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PATENT SPECIFICATION (11) 1 508 977

(21) Application No. 17410/74 (22) Filed 20 April 1974
(21) Application No. 27715/74 (22) Filed 21 June 1974
(21) Application No. 43651/74 (22) Filed 9 Oct. 1974
(21) Application No. 53525/74 (22) Filed 11 Dec. 1974
(23) Complete Specification filed 11 April 1975
(44) Complete Specification published 26 April 1978
(51) INT CL² C07D 498/04; A61K 31/41, 31/43, 31/545; (C07D 498/04, 205/00, 263/00)
(52) Index at acceptance
C2C 1315 1474 200 20Y 213 214 220 226 227 22Y 247 253 255 25Y 28X 292 29X 29Y 306 30Y 311 313 31Y 321 322 323 32Y 332 339 351 352 360 361 366 367 368 36Y 389 431 436 490 491 620 621 623 625 628 630 635 638 650 652 658 65X 660 661 668 66X 678 67X 681 695 699 761 762 767 771 778 790 79Y LQ LZ MM NC NN TR
A5B 213 216 21Y



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(54) NOVEL B-LACTAM ANTIBIOTIC FROM STREPTOMYCES CLAVULIGERUS

(71) We, BEECHAM GROUP LIMITED, a British Company of Beecham House, Great West Road, Brentford, Middlesex, England, 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 clavulanic acid, which is a new antibacterial agent which has been isolated from *Streptomyces clavuligerus*, and to its salts and to processes for their preparation. In addition to being broad spectrum antibiotics of medium potency, clavulanic acid and its salts have the ability to enhance the effectiveness of penicillins and cephalosporins against many B-lactamase-producing bacteria. Thus this invention also relates to pharmaceutical compositions comprising clavulanic acid or its salts.

Streptomyces clavuligerus has been described in detail by Higgens et al, *Int. J. Systematic Bacteriology, 21,* 326 (1971). This streptomycete was of interest because it produced certain β-lactam antibiotics such as penicillin N, 7-(5-amino-5-carboxy-valeramido)-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid and 7-(5 - amino - 5 carboxy - valeramido) - 3 - carbamoyloxymethyl - 7 - methoxy - 3 - cephem-4-carboxylic acid. The streptomycete has been deposited in the Agricultural Research Service Collection as NRRL 3585 and in the American Type Culture Collection as ATCC 27064. *Streptomyces clavuligerus* has also been referred to in United States Patent Specification No. 3770590 and also by Nagarajan et al,. *J. Amer. Chem. Soc., 93,* 2308 (1971), Brannon et al, *Antimicrob. Agents Chemother., 1,* 237 (1972) and *Antimicrob. Agents Chemother., 1,* 247 (1972) and Higgens et al, *J. Antibiotics, 27,* 298 (1974). British Patent Specification No. 1,315,177 also discloses that the cultivation of *Streptomyces clavuligerus* leads to the preparation of 7-(5-amino-5-carboxyvaleramido)-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid and 7-(5-amino-5-carboxyvaleramido)-3-carbamoyloxymethyl-7-methoxy-3-cephem-4-carboxylic acid. None of these aforementioned publications describe clavulanic acid or its salts or esters or the method of their preparation or use in pharmaceutical compositions.

Although clavulanic acid and its salts are broad spectrum antibiotics of medium potency it is envisaged that their greatest interest lies in their ability to inhibit β-lactamases. β-Lactamases are enzymes which open the β-lactam ring of penicillins and cephalosporins to give products which are devoid of antibacterial activity. These enzymes are produced by many bacteria, notably species or strians

of *Escherichia, Klebsiella, Proteus, Pseudomonas, Enterobacter* and *Staphylococcus* and are in many instances the explanation for the resistance of certain strains of such organisms to some penicillins and their cephalosporins. The importance of β-lactamase production may be understood when it is realised that a high proportion of clinically isolated organisms produce β-lactamases [see, for example, M. Wilson and I.A. Freeman, *Bacteriological Proceedings*, 80 (1969) where in a paper entitled 'Penicillin Inactivation by Gram-negative Bacilli' they showed that 84% of the gram-negative organisms isolated in an American hospital produced β-lactamase]. In many cases, some penicillins or cephalosporins are ineffective in treating diseases ascribed to non-β-lactamase-producing organisms because of the common occurrence of co-infection by a β-lactamase producer [see, for example, R. May et al, *Brit. J. Dis. Chest.*, 66, 185 (1972)]. Combination of a β-lactamase-inhibiting substance with a penicillin or cephalosporin might be expected to protect the latter from degradation by bacterial β-lactamase and thereby enhance their antibacterial activity against many infective organisms. This process of enhancement of the antibacterial activity is called synergism when the antibacterial activity of the combination is in excess of the simple addition of the activities of the two separate substances. The β-lactamase-inhibiting component of the mixture is referred to as a synergist.

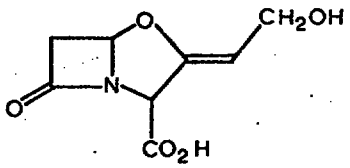
We have discovered that the aerobic cultivation of *Streptomyces clavuligerus* in conventional nutrient media at, for example, about 25—30°C under roughly neutral conditions produces a β-lactamase-inhibitory substance which also possesses antibacterial activity. We have designated this new material 'clavulanic acid'.

Clavulanic acid has the following properties:

(a) It is a carboxylic acid.
(b) It forms a sodium salt which has a characteristic infra-red spectrum substantially as shown in Fig. 1.
(c) It is able to inhibit the growth of strians of *Staphylococcus aureus.*
(d) it is able to synergise the antibacterial effect of ampicillin against β-lactamase-producing strains of *Escherichia coli, Klebsiella aerogenes* and *Staphylococcus aureus.*
(e) It is able to synergise the antibacterial effect of cephaloridine against the β-lactamase-producing strains of *Proteus mirabilis* and *Staphylococcus aureus.*
(f) It forms a methyl ester which has a molecular weight (by mass spectroscopy) of 213.0635 which corresponds to the formula $C_9H_{11}NO_5$.

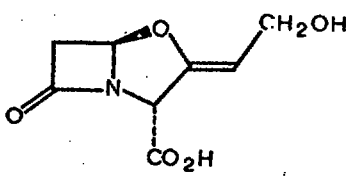
Thus clavulanic acid may be regarded as a monobasic carboxylic acid of the formula $C_8H_9NO_5$ which in the form of a sodium salt has a characteristic infra-red absorption spectrum substantially as shown in Fig. 1.

The compound produced by *Streptomyces clavuligerus* which has the above properties has the formula



Thus clavulanic acid may be named 3-(β-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3,2,0]heptane-2-carboxylic acid.

The stereochemistry at C_5 and C_2 of the clavulanic acid is the same as that found in naturally occurring penicillins so that clavulanic acid may be represented by the structural formula (I):



(I)

Thus a fuller chemical name for clavulanic acid is Z-(2R,5R)-3-(β-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3,2,0]heptane-2-carboxylic acid.

The great usefulness of clavulanic acid and its salts may be readily appreciated when it is realised that certain strains of *Klebsiella aerogenes* A, the growth of which is not inhibited by the presence of 125 μg/ml of ampicillin, amoxycillin, carbenicillin or benzylpenicillin or by the presence of 10 μg/ml of clavulanic acid, are inhibited by the presence of less than 12.5 μg/ml of the previously mentioned penicillins when 5 μg/ml of clavulanic acid is also present. It has also been found that strains of *Staphylococcus aureus* Russell, the growth of which is not inhibited by the presence of 100 μg/ml of ampicillin or by 5 μg/ml of clavulanic acid, are inhibited by the presence of less than 10 μg/ml of ampicillin in the presence of 1 μg/ml of clavulanic acid. In tests on female mice, it has been found that blood and tissue levels of clavulanic acid considerably in excess of 5 μg/ml can readily be achieved by subcutaneous administration of 100 mg/kg of the sodium salt of clavulanic acid and that useful levels of clavulanic acid can be obtained after oral administration of 100 mg/kg of the sodium salt of clavulanic acid.

Accordingly, the present invention provides clavulanic acid as hereinbefore described and its salts.

Salts of clavulanic acid within this invention include the lithium salt, the sodium salt, the potassium salt, the calcium salt, the magnesium salt, the aluminium salt, the silver salt and the ammonium salt of clavulanic acid and salts of clavulanic acid with substituted ammonium compounds such as di- and tri- alkylamines, for example those containing up to 22 carbon atoms or more suitably those containing lower alkyl groups such as trimethylamine, or other amines such as those known to form salts with penicillin such as the benzathine salt, and salts of clavulanic acid with polymeric anion exchange materials.

Salts of clavulanic acid are a favoured aspect of this invention as they tend to be more stable than the parent acid *per se*.

The aforementioned salts may be employed as intermediates in the preparation of esters of clavulanic acid; for example, the benzyl ester of clavulanic acid may be prepared by the reaction of benzyl bromide and a clavulanic acid salt such as the lithium, sodium, potassium, silver or polymeric anion-exchange material salt. Esters of clavulanic acid are described and claimed in our co-pending divisional application No. 36563/77 — 36564/77 — 36565/77 — 36566/77 Serial No. 1508978.

Certain of the aforementioned salts are envisaged primarily as useful in the isolation of clavulanic acid, for example the salts with polymeric anion-exchange materials and with lipophilic secondary or tertiary alkylamines are formed during the isolation of clavulanic acid from fermentation broth as described hereinafter.

An important use of the salts of clavulanic acid is in antibacterial pharmaceutical compositions. It follows that a particularly favoured aspect of this invention is provided by the pharmaceutically acceptable salts of clavulanic acid.

Apt salts of clavulanic acid include the alkali metal and alkaline earth metal salts.

Particularly apt salts of this invention include the alkali metal salts of clavulanic acid such as the sodium and potassium salts of clavulanic acid.

The salts of this invention may be in crystalline or non-crystalline form and when crystalline may contain water of hydration, for example the sodium salt of clavulanic acid may be obtained as a crystalline tetratetrahydrate.

Since the salts of clavulanic acid are intended for use as pharmaceutical agents or intermediates for pharmaceutical agents they are normally used in substantially pure form. The processes hereinafter described may be readily employed to yield such substantially pure materials.

As has been previously stated, clavulanic acid and its salts have valuable therapeutic properties. Accordingly, in a further aspect, this invention provides a pharmaceutical composition which comprises clavulanic acid or a pharmaceutically acceptable salt thereof together with a pharmaceutically acceptable carrier.

