation the antibiotic can be detected in the culture filtrate. Production reaches a peak after 12 to 18 hours.

In order to monitor the course of fermentation, samples were taken under sterile conditions at various times during fermentation. The samples were evaluated as follows:

- a) pH value:  
Measured with a laboratory pH meter (Knick pH mV meter)
- b) Course of growth:  
Growth can be monitored by observing the increase in the number of living bacteria. To do this, 0.5 ml of culture taken under sterile conditions is diluted in saline and 0.1 ml of this dilution is spread onto slides (medium: peptone 10 g, meat extract 8 g, common salt 3 g, disodium hydrogen phosphate 2 g, glucose 10 g per litre). After 18 hours'

incubation at 37°C the individual colonies could be counted.

c) Concentration of antibiotic:

5 The samples were centrifuged in an Eppendorf centrifuge 3200 for 2 minutes and 20 µl of the supernatant were tested in a plate diffusion test. At the same time a calibration curve was plotted with known concentrations.

10 After maximum production had been reached the culture liquid was centrifuged off at 1380 rpm by continuous centrifuging (centrifuge: Type LA 71b-4, Loher & Söhne, Ruhstorf/Rott). For optimum separation of the cells the flow rate had to be kept very low. A first concentration of the active  
15 components was achieved using the adsorption on Amberlite XAD-8 mentioned on page 10, as described in the following Example 1:

Example 1

20 80 litres of culture filtrate was poured over about 8 litres of Amberlite XAD-8. No activity could be detected in the throughflow. When subsequent washing with water was carried out again no activity was eluted. Subsequently, washing was carried  
25 out with 13 litres of methanol. No activity was detected in the washing water. Elution was effected using methanol/hydrochloric acid 99:1, and the following fractions were obtained:

Fraction 1: 0.8 l active  
30 Fraction 2: 4.5 l main activity  
Fraction 3: 2 l active  
Fraction 4: 2 l no activity  
Fraction 5: 1 l no activity

The fractions were tested by thin layer chromatography for their epidermin content, then the active fractions were combined and concentrated by evaporation to dryness in a rotary evaporator (81.2 g). The  
5 residue was taken up in methanol/acetic acid (95/5) (400 ml), centrifuged to remove the insoluble components and subjected to gel chromatography in batches of 50 ml on Sephadex LH-20 (column 100x5 cm, eluant methanol/acetic acid 95:5). Positive fractions  
10 were combined and concentrated by evaporation (14.8 g).

For multiplicative distribution according to Craig, apparatus made by Labortec (Basle) were used. First separation was carried out with a 50 ml apparatus using a system of n-butanol/ethyl  
15 acetate/0.1 M acetic acid (3/1/3). The sample (5 g) dissolved in the lower phase (100 ml) was subjected to the following conditions for separation:

|                                |         |
|--------------------------------|---------|
| Number of stages:              | 160     |
| Vibrating movements per stage: | 70      |
| 20 Intensity of vibration:     | 45      |
| Separating time:               | 20 min. |

For final purification the crude product obtained (4.2 g) was dissolved in batches of 2 g in the lower phase of a 2-butanol/0.05 N ammonium  
25 acetate system (50 ml) and purified in a 10 ml apparatus with 440 elements.

|                                |         |
|--------------------------------|---------|
| Number of stages:              | 440     |
| Vibrating movements per stage: | 50      |
| Intensity of vibration:        | 50      |
| 30 Separating time:            | 10 min. |

The elements containing epidermin were combined, concentrated by evaporation, taken up with water

and lyophilised several times under high vacuum. The antibiotic (2.6 g) proved to be uniform in all the tests carried out.

During the preparation and isolation of the epidermin the plate diffusion test was used for biological characterisation, namely to record the spectrum of activity and to monitor production, working up and concentration. The test was carried out in Petri dishes made by Greiner, Nürtingen, with filter discs 6 mm in diameter (Macherey & Nagel, Düren) as described in Zähner and Maas, Biology of Antibiotics (Springer Verlag, Berlin-Heidelberg-New York, 1972). The filter discs were wetted with 20 µl of the appropriate test liquid, dried on a glass slide at ambient temperature and placed on the test plates. The test plates were incubated at 37°C and the inhibition of growth was evaluated after about 16 hours. The routine test bacteria used were *Streptococcus pyogenes* ATCC 8668 and, owing to its less problematic handling, *Micrococcus luteus* ATCC 9341.

Test plates with *Streptococcus pyogenes* ATCC 8668: Before preparation of the test plates the test bacteria had to be freshly inoculated on blood plates with mucin [medium: 500 ml of agar pH 7.4, 160 ml of mucin solution (10% by weight), 3.5 ml of glucose solution (50% by weight) and 70 ml of sheep's blood]. After 15 to 18 hours' incubation at 37°C a saline suspension ( $E_{578}$  0.5) was prepared and 1 ml of this suspension was pipetted into 100 ml of nutrient base (medium: peptone 10 g, meat extract 8 g, common salt 3 g,  $\text{Na}_2\text{HPO}_4$  2 g, glucose 10 g per litre, pH 7.2). The plates carrying 10 ml batches could be stored for several days at 4°C.

