

PATENT SPECIFICATION

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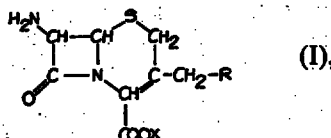
(54) PROCESS FOR THE PREPARATION OF 7-AMINO- Δ^3 -CEPHEM DERIVATIVES

(71) We, BAYER AKTIENGESELLSCHAFT, a body corporate organised under the laws of Germany, of Leverkusen, Germany, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a new process for the preparation of certain 7-amino- Δ^3 -cephem derivatives which can be used as intermediates for the synthesis of anti-microbial active compounds.

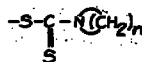
It has already been disclosed that 7-amino-cephalosporanic acid is obtained, for example, by the splitting of cephalosporin C with cultures of various micro-organisms (see, for example, U.S. Patent Specification 3,239,394) or by chemical splitting according to various methods (see, for example, German Auslegeschrift (German Published Specification) 1,176,147, German Offenlegungsschrift (German Published Specification) 1,145,615 or U.S. Patent Specification 3,272,809). Desacetoxy-cephalosporin C can, after catalytic reduction (see U.S. Patent Specification 3,124,576), be converted analogously into 7-amino-desacetoxy-cephalosporanic acid. German Offenlegungsschrift (German Published Specification) 2,212,276 has also disclosed a process for the enzymatic splitting of 7-phenoxyacetamido-desacetoxy-cephalosporanic acid to 7-amino-desacetoxy-cephalosporanic acid with the aid of an adsorptively bound enzyme from *Bacillus megaterium* B 400. Several processes for splitting penicillins with the aid of penicillin acylase bound by covalent bonds to water-insoluble carriers have also been disclosed (see German Offenlegungsschriften (German Published Specifications) 1,907,365; 1,917,057 and 2,215,687).

The present invention provides a process for the preparation of a 7-amino- Δ^3 -cephem derivative of the general formula



in which

R denotes hydrogen, hydroxyl, amino, nitrile, $-\text{O}-\text{CO}-\text{NH}_2$,



, with n denoting an integer from 4 to 6, $-\text{O}-\text{CO}-\text{R}^1$, $-\text{NH}-\text{CO}-\text{R}^1$ or $-\text{S}-\text{CS}-\text{O}-\text{R}^1$, with R^1 denoting straight-chain or branched alkyl with 1 to 4 carbon atoms;

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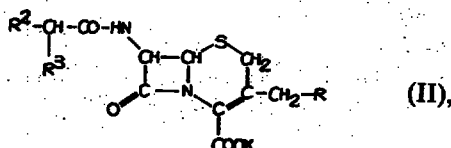
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or in which

R denotes —S—Het or Het, with Het denoting an optionally substituted 5-membered or 6-membered hetero-aromatic ring which can optionally carry a positive charge; and

5 X represents hydrogen or a negative charge, if the radical R contains a positive charge,

in which a compound of the general formula



in which

R and X have the above-mentioned meanings,

R² denotes a phenyl, phenoxy, 2-thienyl or 2-furyl ring which is optionally substituted in the ring by amino, hydroxyl or alkyl with 1 to 3 carbon atoms, and R³ denotes hydrogen, amino, hydroxyl or alkyl with 1 to 3 carbon atoms, or a salt thereof with an inorganic or organic base, is brought into contact with penicillin acylase, which is bound by covalent bonds to a water-soluble carrier, optionally in a form which is enclosed in a water-insoluble carrier material, and the resulting 7-amino-Δ³-cephem derivative of the formula (I) is isolated.

R¹ preferably denotes alkyl with 1 to 3 carbon atoms (namely, methyl, ethyl or n- or isopropyl), especially with 1 or 2 carbon atoms, and n- preferably denotes 4 or 5.

The 5-membered or 6-membered hetero-aromatic ring Het preferably contains 1 to 3, especially 1 or 2, identical or different hetero-atoms. The hetero-atoms are oxygen, sulphur or nitrogen. Furyl, thienyl, pyrazolyl, imidazolyl, 1,2,3- and 1,2,4-triazolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, 1,2,3-, 1,3,4-, 1,2,4- and 1,2,5-oxadiazolyl, azepinyl, pyrrolyl, isopyrrolyl, pyridyl, pyridinium, pyridazinyl, pyrimidinyl and pyrazinyl may be mentioned as examples.

The hetero-aromatic ring Het can be substituted by 1 to 3, preferably 1 or 2, alkyl groups with, preferably, 1 to 3 carbon atoms; methyl and ethyl may be mentioned as examples.

Alkyl in the definition of R² denotes straight-chain or branched alkyl with 1 to 4, especially 1 or 2, carbon atoms, such as methyl, ethyl and n- or isopropyl.

As salts of the compounds of the formula (II) with inorganic or organic bases, which can be employed for the process according to the invention, there may be mentioned the alkali metal salts, such as sodium salts and potassium salts, alkaline earth metal salts, such as calcium salts, ammonium salts and the salts which are formed with aliphatic, araliphatic or aromatic amines. Examples of such amines which may be listed are the primary, secondary and tertiary alkylamines, which contain 1 to 4 carbon atoms per alkyl group and in which the alkyl groups can be substituted by hydroxyl groups, for example ethylamine, triethylamine and hydroxyethylamine, and cyclic amines, for example pyridine, piperidine, morpholine, piperazine and N-methylpiperazine.

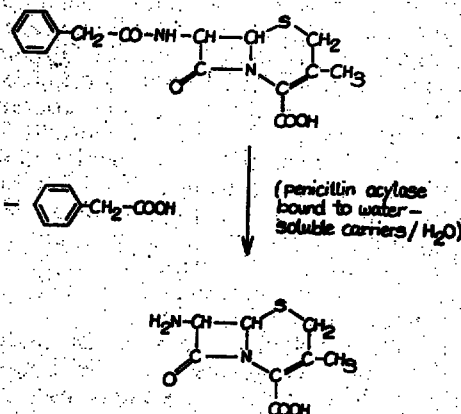
Surprisingly, the penicillin acylase bound by covalent bonds to water-soluble carriers splits the compounds of the formula (II) practically at the same rate as corresponding penicillins.