As previously stated salts of clavulanic acid tend to be more stable than the parent compound so that the compositions of this invention more suitably comprise a pharmaceutically acceptable salt of clavulanic acid, for example one as referred to hereinbefore.

The compositions of the invention include those in a form adapated for oral, topical or parenteral use and may be used for the treatment of infection in mammals including humans.

Suitable forms of the compositions of this invention include tablets, capsules, creams, syrups, suspensions, solutions, reconstitutable powders and sterile forms

suitable for injection or infusion. Such compositions may contain conventional pharmaceutically acceptable materials such as diluents, binders, colourings, flavours, preservatives, and disintegrants in accordance with conventional pharmaceutical practice in the manner well undertood by those skilled in the art of formulating antibiotics. The compositions of this invention may be formed by bringing together the components thereof in known manner, that is in actual use or described in the literature.

Injectable or infusable compositions of the clavulanic acid or its salts are particularly suitable as high tissue levels of the synergist can occur after administration by injection or infusion. Thus, one preferred composition aspect of this invention comprises clavulanic acid or more suitably its pharmaceutically acceptable salt in sterile form, for example a conventional injectable alkali metal salt such as the sterile sodium or potassium salt. Such compositions may consist essentially of said sterile salt, that is the salt *per se* without added lubricants or the like. In accordance with conventional practice such injectable compositions will be made up in a sterile pyrogen-free liquid such as water for injection B.P.

Unit dose compositions comprising clavulanic acid or a salt thereof adapted for oral administration form a further preferred composition aspect of this invention.

Under certain conditions, the effectiveness of oral compositions of clavulanic acid and its salts can be improved if such compositions contain a buffering agent or an enteric coating agent such that the compounds of the invention do not have prolonged contact with highly acidic gastric juice. Such buffered or enterically coated compositions may be prepared in accordance with conventional pharmaceutical practice.

The clavulanic acid or its pharmaceutically acceptable salt may be present in the composition as sole therapeutic agent or it may be present together with a further therapeutic agent such as a penicillin or cephalosporin. Suitable penicillins and cephalosporins for inclusion in such synergistic compositions include not only those known to be highly susceptible to β-lactamases but also those which have a good degree of intrinsic resistance to some β-lactamases.

Naturally if the penicillin or cephalosporin present in the synergistic composition is not suitable for oral administration then the composition will be adapted for parenteral administration.

Penicillins suitable for inclusion in orally administrable compositions of this invention include benzylpenicillin, phenoxymethylpenicillin, propicillin, amoxycillin, ampicillin, epicillin, cyclacillin and other orally active penicillins and their pharmaceutically acceptable salts and *in-vivo* hydrolysable esters and aldehyde and ketone adducts of those penicillins containing a 6-α-aminoacylamido side chain and their pharmaceutically acceptable salts. Suitable penicillin *in-vivo* hydrolysable esters include the acetoxymethyl, pivaloyloxymethyl, α-ethoxycarbonyloxyethyl and phthalidyl esters of ampicillin or amoxycillin or the phenyl, tolyl and indanyl α-esters of carbenicillin and ticarcillin and pharmaceutically acceptable salts thereof. Suitable aldehyde and ketone adducts of penicillins containing a 6-α-aminoacylamido side chain include the formaldehyde and acetone adducts of ampicillin and amoxycillin, such as metampicillin and hetacillin, and their salts. Suitable penicillins for inclusion in injectably or infusably administrable compositions include the pharmaceutically acceptable salts of benzylpenicillin, phenoxymethylpenicillin, carbenicillin, propicillin, ampicillin, amoxycillin, epicillin, ticarcillin and cyclacillin.

Cephalosporins suitable for inclusion in orally administrable compositions of this invention include cephalexin, cephradine, cephalogycine and their pharmaceutically acceptable salts and other known cephalosporins and their pharmaceutically acceptable salts and *in-vivo* hydrolysable esters and aldehyde and ketone adducts of those cephalosporins containing a 7-α-aminoacylamido side chain and their pharmaceutically acceptable salts. Suitable cephalosporins for inclusion in the injectable or infusable compositions of this invention include the pharmaceutically acceptable salts of cephaloridine, cephalothin, cefazolin, cephalexin, cephacetrile, cephamandole, cephapirin, cephradine, cephaloglycine and other known cephalosporins.

When present in a pharmaceutical composition together with a penicillin or cephalosporin, the weight ratio of clavulanic acid or its salt present to penicillin or cephalosporin present may be from, for example, 10:1 to 1:10. for example 3:1 to 1:3.

Compositions of this invention may be used for the treatment of infections of

inter alia, the respiratory tract, the urinary tract and soft tissues in humans.

Compositions of this invention may also be used to treat infections of domestic animals such as mastitis in cattle. Thus this invention provides a method of treating bacterial infections in mammals other than humans which comprises the administration of a composition of this invention. Most suitably this method is the treatment of mastitis in cattle.

The penicillin or cephalosporin in a synergistic composition of this invention will normally be present at approximately the amount conventionally used when that penicillin or cephalosporin is the sole therapeutic agent used in the treatment of infection.

Suitably the weight of clavulanic acid or its salt in a unit dosage form of this invention will be from 50 to 500 mg and more suitably from 50 to 250 mg.

In general the total quantity of antibacterial agents present in a synergistic composition of this invention will not be greater than 1500 mg and will usually be between 100 and 1000 mg.

Normally between 500 and 3000 mg of the synergistic compositions of the invention will be administered each day of treatment (to an average 70 kg adult). However, for the treatment of severe systemic infections or infections of particularly intransigent organisms, higher doses may be used in accordance with clinical practice.

For treatment of infections the synergistic compositions of this invention are normally adapted to produce a peak blood level of at least 0.1 μg/ml, more suitably at least 0.25 μg/ml, and preferably at least 1 μg/ml of clavulanic acid.

Particularly favoured compositions of this invention will contain from 150 to 1000 mg of amoxycillin, ampicillin or an *in-vivo* hydrolysable ester or aldehyde or ketone adduct thereof or a pharmaceutically acceptable salt thereof and from 50 to 500 mg of clavulanic acid or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier therefor.

More suitably the compositions will contain from 200 to 500 mg of amoxycillin or a salt thereof or ampicillin or a salt thereof. More suitably the compositions will contain from 50 to 250 mg of clavulanic acid or a salt thereof.

Most suitably the compositions will contain a salt of clavulanic acid.

The materials present in such compositions may be hydrated. Thus the ampicillin may be present as ampicillin trihydrate and the amoxycillin may be present as amoxycillin trihydrate.

The weights of the antibiotics in such compositions are expressed on the basis of pure free antibiotic equivalent present and not on the basis of salt, ester, adduct or hydrate.

In a process aspect, the present invention provides a process for the preparation or clavulanic acid and salts thereof which process comprises cultivating a strain of *Streptomyces clavuligerus* and recovering clavulanic acid or a salt thereof from the culture medium.

The clavulanic acid or its salt which is recovered by the process of this invention may be the compound which is initially recovered from the culture medium or alternatively it may be obtained subsequent to the initial recovery of the acid, alternative salt or ester as hereinafter described.

Preferably, *Streptomyces clavuligerus* ATCC 27064 or a high-yielding mutant thereof is used in the process of this invention.

When used herein the term 'cultivating' means the deliberate aerobic growth of a clavulanic acid-producing organism in the presence of assimilable sources of carbon, nitrogen and mineral salts. Such aerobic growth may take place in a solid or semi-solid nutritive medium, or in a liquid medium in which the nutrients are dissolved or suspended. The cultivation may take place on an aerobic surface or by submerged culture. The nutritive medium may be composed of complex nutrients or may be chemically defined. Media containing complex nutrients such as yeast extract and soya bean flour [and the like] are particularly suitable.

The nutrient media which may be used for the cultivation of *Streptomyces clavuligerus* may contain 0.1—10% a complex organic nitrogen source such as yeast extract, corn steep liquor, vegetable protein, seed protein, hydrolysates of such proteins, milk protein hydrolysates, fish and meat extracts and hydrolysates such as peptones. Alternatively chemically defined sources of nitrogen may be used such as urea, amides, single or mixtures of common amino acids such as valine, asparagine, glutamic acid, proline and phenylalanine. Carbohydrate (0.1—5%) may be included in the nutrient media. Starch or starch hydrolysates such as dextrin, sucrose, lactose or other sugars or glycerol or glycerol esters may be used.

Glucose is not a particularly suitable carbohydrate. The source of carbon may also be derived from vegetable oils or animals fats. Carboxylic acids and their salts can be included as a source of carbon for growth and production of β-lactamase inhibitors. A particularly suitable low cost medium is one containing soya bean flour (such as Arkasoy; 'Arkasoy' is a Trade Mark) plus dried malt distillers solubles (such as Scotasol; 'Scotasol' is a Registered Trade Mark) plus dextrin. A futher particularly suitable medium is one containing a triglyceride (such as Prichem; 'Prichem' is a Registered Trade Mark) and soya bean flour (such as Arkasoy).

The addition of an antifoam agent such as Pluronic L81 ('Pluronic' is a Registered Trade Mark) may be necessary to control foaming of certain media in fermenters.

Mineral salts such as NaCl, KCl, $MgCl_2$, $ZnCl_2$, $FeCl_3$, Na_2SO_4, $FeSO_4$, $MgSO_4$ Na^+ or K^+ salts of phosphoric acid may be added to the media described above, particularly if chemically defined. $CaCO_3$ may be added as a source of Ca^{++} ions or for its buffering action. Salts of trace elements such as nickel, cobalt or manganese may also be included. Vitamins may be added if desired.

When used herein the term 'mutant' includes any mutant strain which arises spontaneously or through the effect of an external agent, whether that agent is applied deliberately or otherwise. Suitable methods of producing mutant strains include those outlined by H.I. Adler in Techniques for the Development of Micro-Organisms in 'Radiation and Radioisotopes for Industrial Micro-Organisms', Proceedings of a Symposium, Vienna, 1973, page 241, International Atomic Energy Authority and these include:

i. Ionising radiation (such as X- and γ-rays), uv light, uv light plus a photosensitizing agent (such as 8-methoxypsoralen), nitrous acid, hydroxylamine, pyrimidine base analogues (such as 5-bromouracil), acridines, alkylating agents (such as mustard gas or ethyl methane-sulphonate), hydrogen peroxide, phenols, formaldehyde and heat.

ii. Genetic techniques such as recombination, transformation, transduction, lysogenisation, lysogenic conversion and selective techniques for spontaneous mutants.