Test plates with *Micrococcus luteus* ATCC 9341: An overnight culture (medium: peptone 10 g, meat extract 8 g, common salt 3 g,  $\text{Na}_2\text{HPO}_4$  2 g, glucose

10 g per litre, pH 7.2) was diluted to an extinction of 1.0 at 578 nm. 100 ml of agar was inoculated with 0.25 ml of this suspension and 10 ml batches were poured over slides. The test plates could  
5 be stored for several days at 4°C.

Test plates for the activity spectrum:

Test plates with bacteria:

α) Aerobic bacteria:

10 Overnight cultures were grown in the relevant test media and the plates were prepared as described for *Micrococcus luteus* ATCC 9341.

β) Anaerobic bacteria:

15 The preliminary cultures for *Clostridium pasteurianum* ATCC 6013 and *Propionibacterium acnes* DSM 1897 were grown in test tubes which had been filled up to the cotton wool stopper with nutrient solution in order to displace the oxygen. 100 ml of agar was inoculated  
20 with 3 ml of the existing culture and 10 ml batches were poured over the plates. Incubation was effected in an anaerobic pot (BBL-Gas Pak 100, Becton, Dickinson GmbH, Heidelberg) at the optimum temperature.

Test plates with yeast-like fungi:

25 The cells were grown in the relevant test medium for 18-20 hours in a shaken culture. After counting had been carried out in a Thoma counting chamber the test plates were inoculated to a density of  $10^6$  organisms per ml of agar.

30 Test plates with fungi and Streptomycetes:

The test organisms were grown on slanting tubes (medium: yeast extract 4 g, malt extract 10 g, glucose 4 g per litre) at the corresponding temperatures until sporulation occurred. The spores

were floated off with 3 ml of saline Tween 80 (1 drop of Tween 80 to 100 ml of saline) and poured into test plates.

5       The substance epidermin has a very good antibacterial activity; it is particularly effective against a series of gram-positive bacteria. The antibacterial effect of epidermin was tested by comparison with that of nisin (for nisin cf. inter alia DE-A-2,000,818 or GB-B-1,182,156); for a few important, clinically  
10 relevant bacteria, fusidic acid was also included in the comparison. The effectiveness was determined by the plate diffusion test and by determining the minimum inhibitory concentrations.

a)     Plate diffusion test:

15       The plate diffusion test tested the sensitivity of various microorganisms to epidermin and nisin. Table 2 shows that both these antibiotics act almost exclusively on gram-positive bacteria. Nisin with an activity of 40,000 units was used.

**Table 2: Sensitivity to epidermin and nisin**  
Given in mm of inhibited area, concentrations 1 mg/ml  
and 0.5 mg/ml.

| Microorganisms                | Culture conditions |        | Epidermin |     | Nisin |     |
|-------------------------------|--------------------|--------|-----------|-----|-------|-----|
|                               | Temp.              | Medium | 1         | 0,5 | 1     | 0,5 |
| <u>Bacteria</u>               |                    |        |           |     |       |     |
| Eubacteriales, gram-positive  |                    |        |           |     |       |     |
| -----                         |                    |        |           |     |       |     |
| Arthrobacter aurescens        | 27°C               | 5      | 8         | Sp  | 9     | 8   |
| Arthrobacter crystallopoietes | 27°C               | 5      | 16        | 15  | 16    | 15  |
| Arthrobacter globiformis      | 27°C               | 5      | 14        | 12  | 14    | 13  |
| Arthrobacter oxydans          | 27°C               | 5      | 12        | 11  | 11    | 10  |
| Arthrobacter pascens          | 27°C               | 5      | 13        | 12  | 14    | 13  |
| Bacillus cereus               | 37°C               | 3      | 8         | Sp  | -     | -   |
| Bacillus pumilus              | 37°C               | 4      | 14        | 12  | 12    | 10  |
| Bacillus subtilis ATCC 6051   | 37°C               | 4      | 12        | 11  | 9     | 8   |
| Bacillus subtilis ATCC 6051   | 37°C               | 6      | 17        | 16  | 14    | 12  |
| Bacillus subtilis ATCC 6633   | 37°C               | 4      | 14        | 11  | 9     | 8   |
| Bacillus subtilis F 24-2      | 37°C               | 4      | 13        | 11  | 8     | Sp  |
| Bacillus subtilis A 14        | 37°C               | 4      | 12        | 10  | 9     | Sp  |
| Brevibacterium flavum         | 37°C               | 4      | 11        | 10  | 8     | 7   |
| Clostridium pasteurianum      | 30°C               | 7      | 17        | 15  | 14    | 13  |
| Clostridium sporogenes        | 37°C               | 8      | 10        | 9   | 8     | Sp  |
| Corynebacterium spec.         | 27°C               | 4      | 15        | 13  | 9     | 8   |
| Corynebacterium insidiosum    | 27°C               | 4      | 19        | 17  | 10    | 8   |
| Corynebacterium rathayi       | 27°C               | 4      | 14        | 12  | 10    | 9   |
| Micrococcus luteus ATCC 381   | 27°C               | 5      | 10        | 9   | -     | -   |
| Micrococcus luteus ATCC 9341  | 27°C               | 2      | 21        | 20  | 17    | 16  |
| Propionibacterium acnes       | 37°C               | 8      | 22        | 19  | 21    | 18  |
| Sarcina lutea                 | 37°C               | 5      | 15        | 14  | 15    | 14  |
| Staphylococcus aureus Tü 202  | 37°C               | 4      | 13        | 10  | 10    | 9   |