The process according to the invention exhibits a number of advantages over the known processes for the preparation of the compounds of the formula (I). Whilst, for example, enzymes from cell cultures can only be used once, it is possible to isolate the water-soluble carrier-bound penicillin acylase by ultra-filtration and to use it repeatedly. Further, if desired, water-insoluble penicillin acylase preparations can also be obtained from the water-soluble enzyme preparations by enclosing penicillin acylase, bound by covalent bonds to water-soluble carriers, in a water-insoluble carrier material, for example by encapsulation or enclosure in polymers. These water-insoluble preparations can in many cases be used in an advantageous manner.

By means of the process of the present invention, the compounds of the formula (I) can be obtained in very good purity and high yields with very little technical effort. As compared to the use of an enzyme which is only bound by

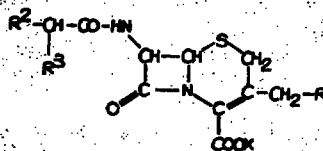
adsorption (see German Offenlegungsschrift (German Published Specification) 2,212,276), the use, according to the invention, of the water-soluble penicillin acylase preparations, bound by covalent bounds, has the advantage that the enzyme remains firmly bound without loss of activity even when using high concentrations of the cephalosporin substrate of the formula (II), resulting in substantially improved economy. The process according to the invention thus represents an enrichment of the art.

If, for example, 7-phenyl-acetamido-desacetoxy-cephalosporanic acid is used as the starting material, the course of the reaction can be represented by the following equation:



The compounds of the formula (II) which can be used according to the invention have already been described in the literature and can be obtained according to known processes (see, for example, E.H. Flynn, Cephalosporins and Penicillins, Chemistry and Biology, Academic Press, New York, 1972).

The following may be mentioned as examples of compounds of the formula (II) which are particularly preferred for the process according to the invention:



	R	R ²	R ³	X
a)	-OCOCH ₃		H	H
b)			H	-
c)	H		NH ₂	H
d)	H		H	H
e)	H		H	H
f)	-O-CH ₂ -CH ₃		H	H
g)	-O-CO-CH ₃		H	H
h)	-O-CO-CH ₃		NH ₂	H

Amongst these compounds, the compounds (d) to (g) are especially preferred for use in the process according to the invention.

The penicillin acylase, which is bound by covalent bonds to water-soluble carriers, can be of very diverse origin.

Penicillin acylase (Enzyme Commission 3.5.1.11) can be obtained from numerous micro-organisms in accordance with generally known methods (see, for example, German Offenlegungsschriften (German Published Specifications) 1,907,365 and 2,217,745), for example from bacteria, especially *Escherichia coli*, *Erwinia* or *Actinomycetes*, such as *Streptomyces*, *Micromonospora*, *Nocardia* or fungi, such as *Fusarium* and yeasts. Penicillin acylase from *Escherichia coli*, for example from a strain of *Escherichia coli* which has been filed under ATCC 11,105, is particularly preferred. The isolation of penicillin acylase from *Escherichia coli* is carried out, for example, according to German Offenlegungsschrift (German Published Specification) 2,217,745 by adjusting a crude extract from *Escherichia coli* cells, which in addition to penicillin acylase also contains cell fragments of the *Escherichia coli* cells, to a pH value of 3.5 to 5.5, preferably 5.0, and centrifuging the extract, then treating the clear solution of the penicillin acylase, obtained as the supernatant liquid, with aluminium silicates, preferably bentonite, separating off the adsorbate in a known manner and eluting, for example with an 0.1–1.0 molar solution of sodium acetate or potassium acetate at about pH 8.5.

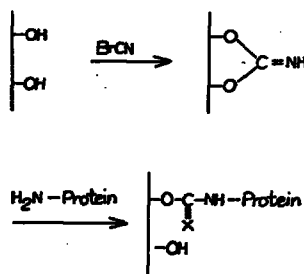
The enriched, clear solution of the penicillin acylase, thereby obtained, can optionally be further purified chromatographically on macroporous ion exchangers, for example sulphotoethyl-cellulose.

Very many water-soluble polymeric compounds can be used as water-soluble carriers to which the penicillin acylase can be bound by covalent bonds. Preferentially, practically all polysaccharides can be used as polymeric carriers. As examples thereof there should above all be mentioned: dextran, water-soluble starch, laevan and carboxymethylcellulose. Dextran and starch are particularly preferred. The polysaccharides used preferably have a molecular weight of 5,000 to 1,000,000, especially 10,000 to 800,000.

The preparation, using polysaccharides, of the penicillin acylase, bound to water-soluble carriers by covalent bonds can be carried out as follows:

When using polysaccharides as water-soluble polymeric carriers, these polysaccharides are first preferably reacted with a cyanogen halide. The active intermediate compound thereby formed is then reacted, optionally after isolation, with the penicillin acylase (see German Offenlegungsschrift (German Published Specification) 1,768,512, and the corresponding U.S. Patent Specification 3,645,852).

The reaction course of this 2-stage reaction (formation of an active intermediate compound and bonding of the penicillin acylate to the polymeric carrier) can be represented by the following equation:



wherein

X can be an NH group or oxygen,



represents a part of the polymeric carrier (polysaccharide), and H_2N -protein denotes the penicillin acylase.

Possible cyanogen halides are cyanogen iodide, chloride and bromide, especially cyanogen bromide.

Both in the first stage (reaction of polysaccharide with cyanogen halide) and in the second stage (reaction of the intermediate compound with penicillin acylase), water is preferentially used as the diluent or solvent.

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Per gram of polysaccharide, preferably 5 to 250, and especially 10 to 200, mg of cyanogen halide are employed when using dextran, whilst preferably 50 to 500, especially 80 to 200, mg of cyanogen halide are employed when using starch.