Cultivation of *Streptomyces clavuligerus* normally takes place in the temperature range 15—40°C, usually 20—35°C and preferably 25—30°C, and at a pH of between 5 and 8.5, preferably between 6 and 7.5.

The *Streptomyces clavuligerus* may be cultivated in the above media in glass conical flasks aerated by shaking on a rotary shaker or in baffled stainless steel fermenters stirred with vaned disc impellers and aerated with a sparger. The fermentation may also be carried out in a continuous fashion.

The starting pH of the fermentation is typically 7.0 and maximum yield of clavulanic acid obtained in 2—10 days at 20—35°C.

In a stirred stainless steel fermenter using the Arkasoy/Scotasol/Dextrin medium previously described the preferred temperature is 26°C and peak yields of clavulanic acid are obtained within 5 days.

Clavulanic acid or its salts may be extracted from the culture medium in various ways but normally the cells of the *Streptomyces clavuligerus* are first removed from the culture medium by such methods as filtration or centrifugation before such extraction procedures are commenced.

Clavulanic acid or its salts may be extracted from clarified culture medium by a variety of methods. Solvent extraction from cold clarified culture medium adjusted to acid pH values and methods which utilize the anionic nature of clavulanic acid at neutral pH such as the use of anion exchange resins have been found to be particularly useful. A further particularly useful method is to form an ester of clavulanic acid, purify the ester and regenerate the acid or its salt therefrom.

The extraction processes for obtaining clavulanic acid or its salts may notionally be divided into a primary isolation process followed by a further purification process.

Suitable primary isolation processes include solvent extraction of the free acid, solvent extraction of an ion pair, adsorption onto anion exchange material, adsorption onto carbon, precipitation, salting out and molecular filtration. The primary isolation processes we prefer to employ are the solvent extraction and anion exchange processes.

In the solvent extraction process the clavulanic acid is extracted into an organic solvent from cold clarified culture medium adjusted to an acid pH value.

In the solvent extraction of the free acid the clarified medium is chilled and the pH lowered into the region of pH 2—3 by the addition of acid while mixing with a water-immiscible organic solvent. Suitable acids used to lower the pH include hydrochloric, sulphuric, nitric, phosphoric or the like mineral acids. Suitable organic solvents include n-butanol, ethyl acetate, n-butyl acetate and methyl isobutyl ketone [and other similar solvents]. n-Butanol is believed to be a particularly suitable solvent for use in the extraction of the acidified culture filtrate. After separation of the phases clavulanic acid is found in solution in the organic phase. The β-lactamase-inhibiting metabolite may be back extracted from the organic phase into a new aqueous phase by making use of the greater water solubility of, for example, the alkali metal or alkaline earth metal salts of clavulanic acid in water than in organic solvents. Thus the β-lactamase-inhibiting metabolite may be back extracted from the organic solvent into an aqueous solution or suspension of an alkali metal or alkaline earth metal base, such as $NaHCO_3$, potassium hydrogen phosphate buffer or calcium carbonate, or water while maintaining the pH at approximately neutrality, for example pH 7. This aqueous extract, after separation of the phases, may be concentrated under reduced pressure. Freeze-drying may also be employed to provide a solid crude preparation of the salt of clavulanic acid. Such solid preparations are stable when stored as a dry solid at −20°C.

An alternative solvent extraction process makes use of ion-pairs of clavulanic acid with lipophilic amines. In this form of solvent extraction process clarified culture medium (usually at approximately neutral pH) containing a salt of clavulanic acid is contacted with an organic phase which contains an acid addition salt of a lipophilic di- or trialkylamine and thereafter separating the organic phase from the aqueous phase. Suitable organic solvents include such conventional water-immiscible polar solvents as methyl isobutyl ketone and trichloroethylene. Suitable amines include di- or trialkyl-amines in which one of the substituent groups is a long chain aliphatic group, for example of 12—16 carbon atoms, and one other is a tertiary alkyl group so that the molecule is lipophilic. Amberlite LA2 has proved a successful amine. Normally the amine is used as its acid addition salt. After this extraction process the clavulanic acid is present in the organic phase as the amine salt. The organic phase is then separated from the aqueous phase. The β-lactamase-inhibiting metabolite (that is, clavulanic acid) may be back-extracted into an aqueous phase by contacting the organic solution with an aqueous solution of an electrolyte, for example a concentrated solution of an alkali or alkaline earth metal salt such as sodium chloride or sodium nitrate. A crude solid preparation of the salte of clavulanic acid may then be obtained from the solution as described above.

In the anion exchange resin primary extraction process, the clarified culture medium, at an approximately neutral or slightly acid pH, that is pH 5.5—7.5, for example pH 6—7, is contacted with a bed of a polymeric anion exchange material such as a weak base anion exchange resin such as Amberlite IR4B or a strong base anion exchange resin such as Zerolit FFIP SRA62 (fomerly called DeAcidite FFIP SRA62 and also called Permutit FFIP SRA62) until the exchange material is substantially saturated, for example as judged when the β-lactamase material emerges from the bed through which the solution percolates. (Amberlite, Zerolit, Permutit and DeAcidite are Registered Trade Marks). Amberlite IRA4B is an example of a weak base anion exchange resin with polyamine active groups and a cross-linked polystyrene-divinylbenzene matrix. Zerolit FFIP SRA62 is an example of a strong base anion exchange resin with quaternary ammonium active groups and a cross-linked polystyrene-divinylbenzene matrix. The bed is then washed to remove unbound soluble impurities. The β-lactamase-inhibiting metabolite is then removed from the anion exchange material by passing therethrough a solution of an electrolyte, for example an alkali or alkaline earth metal salt such as sodium chloride. The β-lactamase-inhibiting fractions may be collected and bulked to yield a solution containing the salt of clavulanic acid and the electrolyte. This solution is then normally 'desalted' by which is meant the salt of clavulanic acid is separated from the electrolyte. This desalting may be effected by known methods of separating antibiotic salts from electrolytes such as passing the solution through a bed of material through which the antibiotic salt and the electrolyte pass at different rates. Suitable materials for desalting antibiotics include highly lipophilic resins which tend to absorb and thus retard the passage of organic materials in the presence of inorganic salts and gel filtration agents such as polyacrylamide gels which tend to retard the passage of small molecules but allow

the passage of larger molecules. Well known desalting agents of the previously described types used for desalting antibiotics include Biogel P2, Amberlite XAD—2 and XAD—4. (Biogel is a Registered Trade Mark). The desired metabolite may be eluted from the bed of desalting material by elution in normal manner, for example with water or water containing small quantities of a lower alkanol such as n-butanol. If desired the solid salt of clavulanic acid may be recovered from the desalted solution (that is the eluate) in conventional manner such as evaporation of the solvent under reduced pressure or by freeze-drying.

Many materials suitable for use in the above described processes are commercially available, for example from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Further purification of the crude solids obtained by the primary isolation methods listed above may be obtained by further application of such methods but ion exchange chromatography is particularly useful.

The ion exchange chromatography may take place in a bed of polymeric anion exchange material. Suitable exchange materials include the polymeric anion exchange materials hereinbefore described, for example a strong or weak base anion exchange resin such as the FFIP or IRA4B types of resin. Suitable medium base ion exchange materials include β-dimethylaminoethyl cellulose (such as Whatman DEAE cellulose DE52; Whatman is a Registered Trade Mark).

The ion exchange material may be loaded by applying to the bed a solution of the crude salt obtained from the primary isolation process. The β-lactamase-inhibiting metabolite may be obtained from the ion exchange material by elution with a solution of an electrolyte. Since the ion exchange materials are generally used in the chloride form it is usual that the electrolyte is the chloride of the cation required in the clavulanic acid salt. Thus to obtain the sodium salt of clavulanic acid from a FFIP column, the column is gradiently eluted with an aqueous solution of sodium chloride (0 to 0.5M). Similarly to obtain the sodium salt of clavulanic acid from a diethylaminoethylcellulose column the column may be gradiently eluted with an aqueous solution of sodium chloride; however since it is common practice to buffer diethylaminoethylcellulose columns we prefer to prepare the column in 0.01M phosphate buffer at pH 7 and gradiently elute with buffered sodium chloride solution (0—0.2M NaCl in 0.01M phosphate buffer, pH 7). In the aforementioned process we have generally gradiently eluted the column, that is, a solution of the electrolyte which gradually increases in concentration during the elution has been employed, rather than the conventional alternative of eluting using a solution in which a stepwise increase in concentration occurs.

Since the process of this invention is aptly adapted to the preparation of an alkali or alkaline earth metal salt of clavulanic acid, the electrolyte used in the proceeding process will aptly be an alkali metal or alkaline earth metal salt.

The active fractions emerging from the bed of ion exchange material may be detected by their β-lactamase inhibiting activity and their antibacterial activity against *Klebsiella aerogenes* in an agar diffusion assay. The fractions containing the bulk of the activity are combined, concentrated to small volume under reduced pressure, and, if desired, desalted. The desalting may be performed as described hereinbefore. The salt of clavulanic acid obtained from this process is normally of acceptable purity but if a yet purer product is required this may be obtained by subsequent purification procedures described hereinafter.

A variation of the process for the recovery of a pure form of clavulanic acid or its salt comprises isolating from a clarified culture medium an impure form of clavulanic acid or salt thereof, forming an ester of clavulanic acid, purifying the ester and thereafter regenerating pure clavulanic acid or a salt thereof from the ester.

The impure clavulanic acid or its salts used in this process will normally contain at least 1% by weight of the antibiotic.

Suitable esters for use in this process include those which may be cleaved by hydrogenolysis, enzymatic methods or by hydrolysis under very mild conditions.

The impure form of clavulanic acid or more usually a salt thereof which is to be purified in this process may be in the form of a solid or solution which will usually also contain considerable quantities of organic or inorganic impurities.