|                               |      |   |    |    |    |    |
|-------------------------------|------|---|----|----|----|----|
| Staphylococcus aureus DSM 683 | 37°C | 4 | 11 | 9  | Sp | -  |
| Staphylococcus aureus Pen.res | 37°C | 4 | 12 | 11 | 9  | 8  |
| Staphylococcus cohnii         | 37°C | 2 | 14 | 12 | 9  | -  |
| Streptococcus pyogenes        | 37°C | 2 | 22 | 20 | 18 | 17 |

Eubacteriales, gram-negative:

|                   |      |   |    |   |   |    |
|-------------------|------|---|----|---|---|----|
| Proteus mirabilis | 37°C | 4 | 11 | 9 | 9 | 7  |
| Proteus vulgaris  | 37°C | 5 | 9  | 8 | 8 | Sp |

Actinomycetales:

|                                     |      |   |    |    |    |   |
|-------------------------------------|------|---|----|----|----|---|
| Streptomyces glaucescens            | 27°C | 3 | 10 | 8  | 10 | 9 |
| Streptomyces violaceoruber          | 37°C | 3 | 9  | 8  | -  | - |
| Streptomyces virido-<br>chromogenes | 37°C | 3 | 11 | 10 | 9  | 8 |

---

(-) indicates that there was no activity;

Sp = traces

b) Minimum inhibitory concentration:

The minimum inhibitory concentration for clinically relevant bacteria given in µg/ml was

5 tested in microtitre plates in the following medium:

5 ml Na-lactate, 5 g Na<sub>2</sub>SO<sub>4</sub>, 0.5 g KH<sub>2</sub>PO<sub>4</sub>, 0.1 g MgCl<sub>2</sub>,  
 5 g NH<sub>4</sub>Cl, 10 g glucose, 50 µg calcium pantothenate,  
 50 µg thiamine, 0.25 µg folic acid, 50 µg niacin,  
 25 µg p-aminobenzoic acid, 50 µg pyridoxine hydrochloride,  
 10 25 µg riboflavin in 1000 ml of distilled water.

Table 3 shows the minimum inhibitory concentrations ( $\mu\text{g/ml}$ ) of epidermin, nisin and fusidic acid in a comparative study. The nisin used here had an activity of 2500 units.

5

Table 3:

Minimum inhibitory concentration in  $\mu\text{g/ml}$ :

|                                    | Epidermin   | Fusidic acid | Nisin  |
|------------------------------------|-------------|--------------|--------|
| <i>St. epidermidis</i> WG 99       | 2           | 4            | 128    |
| <i>Sc. pyogenes</i> ATCC 8668      | 0,125       | 1            | 4      |
| <i>Sc. pneumoniae</i> ATCC 6302    | 2           | 16           | 32     |
| <i>Mc. luteus</i> ATCC 15957       | 0,25        | 0,5          | 16     |
| <i>Mc. luteus</i> ATCC 9341        | $\leq 0,06$ | 0,5          | 8      |
| <i>Cb. xerosis</i> NCTC 9755       | 0,125       | 0,125        | 16     |
| <i>E. coli</i> ATCC 11775          | 128         | $>128$       | $>128$ |
| <i>E. coli</i> ATCC 9637           | 128         | $>128$       | $>128$ |
| <i>Propionib. acnes</i> PC 904     | 0,06        | 0,5          | 32     |
| <i>Propionib. acnes</i> ATCC 25746 | 0,25        | 1            | 64     |

The media mentioned in Table 2 are described hereinafter. The media were autoclaved for 20  
10 minutes at  $121^{\circ}\text{C}$  after the pH value had been adjusted.

The quantities given all refer to 1 litre of water. In the case of chemically defined media, deionised water was used.