5 The reaction temperatures in both stages are from 0° to 50°, preferably from 10° to 25°C. The reactions are preferably carried out under normal pressure, although they can be effected under elevated pressure. 5

10 When producing the penicillin acylase preparation, the reaction medium is kept in the range of pH 8—13, preferably at pH 11.0, by adding a base, for example sodium hydroxide solution. The reaction of the cyanogen halide with the polysaccharide is, in many cases, complete after 10—20 minutes. The solution of the active intermediate compound formed from the polysaccharide and the cyanogen halide should not contain any free cyanogen halide when used for the reaction with the penicillin acylase. The reaction should be carried out as soon as possible since the reactive derivative of the polymer easily becomes inactivated owing to hydrolysis. Before the reaction with the penicillin acylase, the pH value of the solution containing the active polymer derivative is preferably adjusted to about 8.5. After addition of the penicillin acylase, the mixture is stirred at from 0° to 50°C, preferably 5° to 10°C. The high-molecular-weight enzyme derivative can subsequently be separated from the native unconverted enzyme in the customary manner, for example by ultra-filtration, using membranes which are impermeable to the enzyme-polymer preparations. When using dextran with a molecular weight of 500,000 it is possible, for example, to employ membranes which are permeable to materials of molecular weight of more than 100,000. 10

15 The ratio of polysaccharide, cyanogen halide and penicillin acylase can be varied within wide limits. Complete conversion of the enzyme is achieved reliably if at least 5 parts by weight of polysaccharide are used per part by weight of enzyme protein. 15

20 It has been found that the ratio of the amounts of cyanogen halide and polysaccharide can substantially influence the keeping qualities of the enzyme derivative. Thus, the native penicillin acylase is inactivated to the extent of up to 95% within 24 hours in aqueous solution at pH 7.8 and 45°C. Under identical conditions, carrier-bound, water-soluble penicillin acylase which has been coupled, for example, to dextran of molecular weight 500,000 only loses 47% of activity in 24 hours if 17 mg of cyanogen bromide are employed per gram of dextran. If, on the other hand, 80 mg of cyanogen bromide are used per gram of dextran, the enzyme activity only decreases by 5% in 24 hours. It is surprising that for the same coupling yield, the stability of the water-soluble, carrier-bound penicillin acylase can be increased substantially by increasing the proportion of cyanogen halide. Possibly, more covalent bonds are formed between the carrier molecule and the enzyme molecule by increasing the amount of cyanogen halide. This could increasingly stabilise the tertiary structure of the enzyme, the more covalent bonds have been formed between the carrier and the enzyme. However, there is a limit to the amount of cyanogen halide which can be employed, since sparingly soluble and/or insoluble enzyme derivatives can be produced as a result of transverse crosslinking. Thus, when using polysaccharides, for example dextran of molecular weight about 500,000, it is not advisable to use more than 150 mg of cyanogen halide, for example cyanogen bromide, per gram. 20

25 However, if a polysaccharide, for example dextran, having a lower molecular weight, for example 20,000, is used, there is no problem in allowing more than 300 mg of cyanogen halide, for example cyanogen bromide, to react per gram of polysaccharide. However, when using carriers of lower molecular weights, the stability of the enzyme is reduced. 25

30 Exceptionally stable derivatives of penicillin acylase can also be prepared using starch as the water-soluble carrier. Here again the stability of the enzyme derivative depends on the amount of cyanogen halide employed. Preferably, at least 150 mg of cyanogen halide, for example cyanogen bromide, are allowed to react per gram of starch. 30

35 The most advantageous molecular weights of the carriers and the nature and amount of the cyanogen halides and of the penicillin acylase can easily be determined for each case by anyone skilled in the art. 35

40 To carry out the process according to the invention namely the conversion of the compounds of the formula (II) into compounds of the formula (I), the penicillin acylase which is bound to the water-soluble carrier by covalent bonds can be used direct, that is to say without further modification, or be enclosed in a water-insoluble carrier material, for example capsules or polymers of very diverse 40

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kinds, or can, particularly preferentially, be included in fibres by spinning (see German Offenlegungsschrift (German Published Specification) 1,932,426 and the corresponding U.S. Patent Specification 3,715,277). The inclusion of the enzyme bound to the water-soluble carrier by covalent bonds can take place without significant losses in activity, in contrast to the free enzyme which is not bound by covalent bonds.

The penicillin acylase bound by covalent bonds to water-soluble carriers can, as has already been mentioned, be included in fibres by spinning. For this purpose the enzyme preparation is, for example, dispersed with the aid of an emulsifier and spun, in polymers, to give fibrous structures, using the wet spinning process or the dry spinning process. Polymers which can be used for this purpose are polymers are copolymers of very diverse kinds, such as cellulose derivatives, especially cellulose triacetate.

A penicillin acylase bound to dextran of molecular weight 500,000 can, for example, be entrapped in yields of 60% of the original enzyme activity, in accordance with processes described in the literature (H. Nilson, R. Mosbach and K. Mosbach, *Biochim. Biophys. Acta* 268 (1972), 253—256). It is possible, with particular advantage, to entrap the penicillin acylase, bound to a water-soluble carrier by covalent bonds, in the customary manner in a copolymer produced, for example, from acrylamide, N,N'-methylene-bis-acrylamide and N,N,N',N'-tetramethylethylenediamine. In a control experiment with native penicillin acylase, that is to say with penicillin acylase not bound to a carrier by covalent bonds, only 10% of the originally present enzyme activity was obtained after copolymerisation under the same experimental conditions. The entrapped carrier-bound enzyme proved to be exceptionally stable even after repeated use for the preparation of compounds of the formula (I). The entrapped enzyme derivative can be isolated rapidly and simply from the reaction medium, since the polymer can be prepared in particle sizes of more than 1 mm in diameter. The new materials exhibit a powerful enzymatic action, which renders them highly suitable.