The clavulanic acid or salt thereof may be converted into an ester by conventional esterification reactions such as the reaction of clavulanic acid with a diazo compound, the reaction of clavulanic acid with an alcohol in the presence of a condensation-promoting agent such as dicyclohexylcarbodiimide or the reaction of a salt of clavulanic acid with an esterifying agent. Such reactions are described

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in our co-pending Application No. 36563/77 — 36564/77 — 36565/77 — 36566/77 Serial No. 1508978. The preferred method of forming the required ester of clavulanic acid is by the reaction of a salt of clavulanic acid with an esterifying agent such as a reactive chloride, bromide or iodide or a reactive sulphonate ester such as a $O.SO_2.CH_3$ or $O.SO_2C_6H_5CH_3$ containing moiety. Such reactions are frequently carried out in an organic solvent such as tetrahydrofuran, dimethylformamide, dimethylformamide/acetone, acetone, dimethylsulphoxide, N-methylacetamide or hexamethylphosphoramide. Dimethylformamide is a particularly suitable solvent. The esterification is performed at a non-extreme temperature, for example 5° to 30°C, and is conveniently performed at ambient temperature. Suitable salts for use include the alkali metal salts such as the sodium and potassium salts and those of polymeric anion exchange materials.

If desired the salt of clavulanic acid may be dissolved in the solvent in conventional manner or it may be bound to a polymeric support. Suitable supports for use in this process include strongly basic anion exchange materials, especially those possessing a macro-reticular nature which permits the use of non-aqueous solvent systems. We have found Amberlyst A26 to be suitable for this purpose. ('Amberlyst' is a Registered Trade Mark). The clavulanic acid salt may be adsorbed onto the resin from the culture filtrate and the resin then suspended in dimethylformamide containing the esterifying agent and, if desired, sodium iodide or alternatively the salt may be eluted off the resin with a solution of sodium iodide in dimethylformamide or in a mixture of dimethylformamide and acetone and the esterifying agent then added to the fractions containing the clavulanic acid salt. The sodium iodide which may be employed in these procedures aids elution from the resin.

Once formed, the impure ester of clavulanic acid is normally purified chromatographically. In such procedures the ester is normally dissolved in an organic solvent such as ethyl acetate, methylene chloride or chloroform. The solid phase used in the chromatographic process may be a material such as silica gel or hydroxypropyl derivatives of cross-linked polydextran gels such as Sephadex LH20 ('Sephadex' is a Registered Trade Mark).

The fractions emerging from the column may be tested for the presence of clavulanic acid ester by making use of its synergistic properties. Active fractions are normally combined and the organic solvent evaporated off under reduced pressure.

The ester resulting from this process is generally of acceptable purity, but the material may be re-chromatographed if desired.

This purified ester of clavulanic acid may be converted to clavulanic acid or a salt thereof by the before mentioned methods.

Many esters of clavulanic acid differ from analogous esters of penicillins in that they show an enhanced tendency to hydrolyse under mild conditions. Thus for example simple alkyl esters such as the methyl ester slowly hydrolyse to yield a salt of clavulanic acid in water buffered to pH 7. Esters which undergo base hydrolysis under mild conditions include alkyl esters of up to 6 carbon atoms optionally substituted by one chlorine, bromine or iodine atom or one methoxy or hydroxyl group. Other readily hydrolysable esters are described in our co-pending Application No. 36563/77 — 36564/77 — 36565/77 — 36566/77 Serial No. 1508978.

The esters used in the purification of clavulanic acid and its salts are suitably those which are cleaved by hydrogenolysis. One suitable group of esters of clavulanic acid for use in this process are those containing a $CO.O.CHR^1R^2$ moiety wherein R^1 is a hydrogen atom or an optionally substituted phenyl group and R^2 is an optionally substituted phenyl group. More suitably R^1 is a hydrogen atom or a phenyl, tolyl, chlorophenyl or methoxyphenyl group and more suitably R^2 is a phenyl, tolyl, chlorophenyl or methoxyphenyl group. Preferably R^1 is a hydrogen atom. Preferably R^2 is a phenyl group. Thus the benzyl ester of clavulanic acid is a preferred ester for hydrogenolysis.

Hydrogenolysis of such esters normally takes place in the presence of a transition metal catalyst such as a palladium catalyst, for example palladium on charcoal such as 10% palladium on charcoal (about 1/3 weight of catalyst per weight of ester). The reaction will generally employ a low or medium pressure of hydrogen and more suitably will employ a slightly super-atmospheric pressure of hydrogen, that is a pressure just sufficient to prevent leakage of atmospheric oxygen into the apparatus. The reaction may be carried out at an elevated, ambient or depressed temperature, but more suitably carried out at an approximately ambient temperature, for example at about 12—20°C. The reaction

may be carried out in a solvent conventionally used for hydrogenation. Suitable solvents for dissolving esters of clavulanic acid include optionally aqueous alkanols of 1—4 carbon atoms, for example ethanol or aqueous ethanol, or tetrahydrofuran or dioxane [or the like].

If it is desired to produce clavulanic acid *per se* no base need be present during the hydrogenation.

It if it desired to produce a salt of clavulanic acid the reaction may be carried out in the presence of a base, for example a carbonic acid anion base such as sodium or potassium hydrogen carbonate if it is desired to form, for example, the sodium or potassium salt. If the solvent used for the hydrogenation allows the base to go into solution then the acid is neutralised as it is formed by the hydrogenation. A suitable solvent for this form of the process is aqueous ethanol. An alternative method of producing a salt of clavulanic acid is to prepared clavulanic acid and thereafter neutralise it. One suitable method of doing this is to carry out the hydrogenation in the presence of a base but in a solvent in which the base is not soluble, for example in order to form the sodium salt the hydrogenation may be performed in ethanol employing sodium hydrogen carbonate as base. The clavulanic acid produced is then neutralised when a little water is added to the mixture to allow the base to dissolve and react with the acid.

The salts of clavulanic acid produced by the processes described hereinbefore are normally of good purity but if it is desired to purify them yet further the following subsequent purification processes may be employed.

Salts of clavulanic acid may be further purified by chromatography over cellulose using butanol/ethanol/water 4/1/5 v/v top phase as solvent. The active fractions from such chromatography may be combined and evaporated under vacuum until a solid is formed. Freeze-drying may also be employed to yield the solid salt.

Salts of clavulanic acid can be obtained in crystalline form by concentrating a solution of substantially pure salt in an aqueous alcohol such as aqueous ethanol. Such concentration may be effected by evaporation under reduced pressure at room temperature. Trituration under or crystallisation or recrystallisation from a suitable moist organic solvent such as moist acetone may be employed.

The following Descriptions 1—3 illustrate techniques useful for determining the presence of clavulanic acid or its salts.

The following Examples illustrate the invention.

DESCRIPTION 1.
ASSAY SUITABLE FOR DETECTION OF CLAVULANIC ACID.

Principle of the Assay.

Solutions containing clavulanic acid (culture filtrate, samples from isolation procedure and the like) are incubated for 15 minutes with a β-lactamase preparation in 0.05M phosphate buffer at pH 7 and 37°C. During this time, enzyme inhibition or inactivation occurs. Substrate (benzylpenicillin) is then added and incubation continued for 30 minutes at 37°C. The amount of enzymic degradation of the substrate to penicilloic acid is determined by the hydroxylamine assay for penicillin. The amount of β-lactamase used is such as to give 75% hydrolysis of the benzylpenicillin in 30 minutes at 37°C.

The extent of hydrolysis is a reflection of the amount of enzyme remaining uninhibited. The results are expressed as per cent inhibition of the enzyme activity by a given dilution of the clavulanic acid — containing solution (e.g. culture filtrate) or the concentration of clavulanic acid (μg/ml) giving 50% inhibition of the enzyme under the above stated conditions (I_{50}).

β-lactamase Enzyme.

The β-lactamase produced by *Escherichia coli JT4* is used as an enzyme. This culture is an ampicillin resistant strain and owes its resistance to the production of an R-factor controlled β-lactamase. Other similar R-factor controlled β-lactamases may be used if desired.

The culture, maintained on nutrient agar slopes, is inoculated into 400 ml. of sterile Tryptone medium contained in a 2 liter conical flask. This medium has the following composition Tryptone (Oxoid) 32 g/l, (Oxoid is a Registered Trade Mark) yeast extract (Oxoid) 20 g/l, NaCl 5 g/l and $CaCl_2.6H_2O$ 2.2 g/l. The final pH was adjusted to 7.4 with dilute NaOH. The flask is shaken at 25°C for 20 hours on a rotary shaker at 240 r.p.m.

The bacterial cells are collected by centrifugation, washed with 0.05M

phosphate buffer pH 7 (resuspended and centrifuged) and resuspended in water to give a cell concentration 25 times that in the cultivation medium. This cell suspension was then disrupted in an MSE ultrasonic disintegrator at 4°C. The cell debris was removed by centrifugation and aliquots of the supernatant stored deep-frozen. For use in the assay procedure, the supernatant is diluted in 0.005M phosphate buffer until it gives about 75% hydrolysis of a 1 mg/ml. solution of benzylpenicillin in 30 minutes at 37°C.

Assay Procedure.

Suitable dilutions of the inhibitor preparation and β-lactamase solution are mixed and incubated at 37°C for 15 minutes (Test). A control with buffer in place of inhibitor preparation is also incubated. Benzylpenicillin solution (substrate) is then added to test and control mixtures and incubation is continued for a further 30 minutes at 37°C. The residual benzylpenicillin in each mixture is then estimated using the hydroxylamine assay as described by Batchelor et al, *Proc. Roy. Soc., B 154.* 498 (1961). 6 ml. of hydroxylamine reagent are added to all tests, controls and blanks and are allowed to react for 10 minutes at room temperature prior to the addition of 2 ml of ferric ammonium sulphate reagent. The absorption of the final solutions is measured in an E.E.L. Colorimeter or a Spectrophotometer at 490 nm against the reagent blank. The composition of the reactions, tests and blanks prior to the hydroxylamine assay are as follows:

Components (all dissolved in or diluted with 0.005M pH 7 phosphate buffer)	Test ml.	Benzyl-penicillin Blank ml.	Control ml.	Reagent Blank ml.
Escherichia coli β-lactamase solution	1.9	0.0	1.9	1.9
Inhibitor solution	0.1	0.0	0.0	0.0
Benzylpenicillin 5 mg/ml.	0.5	0.5	0.5	0.0
0.005M pH 7 phosphate buffer	0.0	2.0	0.1	0.6

Calculation of Results.

The percentage inhibition of the β-lactamase is calculated as follows:

Absorption of benzylpenicillin blank minus absorption of control (uninhibited reaction) = x

Absorption of test (inhibited reaction) minus absorption of control (uninhibited reaction) = y

$$\% \text{ inhibition} = \frac{y}{x} \times 100$$

To obtain the I_{50} value, the inhibitor preparation is diluted until 50% inhibition of the β-lactamase inactivation of benzylpenicillin is obtained in the above procedure.