Medium:

- |    |     |                                                            |                              |
|----|-----|------------------------------------------------------------|------------------------------|
|    | (2) | Glucose-agar medium:                                       |                              |
|    |     | Peptone                                                    | 10 g                         |
|    |     | Meat extract                                               | 8 g                          |
| 5  |     | NaCl                                                       | 3 g                          |
|    |     | Na <sub>2</sub> HPO <sub>4</sub>                           | 2 g                          |
|    |     | Glucose                                                    | 10 g (autoclaved separately) |
|    |     | Agar                                                       | 20 g                         |
|    |     | pH 7.2                                                     |                              |
| 10 | (3) | Yeast-malt medium:                                         |                              |
|    |     | Yeast extract                                              | 4 g                          |
|    |     | Malt extract                                               | 10 g                         |
|    |     | Glucose                                                    | 4 g                          |
|    |     | Agar                                                       | 20 g                         |
| 15 |     | pH 7.3                                                     |                              |
|    | (4) | Oxoid medium for bacteria:                                 |                              |
|    |     | Meat extract                                               | 10 g                         |
|    |     | Peptone                                                    | 10 g                         |
|    |     | NaCl                                                       | 5 g                          |
| 20 |     | Agar                                                       | 20 g                         |
|    |     | pH 7.2                                                     |                              |
|    | (5) | Nutrient broth                                             | 8 g                          |
|    |     | (Difco)                                                    |                              |
|    |     | Agar                                                       | 20 g                         |
| 25 |     | pH 7.2                                                     |                              |
|    | (6) | Minimal medium for bacteria (HÜTTER <u>et al.</u> , 1966): |                              |
|    |     | D-Glucose                                                  | 8 g                          |
|    |     | Di-ammonium tartrate                                       | 4 g                          |
|    |     | NaCl                                                       | 5 g                          |
| 30 |     | K <sub>2</sub> HPO <sub>4</sub>                            | 2 g                          |
|    |     | MgSO <sub>4</sub> ·7H <sub>2</sub> O                       | 1 g                          |
|    |     | CaCl <sub>2</sub>                                          | 0.2 g                        |
|    |     | MnSO <sub>4</sub> ·H <sub>2</sub> O                        | 0.01 g                       |
|    |     | Ferrioxamine B                                             | 0.02 g                       |
| 35 |     | Agar                                                       | 20 g                         |
|    |     | pH 7.2                                                     |                              |

(7) Medium for *Clostridium pasteurianum*:

|   |               |     |   |
|---|---------------|-----|---|
|   | Meat extract  | 3   | g |
|   | Yeast extract | 3   | g |
|   | Malt extract  | 3   | g |
| 5 | Peptone       | 20  | g |
|   | D-Glucose     | 5   | g |
|   | Ascorbic acid | 0.2 | g |
|   | Agar          | 20  | g |
|   | pH 7.0        |     |   |

10 (8) Brewer thioglycolate medium for *Propionibacterium acnes*:

|  |                      |      |   |
|--|----------------------|------|---|
|  | Thioglycolate medium | 40.5 | g |
|  | Agar                 | 20   | g |
|  | pH 7.2               |      |   |

15        Epidermin is very well tolerated and when used topically no toxic effects are detected.

         A major advantage is the fact that the resistant mutant *Staphylococcus epidermidis* DSM 3095 is resistant to the epidermin which it produces. The strain

20 NCIB 11536 has no such resistance to the low-molecular antibiotic which it produces.

         The new clone DSM 3095 was obtained as follows:

         Adaptation was carried out in 100 ml Erlenmeyer  
flasks with a lateral insert and 10 ml of nutrient  
25 solution (Brain Heart Infusion). The starting  
culture was inoculated with 0.5 ml of a culture  
12 hours old. For the flasks which contained increasing  
concentrations of epidermin, 0.5 ml of the previous,  
well-grown culture were used as inoculant. The  
30 growth was assessed photometrically at 578 nm.  
The culture, which was still well-grown at a concentration  
of 1.0 mg/ml of epidermin in liquid culture, was  
diluted in saline and spread onto a slide into  
which 0.5 mg/ml of epidermin had been poured.  
35 As a comparison, an undiluted 12-hour-old culture

of the comparison strain NCIB 11536, known from the literature, was spread on a plate. After 24 hours' incubation at 37°C, 94 individual colonies were visible in the adapted culture diluted to  
5  $10^{-6}$ . The comparison strain spread out undiluted on plates containing 0.15 mg/ml did not grow even after 48 hours' incubation. The resistant colonies were selected and spread onto plates of medium  
10 2 in a grid pattern. After a preliminary test for production by pricking out fragments with a diameter of 5 mm and testing the activity on *Micrococcus luteus* ATCC 9341, productive clones were tested in a liquid medium.

15 Comparison of the resistant strain with the strain known from the literature:

a) Minimum inhibitory concentration in the plate diffusion test

The minimum inhibitory concentrations shown  
20 in Table 4 were obtained on test plates containing the two strains.

Table 4: Minimum inhibitory concentrations of epidermin in the plate diffusion test with NCIB 11536  
25 and DSM 3095.

| Strain      | $\mu\text{g/ml}$ |
|-------------|------------------|
| NCIB 11536  | 10               |
| 30 DSM 3095 | > 1000           |

b) Growth and production:

The growth and production patterns of the two strains were compared in a nutrient solution [3% meat extract,  
35 2% malt extract, 0.37%  $\text{Ca}(\text{OH})_2$ ]. See Figure 12.

Growth pattern: determination of number of living bacteria.