Usually, enzymatic splitting reactions for the preparation of compounds of the formula (I) are carried out with the aid of penicillin acylase bound to a water-soluble carrier by covalent bonds and optionally enclosed in a water-insoluble polymeric carrier material, in dilute solutions which preferably contain 1 to 20, especially 3 to 6, per cent by weight of the compounds of the formula (II). Before isolating the end products, it is desirable to evaporate off approx. 50% or more of the water used as the diluent. Since the carrier-bound penicillin acylase which can be used according to the invention also permits splitting reactions at higher substrate concentrations, less water requires to be evaporated, corresponding to the greater amount of starting material employed. The use, according to the invention, of the water-soluble derivatives of penicillin acylase for the preparation of compounds of the formula (I) is therefore very economical, and from the point of view of the re-usability of the enzyme derivative and the yield of compounds of the formula (I) is of little importance whether the water-soluble enzyme derivative is used direct in aqueous solution or is used in the insoluble form after copolymerisation or inclusion in polymers.

In carrying out the process according to the invention, the compound of the formula (II) which is to be split is preferably dissolved or suspended in water or a mixture of water and up to 10 per cent by volume of an organic solvent, preferably of a polar water-miscible organic solvent, preferably C₁—C₄ alkyl alcohols such as methanol and ethanol, di(C₁—C₄-alkyl) ketones, such as acetone, or di(C₁—C₄-alkyl)-formamides, such as dimethylformamide.

Preferably, from 1 to 20, especially 3 to 6, parts by weight of the compound of the formula (II) are used.

The solution and/or suspension is brought into contact with the water-soluble enzyme derivative in any desired form. For example, the enzyme derivative can be dissolved in water or can be suspended in the solution after having been enclosed in a polymer.

The splitting of compounds of the formula (II) to give compounds of the formula (I) is carried out at from 20° to 45°C, preferably at from 35° to 40°C.

To neutralise the acyl radical split off during the reaction, constant monitoring and adjustment of the pH value is desirable. During the reaction, the pH should be kept at a value of, preferably, 6 to 9, especially 7.5 to 8.5. This can be done, if required, by adding a base to the reaction mixture. Bases which can be used for this purpose are practically all inorganic and organic bases, such as trisodium phosphate, alkali metal hydroxides, alkali metal carbonates, alkali metal

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bicarbonates, alkaline earth metal carbonates, alkaline earth metal hydroxides, ammonia, primary, secondary and tertiary aliphatic and aromatic amines and heterocyclic bases. Sodium hydroxide, potassium hydroxide, sodium carbonate, potassium carbonate, sodium bicarbonate, potassium bicarbonate, calcium carbonate, ethylamine, methylethylamine, triethylamine, monohydroxyethylamine, dihydroxyethylamine, trihydroxyethylamine, aniline, pyridine and piperidine may be mentioned as examples. The rate of reaction and the end of the reaction can readily be seen from the consumption of the base.

5
The rate of reaction depends above all on the ratio of the amount of available penicillin acylase relative to the amount of the formula (II) employed, and on the specific activity of the penicillin acylase, bound by covalent bonds, which is employed.

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The specific activity of the penicillin acylase is specified in units/ml of solution or units/g of moist weight. 1 unit is defined as the penicillin acylase activity which in one minute hydrolyses 1 micromol of penicillin G, at 37°C and pH 7.8, to 6-aminopenicillanic acid and phenylacetic acid.

15
The complete splitting of 1 kg of 7-phenylacetamidodesacetoxy-cephalosporanic acid within 2 hours requires, for example, about 120,000 units of water-soluble carrier-bound penicillin acylase. In the case of longer splitting times, correspondingly less penicillin acylase is required. The most advantageous ratios and reaction times can easily be determined by anyone skilled in the art.

20
After completion of the splitting reaction, the water-soluble penicillin acylase derivative is isolated by ultra-filtration and can immediately be used for further splitting batches. If the water-soluble enzyme derivative bound by covalent bonds has been encapsulated, copolymerised or incorporated into fibres by spinning in accordance with German Offenlegungsschrift (German Published Specification) 1,932,426, it can be isolated particularly simply by filtration, decanting or centrifuging.

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Commercially available modules with membranes which are impermeable to molecules of molecular weight in the range of from 20,000 to 100,000 are used particularly advantageously for the ultra-filtration.

30
The compound of the formula (I) which has been formed is isolated from the clear solution, which has been freed from the particular penicillin acylase preparations, in accordance with generally customary processes. Customary processes are, for example, precipitation with inorganic mineral acids, for example hydrochloric acid, sulphuric acid or phosphoric acid, or organic carboxylic acids, for example lower alkylcarboxylic acids such as acetic acid, at the isoelectric point, or the customary chromatographic processes, such as thin layer chromatography or column chromatography.

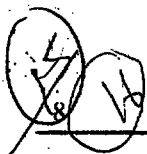
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In many cases it is also advantageous, before precipitating the compound of the formula (I), to remove the split-off acyl radical by extraction at a weakly acid pH with an organic water-immiscible solvent such as ethyl acetate, methyl isobutyl ketone or isobutyl acetate. The extraction is preferably carried out below pH 6.0. Since, however, the solubilities of the compounds of the formula (I) in water decrease at lower pH values, the mixture may have to be diluted with water before acidification. Furthermore, it can also be advantageous, from the point of view of achieving good yields, to concentrate the aqueous solutions *in vacuo* before precipitating the product.

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The product which has been precipitated or been obtained by chromatography can be purified according to customary methods. For example, in order completely to remove the radical which has been split off, the product can be washed with a suitable solvent, for example butyl acetate or acetone, and be dried *in vacuo*, whereby the pure compound of the formula (I) is obtained direct.

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The compounds of the formula (I) obtainable according to the invention can be converted into valuable anti-microbial active compounds in accordance with generally known methods, for example by acylation at the 7-amino group (see, for example, E. H. Flynn, Cephalosporins and Penicillins, Chemistry and Biology, Academic Press, New York, 1972).