With benzylpenicillin as substrate the sodium salt of clavulanic acid has the following I_{50} values against the β-lactamase obtained from the named bacteria:

Source of β-lactamase	I_{50} (μg/ml)
Staphylococcus aureus (Russell)	0.06
Escherichia coli JT4	0.06
Escherichia coli B11	0.12
Klebsiella aerogenes A	0.036
Pseudomonas aeruginosa 1822	0.3
Pseudomonas dalgleish	0.006

DESCRIPTION 2.
PAPER CHROMATOGRAPHIC DETECTION OF CLAVULANIC ACID.

Culture filtrate and a reference solution of clavulanic acid (250 μg/ml partially purified preparation), are spotted (20 μl/origin) onto Whatman No. 1 paper strips 1 cm. wide ("Whatman" is a Registered Trade Mark). The chromatograms are run by descending chromatography for 16 hours at 5°C using n-butanol/isopropanol/water, 7/7/6 v/v, as solvent. The strips are dried at 40°C and laid on agar plates containing 6 μg/ml benzylpenicillin and seeded with a β-lactamase-producing strain of *Klebsiella aerogenes* (synergism system). The plates are incubated overnight at 30°C and clavulanic acid revealed as a zone of inhibited growth. The R_f value of the zone was 0.46. The 6 μg/ml benzylpenicillin alone is below the concentration required to kill the *Klebsiella aerogenes* but in the presence of a β-lactamase inhibitor, this concentration becomes toxic, that is to say there is synergism.

Use of the above synergism system enables clavulanic acid to be detected at concentrations below those at which it shows antibacterial activity.

DESCRIPTION 3.
THIN LAYER CHROMATOGRAPHIC DETECTION OF CLAVULANIC ACID SODIUM SALT.

Solutions of clavulanic acid sodium salt preparations are spotted (5 μl of 1mg/ml) onto glass plates coated with a 0.25 mm layer of silica gel (F254) as supplied by E. Merck, Darmstadt, Germany. The chromatograms are run at 22°C using the top phase of the mixture n-butanol/ethanol/water 4/1/5 v/v. The chromatogram plates are dried at 40°C and clavulanic acid sodium salt located by bioautography on agar plates containing 6 μg/ml. benzylpenicillin and seeded with *Klebsiella aerogenes* (synergism system — see section on paper chromatography above). The agar surface is covered by a fine filter cloth before laying the TLC plate onto it. After allowing 15—30 minutes for wetting and diffusion, the TLC plate is lifted off with the aid of the filter cloth and the agar plate incubated overnight at 30°C to reveal the zones of inhibited growth. The R_f value of clavulanic acid sodium salt in the above solvent is approximately 0.37. Two spray reagents, Ehrlich and triphenyltetrazolium chloride, are also used to reveal the clavulanic acid sodium salt zone. The former reagent consists of 300 mg of *p*-dimethylaminobenzaldehyde dissolved in 9 ml. of ethyl alcohol, 54 ml. of n-butanol and 9 ml of concentrated HCl. On heating the sprayed TLC plate at 120°C for 1—2 minutes, clavulanic acid sodium salt appears as a pink spot. The triphenyltetrazolium chloride reagent consists of a mixture of 1 volume of a 4% solution of 2,3,5-triphenyl-2H-tetrazolium chloride monohydrate in methanol with 1 volume of methanolic sodium hydroxide. After spraying, the TLC plates are heated at 80°C. Clavulanic acid sodium salt appears as a red spot on a white background.

EXAMPLE 1.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

Streptomyces clavuligerus was cultivated at 26°C on agar slopes containing 1% Yeatex (yeast extract) ("Yeatex" is a Registered Trade mark), 1% glucose and 2% Oxoid agar No. 3, pH 6.8. A sterile loop was used to transfer mycelium and spores from the slope into 100 ml of a liquid medium in a 500 ml Ehrlenmeyer flask. The liquid medium had the following composition:—

Oxoid Malt Extract	10 g/l
Oxoid Bacteriological Peptone	10 g/l
Glycerol	20 g/l
Tap water	1 liter

The medium was adjusted to pH 7.0 with sodium hydroxide solution and 100 ml. volumes dispensed into flasks which were closed with foam plugs prior to autoclaving at 15 lb/sq.in. for 20 minutes. An inoculated seed flask was shaken for 3 days at 26°C on a rotary shaker with a 2 inch throw and a speed of 240 r.p.m. Production stage flasks containing the liquid medium described above were inoculated with 5% vegetative inoculum and grown under the same conditions as the seed flask. Samples of culture filtrate were assayed for inhibitor action against the β-lactamase of *Escherichia coli* JT4. Optimum activity was obtained after 3 days. The results are shown in Table 1. A zone of clavulanic acid at R_f 0.46 was seen when the culture filtrate was examined by the paper chromatographic method previously described. The increase in size of the zone paralleled the increase in the β-lactamase inhibitor assay.

Streptomyces clavuligerus was also cultivated in 2 litre shaken flasks containing 400 mls. of medium (Production stage) using the same medium and cultural conditions as described earlier in this Example. In these larger vessels, growth of the organism was slower and optimum β-lactamase inhibitory activity was achieved 7—9 days after inoculation with the vegetative seed. The results are also shown in Table 1.

TABLE 1

β-Lactamase Inhibiting Activity of Streptomyces clavuligerus Grown in 500 ml. and 2000 ml. Flasks

Fermentation Time (Days)	% Inhibition of Escherichia coli β-lactamase at a final dilution of 1/2500 of culture filtrate	
	500 ml. Shaken Flask	2000 ml. Shaken Flask
1	15	–
2	30	–
3	55	–
4	50	10
5	51	21
6	57	36
7	–	51
8	–	53
9	–	50

EXAMPLE 2.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

A seed flask prepared as in Example 1 was used to inoculate 500 ml. conical flasks containing 100 ml. aliquots of the following medium in deionised water:—

Soluble Starch	2% w/v
Glycerol	0.3% w/v
Scotasol	0.1% w/v
Arkasoy	1% w/v
$FeSO_4.7H_2O$	0.01% w/v

The medium was sterilized by autoclaving at 15 p.s.i. for 20 minutes and inoculated by the addition of the 5% vegetative seed stage. The flasks were shaken at 26°C on a rotary shaker as in Example 1. Optimum titre of clavulanic acid was achieved between 3—5 days. A dilution of 1/2500 of the culture filtrate gave 60% inhibition in the β-lactamase inhibition assay. A zone of clavulanic acid was seen at R_f 0.46 when using the paper chromatographic (bicautographic) method previously described. This zone increased in size in parallel with the increase of the activity in the β-lactamase inhibitor assay.

[Soluble starch supplied by British Drug Houses Ltd., Poole, U.K.;
Scotasol is dried distillers solubles supplied by Thomas Borthwich Ltd., 60 Wellington Street, Glasgow, U.K.;
Arkasoy is soya bean flour supplied by British Arkady Co., Old Trafford, Manchester, U.K.].

EXAMPLE 3.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

A seed flask as produced in Example 1 was used to inoculate 500 ml. conical flasks containing 100 ml aliquots of the following medium prepared in deionised water and sterilised as previously described. The inoculum level was 5%.

Dextrin	2% w/v
Arkasoy	1% w/v
Scotasol	0.1% w/v
$FeSO_4$ $7H_2O$	0.01% w/v

The inoculated flasks were shaken at 26°C. Optimum β-lactamase inhibitory activity was achieved between 3—5 days. The activity was similar to that achieved in Example 2.

[Dextrin is supplied by C P C (UK) Ltd., Trafford Park, Manchester, U.K.].

EXAMPLE 4.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

The seed stage as described in Example 1 was used to inoculate 500 ml. conical flasks containing the following medium prepared in deionised water.

Dextrose	1% w/v
Soyabean Meal	1% w/v
Scotasol	0.05% w/v
$CaCO_3$	1% w/v

These flasks were treated exactly as in previous Examples and cultured under identical conditions. β-lactamase inhibitory activity was produced between 3—5 days. Culture filtrate at a final dilution of 1/2500 gave 35—45% inhibition in the β-lactamase inhibition assay.

EXAMPLE 5.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

β-lactamase inhibitory activity attributable to clavulanic acid was produced using the following medium with identical seed stage and cultivation conditions to Example 1.

Glycerol	2% w/v
Soyabean Meal	1.5% w/v
$Mg\ SO_4$	0.1% w/v
K_2HPO_4	0.1% w/v
Medium prepared in deionised water	

β-lactamase inhibitory activity reached a maximum level between 3—5 days and was of a similar order to that produced in Example 4.

EXAMPLE 6.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

The following medium produced clavulanic acid when using the conditions and vegetative seed inoculum as described in Example 1.

Glucose	2%
Lab Lemco* (Oxoid)	1%
Oxoid Yeast Extract	0.3%
$CaCO_3$	0.3%
Medium prepared in deionised water.	

*("Lab Lemco" is a Registered Trade Mark).

Optimum titres were achieved in 3—5 days and a 1/2500 dilution of the culture filtrate gave 35—45% inhibition in the β-lactamase enzyme inhibition assay.

EXAMPLE 7.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

As in Examples 4, 5 and 6 the following medium produced 35—45% inhibition (1/2500 dilution) in the β-lactamase assay at the optimum titre which is reached 3—5 days after inoculation. All conditions were as previously described.

Glucose	2% w/v
Arkasoy	1% w/v
$CaCO_3$	0.02% w/v
$CoCl_2.6H_2O$	0.0001% w/v
Medium prepared in deionised water.	

EXAMPLE 8.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

The following production stage medium, when used under standard cultivation conditions as described in previous Examples, produced 20—30% inhibition at 1/2500 dilution in the β-lactamase assay between 3—5 days after inoculation. Using the paper chromatographic method previously described, a zone of clavulanic acid was seen at R_f 0.46 when the culture filtrate was examined.

Scotasol	2%
Oxoid Yeast Extract	1%

Medium prepared in tap water.
Final pH 7.0

EXAMPLE 9.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

Under standard cultivation conditions, the following medium produced clavulanic acid 3—5 days after inoculation with the vegetative seed. A 1/2500 dilution of the culture gave 20—30% inhibition in the β-lactamase inhibition assay.

	g/l
Glycerol	15
Sucrose	20
Proline	2.5
Monosodium Glutamate	1.5
NaCl	5.0
K_2HPO_4	2.0
$CaCl_2$	0.4
$MnCl_24H_2O$	0.1
$FeCl_36H_2O$	0.1
$ZnCl_2$	0.05
$MgSO_47H_2O$	1.0

Medium prepared in deionised water.
Final pH 7.1.