Production pattern: activity against *Micrococcus luteus* ATCC 9341 in the plate diffusion test.

- 5 Better production is observed with the resistant strain.

c) Sensitivity to epidermin in various growth phases:

- 10 Tests on the inherent inhibition of the producing strain by the antibiotic and a test on the resistance of the selected clones were carried out in a biophotometer (Eppendorf photometer with automatic circulating means).

- 15 These tests may, for example, be carried out as follows:

0.5 ml batches of a 12-hour-old culture of *Staphylococcus epidermidis* NCIB 11536 or the selected resistant strain DMS 3095 were inoculated onto fresh medium.

- 20 These cultures were left to grow until an extinction of 0.043 at 578 nm was obtained (dishes with a layer thickness of 1 cm) and were then distributed in batches of 7.5 ml in the biophotometer cuvettes (layer thickness 2 cm).

- 25 The density of the cell suspension was adjusted to a transmission of 90% in the biophotometer. Incubation was carried out at 37°C with maximum ventilation. Epidermin was added as an aqueous solution in various growth phases.

- 30 In both strains, different epidermin concentrations were added at the beginning and in the middle of the logarithmic phase. The results are shown in Figures 13 and 14.

- 35 No lysis was observed in the resistant strain with the concentration tested hitherto.

The antibiotic epidermin according to the

invention, like the culture liquor from which it is isolated, exerts a bactericidal effect which results in lysis of the cells.

In view of its bactericidal activity and  
5 broad spectrum activity against gram-positive bacteria, the polypeptide antibiotic epidermin according to the invention is particularly effective in the treatment of infections caused by gram-positive bacteria. Epidermin is particularly valuable for  
10 combating skin infections such as eczema, impetigo, cellulitis and particularly acne. Its extremely good effectiveness against some important strains of *Propionibacterium acnes* has already been shown in Tables 2 and 3. It can be said that epidermin,  
15 which is normally produced by organisms inhabiting human skin, is more effective at protecting the skin than other conventional antibiotics.

The pharmaceutical preparations mentioned hereinbefore which contain the antibiotic according  
20 to the invention together with pharmaceutically conventional excipients and carriers, may be applied orally, parenterally, enterally or topically. These preparations, particularly topical preparations, may be presented as solutions, emulsions, gels,  
25 lotions, ointments, creams or powders.

The following Examples relate to the preparation of these pharmaceutical forms:

Example 2

30 Tincture

100 g of tincture contains:

|                            |        |
|----------------------------|--------|
| Epidermin                  | 1.0 g  |
| Ethanol (94.5 % by volume) | 56.0 g |
| 1,2-Propylene glycol       | 40.0 g |
| 35 Demineralised water     | 3.0 g  |

Preparation:

Epidermin is dissolved in a mixture of ethanol/1,2-propylene glycol/water and the solution is then filtered sterile.

5

Example 3

Lotion

100 g of lotion contains:

|    |                                                                    |        |
|----|--------------------------------------------------------------------|--------|
|    | Epidermin                                                          | 1.00 g |
| 10 | 1,2-Propylene glycol                                               | 7.00 g |
|    | Alkyldimethylbenzylammonium chloride (Benzalkon A <sup>(R)</sup> ) | 0.15 g |
|    | Sorbitan monopalmitate (Span 40 <sup>(R)</sup> )                   | 0.40 g |
| 15 | Sorbimacrogol palmitate (Tween 40 <sup>(R)</sup> )                 | 1.20 g |
|    | Decyl oleate (Cetiol V <sup>(R)</sup> )                            | 2.40 g |
|    | Mixture of cetyl and stearyl alcohol (Lanette O <sup>(R)</sup> )   | 1.60 g |
| 20 | Cetyl palmitate                                                    | 0.80 g |
|    | Demineralised water ad                                             | 100 g  |

Preparation:

The above quantities of alkyldimethylbenzylammonium chloride, sorbitan monopalmitate, sorbimacrogol palmitate, decyl oleate, cetyl and stearyl alcohol and cetyl palmitate are stirred into 75 ml of water, the filtered solution of epidermin in 1,2-propylene glycol and the remaining water are stirred in, and the tincture is homogenised.

30

Example 4

Gel

100 g of gel contains:

|    |                                                                       |       |
|----|-----------------------------------------------------------------------|-------|
|    | Epidermin                                                             | 1.0 g |
| 35 | Polyethylene glycol ether of lauryl alcohol (Brij 35 <sup>(R)</sup> ) | 1.0 g |

|   |                                                     |    |        |
|---|-----------------------------------------------------|----|--------|
|   | 1,2-Propylene glycol                                |    | 5.0 g  |
|   | Acrylic acid polymer (Carbopol 934 <sup>(R)</sup> ) |    | 1.2 g  |
|   | Methyl p-hydroxybenzoate                            |    | 1.6 g  |
|   | Propyl p-hydroxybenzoate                            |    | 0.4 g  |
| 5 | Perfume                                             |    | q.s.   |
|   | Sodium hydroxide solution                           | ad | pH 6.5 |
|   | Demineralised water                                 | ad | 100 g  |

Preparation:

- The specified quantities of excipient are
- 10 stirred into 75 ml of water; the epidermin is dissolved in a mixture of 1,2-propylene glycol and the remaining water, and this solution is again stirred in; the finished gel is homogenised once more.