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Because of their great stability, the penicillin acylase preparations employed in the process according to the invention can be used many times over a long period, as a result of which their use is economically particularly advantageous.

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The examples which follow are intended to illustrate the production of penicillin acylase/carrier preparations.



Example 1.

5 (a) 5 g of dextran 500 of approximate molecular weight 500,000 were dissolved in 165 ml of water and the solution was adjusted to pH 11.0 with 2 N sodium hydroxide solution. 85 mg of cyanogen bromide were added at a temperature of 20°C, whilst stirring, and the pH value of the solution was kept at 11.0 with 2 N NaOH. 10 minutes after adding the cyanogen bromide, the solution was adjusted to pH 8.5 with 2 N hydrochloric acid and mixed with 170 ml of an aqueous solution of 550 mg of penicillin acylase (enzymatic activity 7.3 U/mg). The solution was stirred for 16 hours in a cold room at 4°C. After ultra-filtration, enzyme activity was no longer detectable in the filtrate. Accordingly, the coupling to the carrier took place completely. The yield of enzymatic activity was 98%, relative to the starting activity. The enzyme activity remained preserved completely after freeze-drying.

15 (b) 5 g of dextran 500 of approximate molecular weight 500,000 were dissolved in 165 ml of water and the solution was adjusted to pH 11.0 with 2 N sodium hydroxide solution. 400 mg of cyanogen bromide were added at a temperature of 20°C, whilst stirring, and the pH value of the solution was kept at 11.0 with 2 N sodium hydroxide solution. 20 minutes after adding the cyanogen bromide, the solution was adjusted to pH 8.5 with 2 N hydrochloric acid and mixed with 170 ml of an aqueous solution of 550 mg of penicillin acylase (enzymatic activity 7.3 u/mg). The solution was stirred for 16 hours in a cold room at 4°C. Under these conditions, the enzyme was bound completely to the carrier. The yield of enzymatic activity was 96%, relative to the starting activity.

Example 2.

25 (a) 5 g of dextran 20 of approximate molecular weight 20,000 were reacted, in the manner indicated in Example 1(b), with 400 mg of cyanogen bromide and then with 550 mg of penicillin acylase. After the reaction, the yield of enzymatic activity was 96%, relative to the starting activity.

30 (b) 5 g of dextran 20 were reacted, as indicated in Example 1(b), with 800 mg of cyanogen bromide and then with 550 mg of penicillin acylase. After the reaction, the yield of enzymatic activity was 87%, relative to the starting activity.

35 (c) 5 g of dextran 60 of approximate molecular weight 60,000 were reacted, in the manner indicated in Example 1(b), with 400 mg of cyanogen bromide and then with 550 mg of penicillin acylase. After the reaction, the yield of enzymatic activity was 99%, relative to the starting activity.

Example 3.

40 (a) 5 g of Zulkowsky-type soluble starch (for example the preparation commercially available from Messrs. E. Merck/Darmstadt) were dissolved in 165 ml of water and the solution was adjusted to pH 11.0 with 2 N sodium hydroxide solution. 400 mg of cyanogen bromide were added at a temperature of 20°C whilst stirring and the pH value of the solution was kept at 11.0 with 2 N NaOH. 20 minutes after adding the cyanogen bromide, the solution was adjusted to pH 8.5 with 2 N hydrochloric acid and mixed with 170 ml of an aqueous solution of 550 mg of penicillin acylase (enzymatic activity 7.3 U/mg). The solution was stirred for 16 hours in a cold room at 4°C. After the reaction, the yield of enzyme activity was 98%, relative to the starting activity. The penicillin acylase bound to starch by covalent bonds could be freeze-dried without loss of activity. Yield 6.2 g, with an enzymatic activity of 0.65 U/mg.

50 (b) 103 g of Zulkowsky-type soluble starch (for example the preparation commercially available from Messrs. E. Merck/Darmstadt) were dissolved in 1,250 ml of water and the solution was adjusted to pH 11.0 with 2 N sodium hydroxide solution. 16.8 g of cyanogen bromide were added at a temperature of 20°C whilst stirring and the pH value of the solution was kept at 11.0 with 2 N NaOH. 20 minutes after adding the cyanogen bromide, the solution was adjusted to pH 8.5 with 2 N hydrochloric acid and mixed with 2,060 ml of an aqueous solution of 10.3 g of penicillin acylase (enzymatic activity 11.9 U/mg). The solution was stirred for 16 hours in a cold room at 4°C. After the reaction, the total enzyme activity was 94.5%, relative to the starting activity.

Example 4.

2.9 g of penicillin acylase bound to starch by covalent bonds and having an activity of 650 U/g, prepared according to Example 3(a), were dissolved in 600 ml of 0.05 M triethanolamine/hydrochloric acid buffer of pH 7.0. 85.5 g of acrylamide, 4.5 g of N,N'-methylene-bis-acrylamide and 5 ml of an aqueous solution of 2.5 g of ammonium peroxidesulphate were added to the solution (giving solution A).

2,900 ml of toluene, 1,100 ml of chloroform, 5 ml of N,N,N',N'-tetramethylethylenediamine and 10 ml of an emulsifier were introduced into a three-necked flask equipped with a stirrer, cooled to 4°C and stirred at a speed of 250 revolutions per minute. Solution A was allowed to drip into the reaction vessel from a dropping funnel under nitrogen protective gas, the mixture was stirred for 30 minutes and the polymer was filtered off. It was washed twice, in each case with 1 l of toluene and 2 litres of 0.5 M sodium chloride solution. The polymer contained 63% of the original penicillin acylase activity.

The following examples are intended to illustrate the process of the present invention:

Example 5.

20 mmols of the compound of the formula (II) which was to be split were dissolved in 200 ml of water (if appropriate with addition of sodium hydroxide solution) at pH 7.5. The solution was stirred in a vessel thermostatically controlled at 37°C, and 150 ml of the solution prepared according to Example 1(b), having a total activity of 1,500 U of dextran-bound penicillin acylase, were added.