EXAMPLE 10.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

A stock Yeatex/glucose agar slope was used to inoculate a Yeatex/glucose agar slope in a Roux bottle by making a mycelium/spore suspension in sterile water. The Roux bottle slope was incubated at 26°C for 10 days. To this slope 100 mls. of sterile water was added and a mycelial suspension prepared. This was used to inoculate 50 litre of a steam-sterilised seed medium of the following composition in tap water.

Oxoid Malt Extract	1% w/v
Oxoid Bacteriological Peptone	1% w/v
Glycerol	1% w/v
10% Pluronic L81 Antifoam in Soyabean Oil	0.5% w/v

[Pluronic supplied by Jacobs and Van den Berg UK Ltd., 231 The Vale, London, W3 containing a polypropylene-polyethylene block polymer, and Soyabean Oil supplied by British Oil and Cake Mills Ltd., Stoneferry Road, Hull, U.K.].

The medium was contained in a 90 litre stainless steel baffled fermenter, agitated by a 5" vaned disc impeller at 240 r.p.m. Sterile air was supplied at 50 l/min and the tank incubated at 26°C.

After 72 hours, the seed fermenter was used to incoulate 150 litre of the same medium using a 5% v/v addition by sterile transfer. This production stage medium was contained in a 300 L stainless steel, fully baffled fermenter agitated by a 8¼" vaned disc impeller at 210 r.p.m. Sterile air was supplied at 150 l/min. The fermentation was maintained at 26°C. An antifoam agent was added when required in 10 ml. shots (10% Pluronic L81 in soyabean oil). Samples were removed for β-lactamase inhibition assay at regular intervals. The fermenter was harvested between 4—5 days at the optimum level of β-lactamase inhibitory activity (Table 2).

TABLE 2

β-Lactamase Inhibitory Activity of Samples of Culture Filtrate taken from a 300 litre Fermentation of *Streptomyces Clavuligerus*

Fermentation Time (days)	% Inhibition in β-Lactamase Inhibition Assay at a Final Dilution of 1/2500
1.0	12
1.5	20
2.0	31
2.5	36
3.0	50
3.5	54
4.0	51
4.5	56
5.0	55

EXAMPLE 11.

CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

The seed fermenter was run exactly as described in Example 10 using the same medium.

After 72 hours, the seed fermenter was used to give a 5% v/v vegetative inoculum into a 300 litre stainless steel fully baffled fermenter containing 150 litre of steam-sterilised medium agitated by an 8½ inch vaned disc impeller at 210 r.p.m. Sterile air was supplied at 150 l/min. The fermentation was maintained at 26°C. An antifoam agent was added when required in 10 ml. shots (10% Pluronic L81 in soya bean oil).

The medium used in the production stage was as described in Example 3 with the addition of 0.05% v/v of 10% Pluronic L81-soyabean oil antifoam prior to sterilisation.

The β-lactamase inhibitory activity of fermentation samples was similar to those of Example 10 (see Table 2). Paper chromatographic examination revealed a zone of clavulanic acid at R_f 0.46 using the bioautographic (synergism) method previously described. The size of the clavulanic acid zone increased in parallel with the increase in the β-lactamase inhibitor assay.

EXAMPLE 12.

CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

100 mls of sterile water was added to a sporing culture which had been grown on Bennetts agar in a Roux bottle for 10 days at 26°C. A mycelium/spore suspension was produced and used to inoculate 75 litres of a steam-sterilised medium of the following composition in tap water.

Dextrin	2% W/V
Arkasoy '50'	1% W/V
10% Pluronic L81 in soyabean oil	0.03% V/V

The pH of the medium was adjusted to 7.0.

The medium was contained in a 100 litre stainless steel baffled fermenter, agitated by a 7½" vaned disc impeller at 140 rpm. Sterile air was supplied at 75 l/minute and the tank incubated for 72 hours at 26°C.

The contents of the seed fermenter were used to inoculate 1500 litres of a steam-sterilised medium of the following composition in tap water.

Arkasoy '50'	1.5% W/V
Glycerol	1.0% W/V
KH_2PO_4	0.1% W/V
10% Pluronic L81 in soyabean oil	

The medium was contained in a 2000 litre stainless steel fully baffled fermenter agitated by two 19" vaned disc impellers at 106 r.p.m.

Sterile air was supplied at 1200 litres per minute. An antifoam agent was added in 25 ml amounts as required. (10% Pluronic L81 in soyabean oil). The fermentation was controlled at 26°C until a maximum yield of clavulanic acid was obtained between 3—5 days when 200—300 μg/ml of clavulanic acid were produced.

EXAMPLE 13.
CULTIVATION OF STREPTOMYCES CLAVULIGERUS.

Inoculum was produced in a seed flask as previously described, but using the medium described in Example 3 (with pH of the medium adjusted to 7.0). This was used to inoculate 500 ml conical flasks containing 100 ml aliquots of the following medium prepared in deionised water and sterilised. The inoculum level was 5%.

Prichem *P224	1% W/V
Arkasoy '50'	1.5% W/V
KH_2PO_4	0.1% W/V

The pH of the medium was adjusted to 7.0.

*("Prichem" is a Registered Trade Mark).

The inoculated flasks were shaken at 26°C and optimum β-lactamase inhibitory activity was achieved between 3—5 days. Levels of 300—500 μg/ml of clavulanic acid were achieved.

Prichem P224 is a triglyceride supplied by Prices Limited, Bromborough, Bebington, Wirral, Cheshire, U.K.

Prichem P224 is based on oleic acid (65%), palmitic acid (11%) and other similar acids.

EXAMPLE 14.
ISOLATION OF CRUDE CLAVULANIC ACID SODIUM SALT.

Harvested culture liquor produced as described in Example 10 was clarified by continuous flow centrifugation and the mycelium discarded. From 150 litre of fermentation liquor 120 litre of clarified culture fluid was obtained. This filtrate gave 58% inhibition in the β-lactamase inhibition assay at 1/2500. The filtrate was chilled to 5°C and 40 litre of n-butanol added. The mixture was stirred and 25% H_2SO_4 added until the pH was 2.0. The acidified mixture was stirred for a further 10 mins. before separating the phases by centrifugation. The aqueous phase was discarded. To the n-butanol extract 0.5% of Norit GSX carbon ("Norit" is a Registered Trade Mark) was added and the mixture stirred for 15 minutes. The carbon was discarded after removal by filtration using diatomaceus earth as a filter aid. To the n-butanol a quarter of its volume of deionised water was added and the mixture stirred while adding 20% NaOH solution until the pH had equilibrated at 7.0. The phases were separated by centrifugation and the n-butanol phase discarded. The aqueous phase was concentrated under reduced vacuum to 800 ml. and then freeze-dried. This yielded 35g. of a crude solid preparation of clavulanic acid with an I_{50} of 1.3 μg/ml in the β-lactamase inhibition assay. This solid preparation was stored dry at −20°C while awaiting further purification.

EXAMPLE 15.
ISOLATION OF CRUDE CLAVULANIC ACID SODIUM SALT.

One litre of culture filtrate giving 53% inhibition at 1/2500 in the β-lactamase inhibition assay and obtained as described in Example 12 was percolated down a 1 inch diameter × 6 inch column of Permutit Isopor ("Isopor" is a Registered Trade Mark) resin FF 1P (SRA 62) in the Cl^- form [supplied by Permutit Co. Ltd., 632—652 London Road, Isleworth, Middlesex, U.K.]. The culture filtrate was followed by 300 ml. of distilled water to wash the column. Elution of the active β-lactamase inhibitor was achieved with 0.2M NaCl solution. Fractions (20 ml.) were collected and assayed at a 1/2500 final dilution in the β-lactamase inhibition assay. Active fractions were combined and concentrated under vacuum to 20 ml. This solution was desalted by gel exclusion chromatography on a Biorad Biogel P2 column 1¼ inches in diameter with a gel bed of 16 inches and eluted with 1% n-butanol in water. [Biogel P2 is supplied by Bio Rad Laboratories, 32nd and Griffin Ave., Richmond, California, U.S.A.]. The active fractions, as determined by the β-lactamase inhibition assay, were combined. Sodium chloride was eluted after clavulanic acid and was detected using silver nitrate solution. The combined active fractions were concentrated and freeze-dried.

One litre of culture filtrate, after the above treatment, yielded 0.45g. of a crude solid preparation of clavulanic acid having an I_{50} of 0.92 μg/ml.

This solid was stored at −20°C while awaiting further purification.

EXAMPLE 16.
ISOLATION OF CRUDE CLAVULANIC SODIUM SALT.

Chilled culture filtrate (5—10°C) containing 300 μg/ml of clavulanic acid salt was pumped to an in-line mixer, at the inlet of which enough 6% (v/v) nitric acid was added to maintain an outlet pH of 2.0 ± 0.1. The acidifed filtrate was passed at 4.1/min through a glycol-cooled plate heat exchanger (A.P.V. Ltd.) to maintain a temperature between 2—5°C. The pH was monitored in a flow cell before passing into a three stage countercurrent separator (Westfalia Separator Ltd., Model EG 1006).

Chilled water-saturated n-butanol (at about 5°C) was pumped at 3 l/min into the countercurrent separator.

The aqueous outlet from the countercurrent separator was run to waste. Entrained water was removed from the butanol outflow of the countercurrent separator using a liquid/liquid centrifugal separator. (Alfa Laval Ltd. Model 3024X—G, "Alfa" is a Registered Trade Mark). The butanol was collected in a stainless steel vessel, fitted with a cooling jacket, in which it was stored at about 5°C.

From the vessel, 40 l aliquots were removed and thoroughly mixed with 2 l of chilled water (5°C), saturated with n-butanol. The pH of this mixture was adjusted to pH 6.8 ± 0.1 using 20% sodium hydroxide solution.

This aqueous extract/butanol mixture was fed to a liquid/liquid centrifugal separator (Sharples Centrifuge Ltd. Model M35PY—5PH) at a pumped rate of 2 l/min.

From 1800 l of culture filtrate, 90 l of aqueous phase was recovered, containing sodium clavulanate equivalent to 39% of the clavulanic acid present in the culture filtrate.

15 l of the aqueous extract was adjusted from 2% to 8% total solids by the addition of 60 g sodium chloride per litre, and spray-dried (Anhydro, Copenhagen, Type Lab S 1). The conditions used were: Feed rate 2l/hr; Atomizer voltage 170 v; Heater setting 6—7; Inlet temp 150°C; Outlet temp 80°C.