Legend to the figures:

- Figure 1: Amino acid chromatogram of the acid total hydrolysate of epidermin; ninhydrin coloration,  $\lambda = 570$  nm.
- 5 Figure 2: Gas chromatogram of the n-propylester of N-pentafluoropropionyl-amino acid of the acid total hydrolysate of epidermin on Chiralsil-Val. Temperature programme 3 minutes at 85°C, isothermic, then 4°C per minute up to 200°C; carrier gas  $H_2$  (0.92 bar).
- 10 Figure 3: UV spectrum of epidermin in water, pH 3 (C=0.15 mg/ml).
- Figure 4: Infra-red spectrum of epidermin in a potassium bromide tablet.
- 15 Figure 5:  $^1H$  nuclear resonance spectrum of epidermin (20 mg/0.5 ml  $D_7$ -dimethylformamide, 400.16 MHz).
- Figure 6:  $^{13}C$  nuclear resonance spectrum of epidermin (40 mg/0.5 ml  $^{12}C$ ,  $^2H$ -dimethylformamide, 100.6 MHz, 45360 pulses).
- 20 Figure 7: HPLC chromatogram of epidermin on  $\mu$  Bondapak  $C_{18}$  (300x3.9 mm). Mobile phase: A=acetonitrile/0.01 M  $KH_2PO_4$  (10/90), B=acetonitrile/0.01 M  $KH_2PO_4$  (70/30); linear gradient of 10% B to 100% B in 30 minutes, flow rate 2 ml/min.
- 25 Figure 8:  $^{13}C$  nuclear resonance spectrum of the fragment P2 (12 mg/0.5 ml  $^{12}C$ ,  $^2H$ -dimethylformamide, 100.6 MHz, 38300 pulses).
- Figure 9: Isolation of epidermin.
- Figure 10: Comparison of the media in terms of
- 30 the number of living bacteria (continuous lines) and activity against *Micrococcus luteus* (dash-dot lines)
- ▲ Brain Heart Infusion
  - 3% meat extract 2% malt extract 0.25%  $CaCO_3$
  - 3% meat extract 2% malt extract 0.37%  $Ca(OH)_2$
- 35 Figure 11: Course of fermentation on a 10 litre scale
- pH curve
  - ▲ number of living bacteria

○ activity against *Streptococcus pyogenes*  
ATCC 8668

5      Figure 12: Comparison of strain known from the  
         literature with resistant clone DSM 3095 by  
         means of the number of living bacteria (continuous  
         lines) and activity against *Micrococcus luteus*  
         ATCC 9341 (broken lines).

○ strain known from the literature

▲ resistant clone

10     Figure 13: Addition of epidermin at various growth  
         phases in *Staphylococcus epidermidis* NCIB 11536.  
         Curve 1: normal growth pattern  
         Curve 2: addition of 10 µg/ml of epidermin at  
         the start of the logarithmic phase results in lysis.  
15     Curve 3: addition of 10 µg/ml of epidermin  
         in the middle of the logarithmic phase also  
         results in lysis.  
         Curve 4: addition of 2.5 µg/ml of epidermin  
         in the middle of the logarithmic phase leads  
20     to retardation of growth.