During the reaction, the pH value was kept constant at pH 7.8 by addition of 0.5 M NaOH, using a pH-stat. The termination of the addition of NaOH indicated the end of the reaction. After the reaction had ended, the solution was filtered through an ultra-filter apart from a residual volume of 30 ml. 100 ml of water were added to the solution which was left and this mixture was again filtered until the residual volume was 30 ml. This washing process was repeated once more and the combined permeates were concentrated to 100 ml *in vacuo* at 30°C.

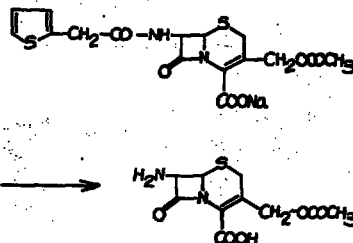
50 ml of butyl acetate were added to the concentrated solution and the resulting compound of the formula (I) was precipitated by adding 5 N hydrochloric acid until the isoelectric point of 3.7 was reached. After the sample had stood in the refrigerator for 2—3 hours, it was filtered and the crystalline product was washed with 100 ml of water and 100 ml of acetone and dried *in vacuo*.

The strength of the products was determined by photometric determination of the liberated 7-amino group in a known manner after reaction with *p*-dimethylaminobenzaldehyde, in a modified version of the Svotek method (Czech. Patent 116,959 (1965)).

The extinction at 415 nm was compared with standard curves for pure 7-aminocephalosporanic acid and 7-amino-desacetoxycephalosporanic acid.

In addition, the identity of the products was demonstrated by infrared and ¹H nuclear resonance spectra and by paper chromatography in the system butanol:glacial acetic acid: water (12:3:5). The following reactions were carried out in this way:

Example 5(a).



Starting compound:

9.08 g of 7-(2-thienyl)-acetamido-cephalosporanic acid (sodium salt).

Reaction time: 85 minutes.

End product: 7 aminocephalosporanic acid.

Yield: 5.30 g (90% of theory). Purity: 97%.

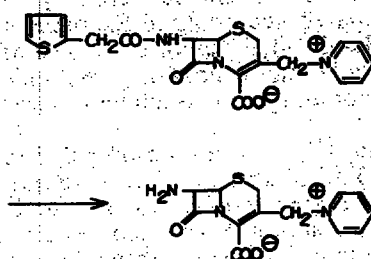
Rf: 0.27 (no desacetyl-7-aminocephalosporanic acid of Rf 0.10).

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Example 5(b).



Starting compound:

8.70 g of 7-(2-thienyl)-acetamido-3-pyridiniummethyl- Δ^3 -cephem-4-carboxylate.

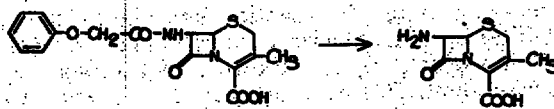
End product: 7-amino-3-pyridiniummethyl- Δ^3 -cephem-4-carboxylate.

Reaction time: 75 minutes

Rf: 0.05.

Because of the pyridine ring in the molecule, the product of this reaction could not be isolated by precipitation at the isoelectric point. The completeness of the reaction, and identity of the product, were demonstrated by paper chromatography.

Example 5(c).



Starting compound:

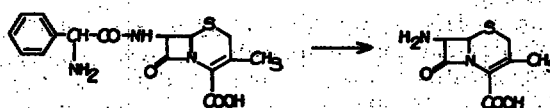
7.01 g of 7-phenoxyacetamido-desacetoxy-cephalosporanic acid.

End product: 7-amino-desacetoxycephalosporanic acid.

Yield: 3.38 g (78% of theory). Purity: 97%.

Melting point: 238–240°C (decomposition).

Example 5(d).



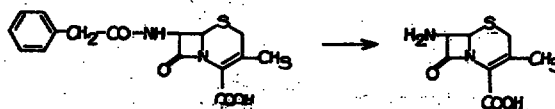
Starting compound:

6.99 g of 7-(α -amino-phenylacetyl)-desacetoxycephalosporanic acid.

End product: 7-amino-desacetoxycephalosporanic acid.

Yield: 3.66 g (84% of theory). Purity: 97%.

Example 6.



500 g of phenylacetamido-desacetoxycephalosporanic acid (purity 98%) were added to 20 l of a solution of starch-bound penicillin acylase which contains a total activity of 115,000 U. The reaction batch was kept at a constant pH of 7.8 ± 0.2 by addition of triethylamine, whilst stirring. The reaction temperature was 38°C. After 65 minutes, no further triethylamine was taken up. The solution was filtered through a paper filter and was ultra-filtered in a laboratory module, using 10 membranes which were permeable to molecules of molecular weight up to 20,000,

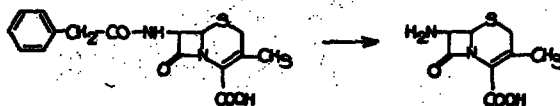
under an operating pressure of 12 kg/cm², until the residual volume was 1,000 ml. The retained material, which in addition to the starch-bound penicillin acylase which had been separated off also contained, *inter alia*, residual 7-aminodesacetoxycephalosporanic acid, was diluted 3 times in succession with 1,000 ml of water at a time and in each case again ultra-filtered until the residual volume was 1,000 ml. The 7-aminodesacetoxycephalosporanic acid formed was isolated from the combined ultra-filtrates according to generally customary processes, for example according to the following process:

The ultra-filtrate, including the wash water, was adjusted to pH 5.5 with 5 N hydrochloric acid and extracted with twice 5 l of isobutyl acetate. The aqueous phase was adjusted to pH 7.0 and concentrated to 8 l *in vacuo* at 30°C. The 7-aminocephalosporanic acid was precipitated from the concentrate at the isoelectric point at pH 3.7 by addition of 5 N hydrochloric acid. After standing for 2–3 hours, the product was filtered off — after the pH value had been checked and, as necessary, readjusted to pH 3.7 — and was rinsed with 1,000 ml of water and 1,000 ml of acetone. It was dried *in vacuo* at 40°C. Yield: 306 g (94% of theory). Purity: 97%.