The dried product, total weight 1 kg., contained 62% of the sodium clavulanate present in the feedstock.

The remaining 75 l of aqueous extract was concentrated by ultrafiltration (De Danske Sukkerfabrikker. Laboratory Module, Membrane Type 900). The operating procedure was to re-circulate the retentate from a stainless steel tank, fitted with a cooling system, with the outlet valve set so as to give a differential pressure across the 40 membranes of 25 atmospheres. The temperature was maintained at 2—5°C and the pH at 6.8 ± 0.1 by addition of 2*N* hydrochloric acid, as necessary. The volume was reduced to 34 l which contained 72% of the clavulanic acid present in the feedstock.

The aqueous concentrate was stored at about 5°C, adjusted to 8% solids, and spray-dried as above. The dried material, which contained 75% of the clavulanic acid sodium salt present in the feed stock, was approximately 2% pure on a weight/weight basis.

(The total spray-dried product, from 90 l of aqueous extract contained 69.4g of sodium clavulanate, which was 72% of the sodium clavulanate in the spray-drying feedstock and 21% of the metabolite present in 1800 l of culture filtrate).

EXAMPLE 17.
PARTIAL PURIFICATION OF CRUDE SODIUM SALT OF CLAVULANIC ACID.

Crude clavulanic acid sodium salt preparations obtained as described in Example 15 were purified by ion exchange chromatography. Eighteen grams of material prepared as described in Example 15 having an I_{50} value of 1.3 μg/ml (final concentration) were dissolved in 25 ml. of distilled water and applied to a 1½" × 16" bed of Permutit FF IP (SRA 62) resin in the chloride form. The column was eluted with a sodium chloride gradient formed by gravity-feeding 0.5M sodium chloride into a mixing reservoir containing 1 litre of distilled water which in turn fed the chromatographic column. 10 ml. cuts were collected and β-lactamase inhibitory activity assayed using a 1/2500 dilution of the fractions.

Activity was eluted after a main band of colour between fractions 24 and 30. The active fractions were combined and concentrated to 30 ml.

This solution was desalted using a 2" x 18" bed of Biorad Biogel P2 and eluting with 1% n-butanol in water. The 20 ml. fractions were assayed for clavulanic acid content using the β-lactamase inhibition assay. The fractions were also spotted onto paper strips and sprayed with either the Ehrlich or the triphenyltetrazolium spray reagents described in Description 3. β-lactamase inhibitory activity correlated with the pink or red spots respectively produced by these reagents. Active cuts were combined, excluding those containing sodium chloride, and concentrated under vacuum to dryness. This yielded 520 mg. of partially purified clavulanic acid sodium salt with an I_{50} of 0.2 μg/ml in the standard β-lactamase inhibitor assay.

Thin layer chromatography (silica gel) of this clavulanic acid preparation gave the following R_f values: n-butanol/ethanol/water 4:1:5 v/v top phase R_f 0.37; n-butanol/acetic acid/water 12:3:5 v/v R_f 0.44; isopropanol/water 7:3 v/v R_f 0.78. The zones were detected by spraying with Ehrlich's reagent. 6-Aminopenicillanic acid, run as a marker and detected with the same spray, had R_f values of 0.38, 0.39 and 0.77, respectively.

EXAMPLE 18.
PURIFICATION OF CLAVULANIC ACID SODIUM SALT.

Culture filtrate produced as described in Example 12 was solvent-extracted as in Example 14 to give a solid preparation which was further purified by ion exchange chromatography using Whatman diethylaminoethyl cellulose DE 52 as follows. The crude solid (10g.) was dissolved in 20 ml. of distilled water and applied to a 1½" x 20" column of DE 52 cellulose previously equilibrated with 0.01M sodium phosphate buffer pH 7.5. The column was eluted with a NaCl gradient. 0.1M NaCl in 0.01M sodium phosphate buffer pH 7.5 was fed into a mixing chamber containing 1 litre of 0.01M phosphate buffer pH 7.5, which in turn was connected to the column. Fractions (10 ml.) were collected and these were assayed for β-lactamase inhibitory activity at a dilution of 1/2500. The fractions were also examined for antibacterial activity by the hole-in-plate assay method using nutrient agar plates seeded with *Klebsiella aerogenes*. The fractions having the highest β-lactamase inhibitory activity and giving zones of inhibition in the hole-in-plate assay were combined, concentrated and then desalted on a Biorad Biogel P2 column. These fractions were shown to contain clavulanic acid by paper and thin layer chromatography.

EXAMPLE 19.
ISOLATION OF SOLID CLAVULANIC ACID SODIUM SALT.

A partially purified solid preparation of the sodium salt of clavulanic acid (500 mg) prepared as in Example 17 was loaded onto a Whatman microgranular CC31 cellulose column with 1" x 20" bed size. The chromatographic solvent was n-butanol/ethanol/water 4:1:5 v/v, top phase. The column was run at 4°C and 4 ml fractions collected. Fractions were tested for the presence of clavulanic acid by spotting onto filter paper and spraying with the Ehrlich (pink spot) or triphenyltetrazolium (red spot) spray reagents. These spot tests were confirmed by β-lactamase inhibition assays at a 1/250 dilution. Active fractions were combined and dried under vacuum on a rotary evaporator to yield solid clavulanic acid sodium salt tetrahydrate in the form of a thin layer adhering to the flask. In order to extract the product from the flask the solid was dissolved in a small volume of distilled water and freeze-dried to yield the sodium salt of clavulanic acid (40 mg) as a fluffy white solid.

EXAMPLE 20.
ISOLATION OF SUBSTANTIALLY PURE CLAVULANIC ACID SODIUM SALT.

Concentrated back extract (6 l) (from ultrafiltration in Example 16) containing 10g of clavulanic acid sodium salt as determined by the β-lactamase inhibition assay of Description 1 was percolated at 1 l/hr onto a 2" x 24" column of Permutit Zerolit FF1P SRA 62 anion exchange resin in the chloride form. The column was then washed with 2 l of deionized water prior to elution with a sodium chloride gradient. The gradient was formed by a reservoir containing 4 l of 1.4M NaCl feeding a stirred reservoir containing 4 l of 0.7M NaCl which in turn was connected to a stirred reservoir containing 4 l of deionized water which was

connected via a pump to the column. The column was eluted at 2.5 ml/min and 25 ml fractions collected. Fractions were assayed by the β-lactamase inhibition assay. Active fractions (nos. 140—230) were combined and vacuum evaporated to near dryness. Ethanol (500 mls) was then added and the solid filtered off after vigorous shaking. The ethanol extract was then vacuum evaporated to dryness on a rotary evaporator and redissolved in deionized water (40 mls). This was loaded onto a 4" x 24" column of Biorad Biogel P_2 and eluted with a 1% n-butanol solution. Fractions were collected (25 ml) and assayed for β-lactamase inhibitory activity at a 1/2500 final dilution. Tests for sodium chloride content on 1/25 dilutions of the fractions were made using silver nitrate solution. Those fractions containing clavulanic acid sodium salt free of sodium chloride were combined, concentrated by evaporation of the solvent under reduced pressure to 20 mls and then freeze dried. This yielded 4.8 g of the sodium salt of clavulanic acid. (I_{50} about 0.06 μg/ml indicating a substantially pure product had been obtained).

EXAMPLE 21.
EXTRACTION OF CLAVULANIC ACID USING LIPOPHILIC AMINE.

Culture filtrate (200 ml, obtained in a similar manner to Example 3 but using a medium containing 0.1% v/v KH_2PO_4 instead of 0.01% $FeSO_4.7H_2O$) was extracted with Amberlite LA2* (chloride form, 15% v/v in methyl isobutyl ketone, 66 ml) for 30 minutes at 5°C.

The phases were separated by centrifugation (1660 g, 20 minutes). The solvent phase (60 ml) was recovered by pipette and divded into four equal portions. Each portion was extracted by stirring at 5°C for 20 minutes with 1/4 volume (3.75 ml) aqueous extractant as indicated in the table below. The resulting mixture was centrifuged (1660 g. 15 minutes). 3.6 ml. aqueous phase was recovered from each extraction.

Sample	Volume (ml)	Clavulanic acid or sodium salt concentration (μg ml^{-1})	Clavulanic acid sodium salt (mg)
clarified brew	200	128	25.4
extracted brew	200	15	3.0
M NaCl extract	3.6	305	1.1
2M NaCl extract	3.6	598	2.5
M $NaNO_3$ extract	3.6	638	2.3
2M $NaNO_3$ extract	3.6	758	2.73

The result obtained with 2M $NaNO_3$ represents a recovery of 43% from clarified brew.

*Amberlite LA2 is obtainable from Rohm and Haas (UK) Ltd. Croydon.

EXAMPLE 22.
EXTRACTION OF CLAVULANIC ACID USING LIPOPHILIC AMINE.

Clarified brew (47 litres, obtained as in Example 12) was extracted with Amberlite LA2 (acetate form, 15% v/v in methyl isobutyl ketone, 12.5 litres) by stirring for 1 hour at 17°C. After adding octan-1-ol (500 ml) the phases were separated in a continuous flow centrifuge yielding 9.2 litres solvent phase, which was then stirred at 5°C for 1½ hours with 1M sodium nitrate (2.3 litres). The mixture was separated by continuous flow centrifugation yielding 2.4 litres aqueous phase (including water used for displacement purposes). Aqueous phase pH (initially 8.0) was adjusted to 7.0 with concentrated hydrochloric acid.

Sample	Volume (l)	Clavulanic acid or sodium salt concentration ($\mu g\ ml^{-1}$)	Clavulanic acid or sodium salt (mg)
clarified brew	47	146	6862
extracted brew	47	19	893
M $NaNO_3$ extract	2.4	1638	3931

Extraction efficiency from clarified brew to sodium nitrate extract is 57%.

EXAMPLE 23.
PREPARATION OF CLAVULANIC ACID BENZYL ESTER.