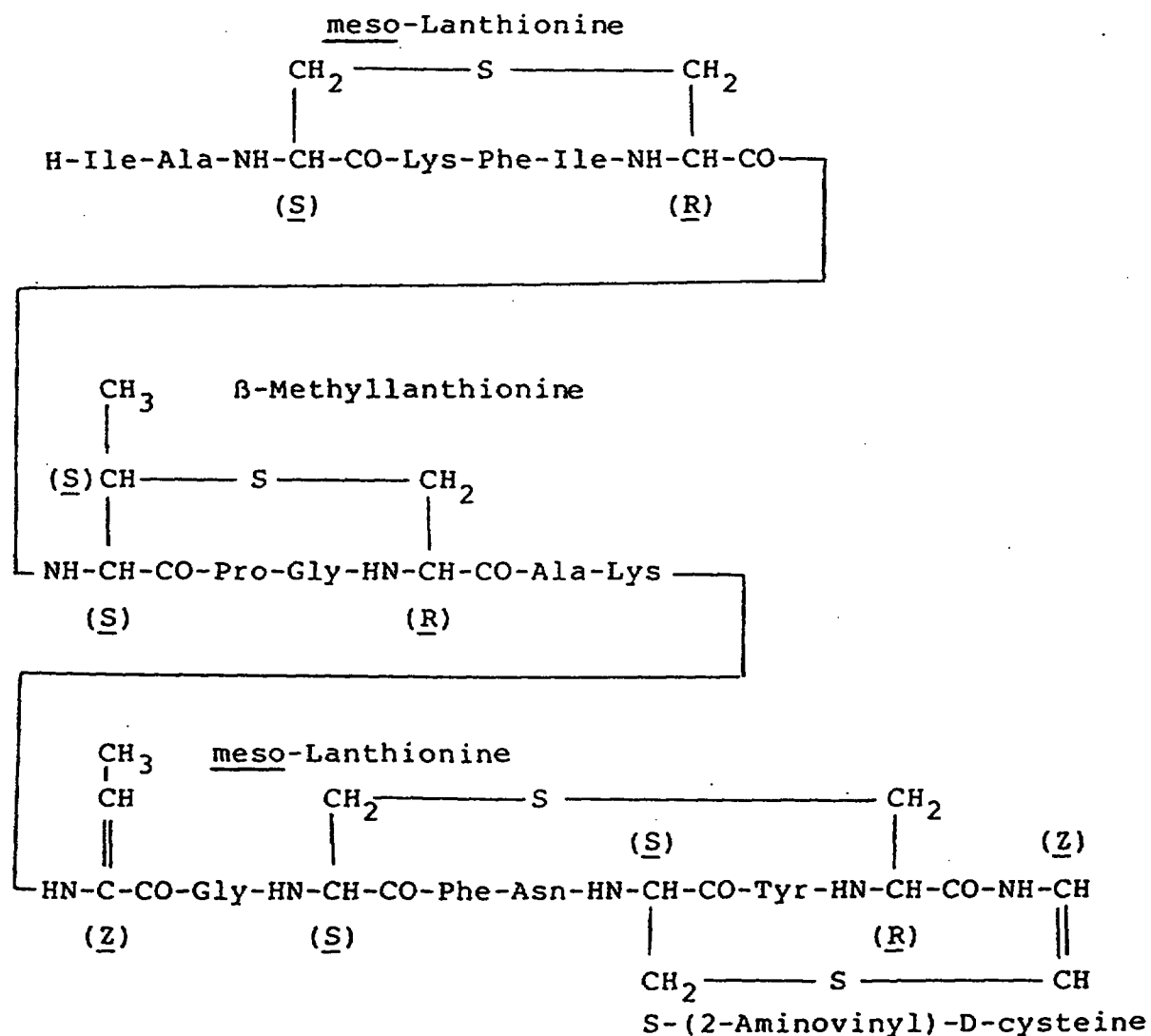
         Figure 14: Addition of epidermin at various growth  
         phases in the resultant clone DSM 3095.  
         Curve 1: normal growth pattern, identical  
         to the strain NCIB 11536.  
25     Curve 2: addition of 120 µg/ml of epidermin  
         at the beginning of the logarithmic phase  
         produces only a slight retardation of growth.  
         Curve 3: addition of 360 µg/ml of epidermin  
         in the middle of the logarithmic phase again  
30     results only in a retardation of growth.

         Figure 15: Gas chromatogram of the N-propylester  
         of N-pentafluoropropionyl amino acid of a  
         total hydrolysate of H1 on Chirasil-Val.  
         Temperature programme 3 minutes at 80°C,  
35     isothermic, then 4°C per min. up to 200°C,  
         carrier gas H<sub>2</sub>.



Patent Claims

1. Antibiotically active polypeptide known as epidermin, characterised by the following amino acid composition: Asn (1), Pro (1), Gly (2), Ala (2), Ile (2), Phe (2), Lys (2), Lan (2),  $\beta$ -Me-Lan (1), Dhb (1), Tyr (1), S-(2-aminovinyl)-D-cysteine (1) with the following primary structure:





whilst the halves of the individual thioetheramino acids closer to the N-terminus are of D configuration.

2. Epidermin as claimed in claim 1, characterised  
5 by the following additional parameters:

1. Nature: colourless powder;
2. Solubility: very readily soluble in mixtures  
10 of water/glacial acetic acid or methanol/glacial  
acetic acid, soluble in lower alcohols, insoluble  
in chloroform, acetone, diethyl ether, petroleum  
ether;
3. Colour reaction on silica gel plates: Ninhydrin,  
15 chlorine/TDM [TDM=4,4'-bis-(dimethylamino)-  
diphenylmethane], orcin/sulphuric acid,  
anisaldehyde/sulphuric acid. Non-destructive  
detection in UV light at 254 nm and by spraying  
with water;
4. Thin layer chromatography: ready-made silica  
20 gel plates 60 F<sub>254</sub> (Merck) were used.  
System A: chloroform/methanol/  
17% ammonia (2/2/1),  $R_F = 0.73$   
System B: chloroform/methanol/  
17% ammonia (70/35/10)  $R_F = 0.30$   
25 System C: n-butanol/glacial acetic acid/  
water (4/1/1)  $R_F = 0.05$
5. HPLC: see Fig. 7.
6. Stability: stable from pH 2 to pH 7, but  
30 a sharp drop in activity occurs at higher  
pH values;
7. Molecular mass: in the region of 2160 (without  
anions);
8. Ultra-violet absorption spectrum: in aqueous  
solution, long-wave maximum at 267 nm (Fig. 3);
- 35 9. Infra-red absorption spectrum: IR spectrum  
of a sample in a potassium bromide tablet  
(Fig. 4);