The same starch-bound penicillin acylase was used 25 times in succession in the same manner without the occurrence of a significant loss of activity.

The yield of the 25 splitting reactions averaged 93.5% of theory.

Example 7.



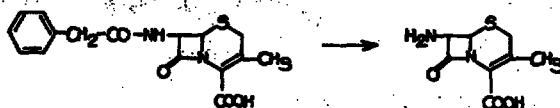
20 g (60 mmols) of 7-phenylacetamido-desacetoxycephalosporanic acid were suspended in 500 ml of water and dissolved by addition of triethylamine until the pH value was 7.8. The solution was warmed to 37°C and 80 ml of the enzyme solution prepared according to Example 1(b), having a total activity of 800 U, were added. The pH value was kept at 7.8 ± 0.3 during the splitting reaction by addition of triethylamine. After 6 hours, the reaction had ended. The reaction mixture was filtered through an ultra-filter until the residual volume was 50 ml and the solution which remained was diluted with 100 ml of water and again concentrated to 50 ml by ultra-filtration. This washing process was repeated once more. All permeates were combined and concentrated to 400 ml *in vacuo* at 30°C.

After addition of 200 ml of methyl isobutyl ketones, the pH was adjusted to 3.7 with 12 N hydrochloric acid and the 7-aminodesacetoxycephalosporanic acid which had crystallised out was filtered off after standing for 1 hour and washed with a little water/methyl isobutyl ketone and acetone.

Yield: 11.9 g (92.5% of theory). Purity: 98%.

Melting point: 238–240°C (decomposition).

Example 8.



20 g (60 mmols) of 7-phenylacetamido-desacetoxycephalosporanic acid were suspended in 350 ml of water and dissolved by adding triethylamine until the pH value was 7.8. The solution was warmed to 37°C and the entrapped penicillin acylase, bound to starch by covalent bonds, prepared according to Example 4 and having a total activity of 1,120 units, was added. During the splitting reaction the reaction mixture was kept at 7.8 ± 0.3 by addition of triethylamine. The reaction had ended after 5 hours. The entrapped penicillin acylase derivative was filtered off and washed with a little water. 200 ml of methyl isobutyl ketone and 12 N hydrochloric acid were added to the filtrate, including the wash water, until the pH was 3.7. After standing for 1 hour at 5°C, the pH was readjusted to 3.7 with hydrochloric acid, as necessary, and the 7-aminodesacetoxycephalosporanic acid which had crystallised out was filtered off and rinsed with a little water acetone. Yield: 12.1 g (94% of theory). Purity: 97.5%. Melting point: 238–240°C (decomposition).

The preparation of the penicillin acylase required for the preparation of the enzyme resin is illustrated in the following Example.

Example 9.

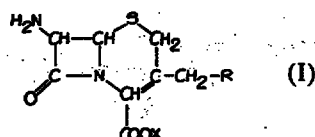
(a) A crude extract which contained penicillin acylase was obtained by culturing *Escherichia coli* in accordance with the process described in German Patent Specification 1,111,778, concentrating the culture by centrifuging to give a sludge, and breaking open the cells according to known mechanical processes, with addition of 3% of methyl isobutyl ketone, as appropriate.

(b) 610 litres of a crude extract thus obtained, containing 5.2×10^6 U, were diluted to 2,100 litres with dionised water, adjusted to pH 5.0 with sulphuric acid and centrifuged. The clear supernatant liquid contained 5.7×10^6 U, with a specific activity of 1.7 U/mg.

While readjusting the pH to 5.0, 13.8 kg of bentonite were stirred in and after 30 minutes the latter was separated off in a continuous flow centrifuge. The bentonite which had been separated off was eluted with 90 litres of 0.5 M sodium acetate solution of pH 8.0, and filtered off. 92.5 litres, containing 4.5×10^6 (86% of theory) were obtained, with a specific activity of the penicillin acylase of 7.3 U/mg.

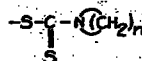
WHAT WE CLAIM IS:—

1. A process for the preparation of a 7-amino- Δ^3 -cephem derivative of the general formula



in which

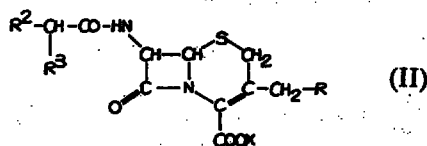
R denotes hydrogen, hydroxyl, amino, nitrile, $-\text{O}-\text{CO}-\text{NH}_2$,



, with n denoting an integer from 4 to 6, $-\text{O}-\text{CO}-\text{R}^1$, $-\text{NH}-\text{CO}-\text{R}^1$ and $-\text{S}-\text{CS}-\text{O}-\text{R}^1$, with R^1 denoting alkyl with 1 to 4 carbon atoms, or in which

R denotes $-\text{S}-\text{Het}$ or Het , with Het denoting an optionally substituted 5-membered or 6-membered hetero-aromatic ring, which can optionally carry a positive charge, and

X denotes hydrogen or a negative charge, if the radical R contains a positive charge, in which a compound of the general formula

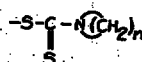


in which

R and X have the above-mentioned meanings,

R^2 denotes a phenyl, phenoxy, 2-thienyl or 2-furyl ring which is optionally substituted in the ring by amino, hydroxyl or alkyl with 1 to 3 carbon atoms, and R^3 denotes hydrogen, amino, hydroxyl or alkyl with 1 to 3 carbon atoms, or a salt thereof with an inorganic or organic base, is brought into contact with penicillin acylase which is bound by covalent bonds to a water-soluble carrier, and the resulting 7-amino- Δ^3 -cephem derivative is isolated.

2. A process according to claim 1 in which R denotes hydrogen, hydroxyl, amino, nitrile, $-\text{O}-\text{CO}-\text{NH}_2$,





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The preparation of the penicillin acylase required for the preparation of the enzyme resin is illustrated in the following Example.

Example 9.

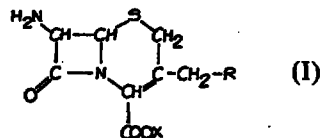
(a) A crude extract which contained penicillin acylase was obtained by culturing *Escherichia coli* in accordance with the process described in German Patent Specification 1,111,778, concentrating the culture by centrifuging to give a sludge, and breaking open the cells according to known mechanical processes, with addition of 3% of methyl isobutyl ketone, as appropriate.

(b) 610 litres of a crude extract thus obtained, containing 5.2×10^6 U, were diluted to 2,100 litres with dionised water, adjusted to pH 5.0 with sulphuric acid and centrifuged. The clear supernatant liquid contained 5.7×10^6 U, with a specific activity of 1.7 U/mg.

While readjusting the pH to 5.0, 13.8 kg of bentonite were stirred in and after 30 minutes the latter was separated off in a continuous flow centrifuge. The bentonite which had been separated off was eluted with 90 litres of 0.5 M sodium acetate solution of pH 8.0, and filtered off. 92.5 litres, containing 4.5×10^6 (86% of theory) were obtained, with a specific activity of the penicillin acylase of 7.3 U/mg.

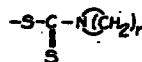
WHAT WE CLAIM IS:—

1. A process for the preparation of a 7-amino- Δ^3 -cephem derivative of the general formula



in which

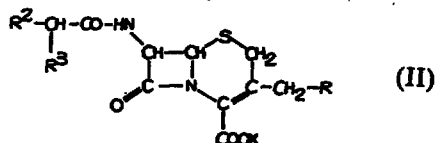
R denotes hydrogen, hydroxyl, amino, nitrile, $-\text{O}-\text{CO}-\text{NH}_2$,



, with n denoting an integer from 4 to 6, $-\text{O}-\text{CO}-\text{R}^1$, $-\text{NH}-\text{CO}-\text{R}^1$ and $-\text{S}-\text{CS}-\text{O}-\text{R}^1$, with R^1 denoting alkyl with 1 to 4 carbon atoms, or in which

R denotes $-\text{S}-\text{Het}$ or Het , with Het denoting an optionally substituted 5-membered or 6-membered hetero-aromatic ring, which can optionally carry a positive charge, and

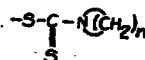
X denotes hydrogen or a negative charge, if the radical R contains a positive charge, in which a compound of the general formula



in which

R and X have the above-mentioned meanings, R^2 denotes a phenyl, phenoxy, 2-thienyl or 2-furyl ring which is optionally substituted in the ring by amino, hydroxyl or alkyl with 1 to 3 carbon atoms, and R^3 denotes hydrogen, amino, hydroxyl or alkyl with 1 to 3 carbon atoms, or a salt thereof with an inorganic or organic base, is brought into contact with penicillin acylase which is bound by covalent bonds to a water-soluble carrier, and the resulting 7-amino- Δ^3 -cephem derivative is isolated.

2. A process according to claim 1 in which R denotes hydrogen, hydroxyl, amino, nitrile, $-\text{O}-\text{CO}-\text{NH}_2$,





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- with n denoting 4 or 5, $-\text{O}-\text{CO}-\text{R}^1$, $-\text{NH}-\text{CO}-\text{R}^1$ or $-\text{S}-\text{CS}-\text{O}-\text{R}^1$ with R^1 denoting methyl or ethyl, or $-\text{S}-\text{Het}$ or $-\text{Het}$ with Het denoting a 5-membered or 6-membered heterocyclic ring containing 1 or 2 hetero-atoms selected from oxygen, sulphur and nitrogen, the ring being optionally substituted by 1 to 3 alkyl groups each having 1 to 3 carbon atoms.
- 5 3. A process according to claim 1, in which R , R^3 and X each denote hydrogen and R^2 denotes phenyl. 5
4. A process according to claim 1, in which R , R^3 and X each denote hydrogen and R^2 denotes phenoxy.
- 10 5. A process according to claim 1, in which R denotes $-\text{O}-\text{COCH}_3$, R^2 denotes phenoxy and R^3 and X each denote hydrogen. 10
6. A process according to claim 1, in which R denotes $-\text{O}-\text{COCH}_3$, R^2 denotes phenyl and R^3 and X each denote hydrogen.
- 15 7. A process according to claim 1 or 2, in which the compound (II) is employed in the form of an alkali metal salt, an alkaline earth metal salt or a salt formed with an aliphatic, araliphatic or aromatic amine. 15
8. A process according to any of claims 1 to 7, wherein the penicillin acylase is bound to a polysaccharide by covalent bonds.
- 20 9. A process according to claim 6, wherein the penicillin acylase is bound to starch by covalent bonds. 20
10. A process according to claim 6, wherein the penicillin acylase is bound to dextran by covalent bonds.
11. A process according to any of claims 1 to 10, wherein the penicillin acylase which is bound by covalent bonds to a water-soluble carrier is enclosed in a water-insoluble carrier material.
- 25 12. A process according to any of claims 1 to 11, wherein the reaction is effected at from 20° to 45°C . 25
13. A process according to claim 12, wherein the reaction is effected at from 35° to 40°C .
- 30 14. A process according to any of claims 1 to 13, wherein the pH of the reaction mixture is maintained at from 6 to 9. 30
15. A process according to claim 14, wherein the pH is maintained at 7.5 to 8.5.
- 35 16. A process according to claim 1 for the preparation of a 7-amino- Δ^3 -cepham derivative, being a process substantially as described in any one of Examples 5(a) — 5(d), 6, 7 and 8. 35
17. A 7-amino- Δ^3 -cepham derivative whenever prepared by a process according to any of claims 1 to 16.

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