10. Nuclear magnetic resonance spectra  
    <sup>1</sup>H-NMR spectrum: Fig. 5, <sup>13</sup>C-NMR spectrum:  
    Fig. 6.
3. Process for preparing, isolating and purifying  
5 the antibiotic epidermin, as claimed in claims  
1 and 2, characterised in that Staphylococcus epidermidis  
DSM 3095 is grown aerobically at 34-37°C in a complex  
nutrient solution consisting of 2-4% of a nitrogen  
source such as meat extract, 1-3% of a sugar or  
10 sugar alcohol as the source of carbon and  
0.25 to 1% of a carbonate and/or 0.25 to 0.5% of a hydroxide  
of an alkaline earth metal, then, after removal  
of the cells and inorganic salts, in order to isolate  
the active substance formed, the culture liquor  
15 is either extracted with n-butanol at pH 8, the  
butanol extract is evaporated, the residue is dissolved  
in methanol and the solution is freed from lipid  
contaminants by ether precipitation or it is treated  
with polymers based on acrylate or polystyrene,  
20 whilst the adsorbed epidermin is released from  
the resin with methanol/concentrated hydrochloric  
acid (99:1), the solution is then neutralised with  
ammonia and concentrated by evaporation in vacuo,  
and the isolated material thus obtained is subjected  
25 to gel chromatography [Sephadex LH-20 with methanol/acetic  
acid (95:5)] in order to separate off other low-  
molecular weight peptides, amino acids and salts, and  
then subjected to multiplicative countercurrent distribution  
according to Craig, in which first a liquid-liquid  
30 distribution is effected using a system of n-butanol/ethyl  
acetate/0.1 N acetic acid (3:1:3) in which epidermin  
is left on the baseline, and subsequently  
in a second distribution using the neutral system  
2-butanol/0.05 N ammonium acetate (1:1), the pure  
35 epidermin is isolated as a colourless powder from the  
eluate by freeze-drying.



4. Process as claimed in claim 3, characterised in that, as nitrogen source, 2-4% by weight of a meat extract, a mixture of all 20 amino acids in amino acid concentrations of 2 g/l, or 2 to 4% by weight of casamino acid in the presence of tryptophan, and as carbon source 1 to 3% by weight of malt extract, maltose, galactose, lactose, mannitol, glucose, glycerol or combinations of lactose with maltose, or galactose with maltose, and 0.25 to 1% by weight of calcium carbonate or 0.25 to 0.5% by weight of calcium hydroxide are introduced into the fermentation medium and, if casamino acid is used, vitamins such as biotin, nicotinic acid, thiamine, pyridoxine.HCl and calcium pantothenate are added, the pH value is adjusted to 6.0 to 7.0 before fermentation and the course of production is monitored by continuous sampling.

5. Epidermin obtained by the process claimed in claim 3.

6. As a new epidermin-producing strain which is resistant to epidermin, *Staphylococcus epidermidis* DSM 3095.

7. *Staphylococcus epidermidis* DSM 3095 with the following characteristics:

|                        |                                                             |
|------------------------|-------------------------------------------------------------|
| Gram coloration        | : positive                                                  |
| Cell size              | : 0.5-0.8 $\mu$ m in diameter                               |
| Colony size            | : about 1 mm in diameter                                    |
| Appearance of colonies | : smooth, glossy, slightly raised in the centre             |
| Colour of colonies     | : greyish-white                                             |
| Cells in culture       | : often single cells or groups of two, seldom larger clumps |



- 40 -

|    |                         |                                                                                                                        |
|----|-------------------------|------------------------------------------------------------------------------------------------------------------------|
|    | Haemolysis              | : cultures spread on blood slides showed strong haemolysis                                                             |
| 5  | Anaerobic growth        | : the strain grows even under anaerobic conditions                                                                     |
|    | Lysozyme sensitivity    | : the cells are resistant to lysozyme in quantities of up to 2 mg/ml                                                   |
| 10 | Lysostaphin sensitivity | : the cells are sensitive to lysostaphin even in quantities of 20 µg/ml                                                |
|    | Epidermin resistance    | : demonstrated up to 1 mg/ml                                                                                           |
| 15 | Other resistances       | : in liquid culture the strain shows a marked resistance to Streptomycin (up to 1 mg/ml) and Spectinomycin (0.5 mg/ml) |
| 20 | Increased NaCl content  | : the strain still grows well with a sodium chloride content of up to 15% by weight,                                   |

the colonies or the spread-out cultures being adhesive.

25 8. Antibacterial pharmaceutical preparations, characterised in that they contain epidermin as claimed in claims 1 and 2 together with conventional carriers and/or excipients.

30 9. Epidermin for use as an antibacterial agent.