Culture filtrate (20 l.) obtained as described in Example 10 was vacuum evaporated using a climbing film evaporator to 5 l. The concentrate was then freeze-dried using an Edwards E.F.6 shelf freeze drier manufactured by Edwards High Vacuum Ltd. The 300g. of solid so obtained contained 3 g. of the sodium salt of clavulanic acid as determined by the enzyme inhibition assay. The solid was suspended in 900 ml. of dry dimethylformamide and 150 ml. of benzyl bromide was added. The mixture was stirred for 2 hours at room temperature and then diluted with 1 l. of ethyl acetate. The reaction mixture was filtered and the filtrate concentrated to as low a volume as possible. The oily residue was extracted with a further 1 l. of ethyl acetate and the extract filtered. The filtrate was again concentrated and the resulting oily residue loaded onto a 3" x 14" silica gel column (Biogel Biosil A 100 mesh) in cyclohexane. The column was eluted with cyclohexane to remove benzyl bromide and the solvent was then changed to ethyl acetate and 20 ml. fractions collected. Fractions were tested for the presence of the benzyl ester of clavulanic acid by spotting onto glass backed silica gel t.l.c. plates (Merck precoated silica gel 60 F 254) (Merck is a Registered Trade Mark) and spraying with 2,3,5-triphenyl-tetrazolium chloride (TTC) spray reagent. Fractions giving intense red spots with this reagent were further examined by t.l.c. on silica gel plates using chloroform-ethyl acetate (8:2) as the solvent and spraying the developed plates with TTC spray. The benzyl ester of clavulanic acid runs at R_f 0.31 at 22°C. Fractions containing this ester were combined and concentrated to 15 ml. and this solution was further chromatographed on a 1½" x 16" silica gel column (Merck silica gel H, type 60) with chloroform/ethyl acetate 8:2 as the solvent. 15 ml. fractions were collected and tested for the benzyl ester as described above. Those fractions containing the ester were concentrated to 8 ml. and finally purified by column chromatography on a 1" x 16" silica gel column (Merck silica gel H, type 60) with ethyl acetate-cyclohexane (8:2) as the solvent. Selected fractions were combined and vacuum evaporated to give pure benzyl ester as an oil, 160 mg.

EXAMPLE 24.
PREPARATION OF CLAVULANIC ACID BENZYL ESTER.

Spray-dried solid (3.3 kg) containing 69.4 g of clavulanic acid sodium salt (as determined by enzyme inhibition assay) was obtained as described in Example 16. The solid was then slurried in 5.5 l. of dimethylformamide and 500 mls. of benzyl bromide added. After stirring at room temperature for 2 hours, 12 l. of ethyl acetate were added and the solids removed by filtration. The filtrate was vacuum evaporated to an oily residue (212 g). The residue was loaded onto a column containing a 4" x 13" bed of silica gel (Hopkins & Williams MFC) in cyclohexane. The column was eluted with 12 l. of cyclohexane to remove excess benzyl bromide. The eluent was then changed to ethyl acetate and 500 ml. fractions collected. These were tested for benzyl clavulanate content by spotting onto silica gel t l c plates (Merck precoated silica gel 60 F 254) and spraying with 2,3,5-triphenyltetrazolium chloride (TTC) spray reagent. Fractions giving intense red spots were further examined by t l c on silica gel with chloroform/ethyl acetate (8:2) as the solvent and spraying the developed plates with T T C spray. Fractions 5—13 contained the bulk of the ester, and these were combined and vacuum concentrated to an oil (79.3 g). This preparation was then chromatographed on a

4" x 18" column of silica gel (Merck silica gel H type 60) with chloroform/ethyl acetate (8:2) as the solvent. Fractions were selected as described above and yielded on concentration 45.9g. of oil which was 62% purity as adjudged by NMR spectroscopy.

This product was finally chromatographed on a 2¼" x 18" column of Sephadex LH 20 in cyclohexane/chloroform 1:1. After selection of fractions and concentration a colourless oil (27.6 g) was obtained which proved to be 95% pure benzyl ester of clavulanic acid as determined by NMR spectroscopic examination.

(Sephadex LH20 is a hydroxypropyl derivative of Sephadex Q25 supplied by Pharmacia Great Britain, 75 Uxbridge Road, London, W.5, U.K.; Sephadex is a Registered Trade Mark).

EXAMPLE 25.
PREPARATION OF CLAVULANIC ACID BENZYL ESTER.

Culture filtrate (150 l) at pH 7.0 containing 16.2 g. of clavulanic acid sodium salt as determined by the enzyme inhibition assay was stirred with 5 kg. of Amberlyst A.26 anion exchange resin in the chloride form (Rohm & Hass Company, Philadelphia, USA) for 1 hour at room temperature. The resin was then filtered and the filtrate reassayed, showing that 6.4 g of clavulanic acid had been removed. The resin was washed with 20 l. of deionised water, followed by 20 l. of acetone and 10 l. of dimethylformamide (DMF). After refiltering the resin was suspended in 2.3 l. of DMF/0.2M NaI. To this was added 200 mls. of benzyl bromide and the suspension stirred thoroughly. After standing at room temperature for 16 hours, ethyl acetate (2 l) was added, the resin was then filtered and further washings (ethyl acetate) of the resin were combined with the filtrate. The extract was then concentrated to a small volume and chromatographed on 3" x 18" silica gel column (Merck silica gel H type 60) with ethyl acetate/cyclohexane (8:2) as the solvent. Fractions containing benzyl clavulanate were selected by spotting onto silica gel t l c plates and spraying with TTC reagent as described in Example 23. Those selected were concentrated to 20 mls and then chromatographed on a 1½" x 18" silica gel column (Merck silica gel H type 60) with chloroform/ethyl acetate (8:2) as the solvent. Selected fractions were combined and evaporated to a colourless oil (440 mgs) which was 90% benzyl clavulanate as determined by NMR spectroscopy.

EXAMPLE 26.
PREPARATION OF CLAVULANIC ACID BENZYL ESTER.

An aliquot of aqueous back extract of the butanol extract of culture filtrate obtained as described in Example 14 was freeze-dried using an Edwards chamber drier. A 24 g. portion of the solid obtained contained 0.96 g. of the sodium salt of clavulanic acid as determined by the enzyme inhibition assay. This solid was suspended in 75 ml. of dry dimethylformamide and 75 ml. of benzyl bromide was added. The mixture was stirred for 2 hours at room temperature. The suspension was then diluted with 500 ml. of ethyl acetate and the mixture filtered. The filtrate was concentrated to an oily residue on a vacuum rotary evaporator. This residue was loaded onto a 2" x 14" silica gel column (Biogel Biosil A.100 mesh) in cyclohexane. Benzyl bromide was eluted from the column and then the solvent was changed to ethyl acetate and 10 ml. fractions were collected. Fractions containing the benzyl ester of clavulanic acid were selected as in Example 23. Further purification was also achieved as described in Example 23 by column chromatography. This process yielded 220 mg. of pure benzyl ester.

EXAMPLE 27.
PREPARATION OF CLAVULANIC ACID BENZYL ESTER.

Impure 3 - (β - hydroxyethylidine) - 7 - oxo - 4 - oxa - 1 - azabicyclo[3,2,0]heptane - 2 - carboxylic acid sodium salt (thought to be roughly 55 mg. of pure material) in dry dimethylformamide (0.64 ml.) was treated with benzyl bromide (0.18 ml.). The solution was kept at room temperature (approx. 17—18°C) for 3 hours under anhydrous conditions. The reaction mixture was fractionated on silica gel, eluting with ethyl acetate, to give in substantially pure form the benzyl ester of 3 - (β - hydroxyethylidine) - 7 - oxo - 4 - oxa - 1 - azabicyclo[3,2,0]heptane - 2 - carboxylic acid 63 mg.) as a colourless oil. i.r. (film) 1800, 1745, 1695 cm^{-1}; n.m.r. ($CDCl_3$), 2.25 (s,l, exchangeable with D_2O), 3.05 (d,l,J = 17Hz), 3.51 (dd,l,J=17 Hz, J_2=2.5 Hz), 4.24 (d,2,J=7.5Hz), 4.92 (dt,l,J=7.5Hz, J_2=1.5Hz), 5.15 (d,l,J=1.5Hz), 5.24 (s,2), 5.71 (d,l,J=2.5 Hz), 7.45 δ (s,5).

EXAMPLE 28.
PREPARATION OF CLAVULANIC ACID.

Benzyl clavulanate (100 mgs) in ethanol (5 ml) was hydrogenated over 10% Pd/C (30 mgs) for 45 minutes at ambient temperature and atmospheric pressure. The catalyst was filtered off, washed with ethanol and the combined filtrates were evaporated *in vacuo* to give clavulanic acid as an unstable, viscous oil (59 mgs). N.m.r. (C_5D_5N): 3.05(d,1,J=18Hz), 3.60(dd,1,J_1=18Hz, J_2=2.5Hz), 4.75 (d,2,J=7.5Hz), 5.58(t,1,J=7.5Hz), 5.66 (S,1), 6.08(d,1,J=2.5 Hz).

EXAMPLE 29.
PREPARATION OF CLAVULANIC ACID SODIUM SALT.

Substantially pure benzyl clavulanate (281 mg) in ethanol (25 ml) containing sodium hydrogen carbonate (82 mg) was hydrogenated over 10% Pd/C (90 mg) for 25 minutes at room temperature and atmospheric pressure. The catalyst was filtered off, washed with water and ethanol, and the combined filtrates evaporated under reduced pressure at room temperature. The residual semi-solid was triturated with acetone, filtered and washed with ether to yield sodium clavulanate (135 mg) (in the form of its crystalline tetrahydrate).

EXAMPLE 30.
PREPARATION OF SODIUM CLAVULANATE.

Benzyl clavulanate (840 mg) in ethanol (30 ml) and water (5 ml) was hydrogenated over 10% Pd/C (267 mg) and sodium bicrabonate (244 mg) for 25 minutes at room temperature and atmospheric pressure. The catalyst was filtered off, washed with water and ethanol and the combined filtrates evaporated under reduced pressure. Sodium clavulanate tetrahydrate crystallised from a water-acetone mixture as micro-needles (565 mg). Recrystallisation from water-acetone gave crystalline sodium clavulanate tetrahydrate in the form of needles.

After drying over P_2O_5 *in vacuo* for 24 hours the following data was obtained:
Found: C 41.01, 40.86; H 3.77, 3.64; N, 5.68, 5.51;
$C_8H_8NO_5Na$ requires: C 43.41; H 3.64; N 6.33; $C_8H_8NO_5Na.4H_2O$ requires: C 32.77; H 5.50; N 4.78; $C_8H_8NO_5Na.\ 0.7\ H_2O$ requires: C 41.10; H 4.05; N 5.99.
I.R (KBr disc) 1785, 1700, 1620 cm^{-1}.
N.M.R. (D_2O) 3.06 (d, 1, J=18.5H_3), 3.57 (dd, 1, J=18.5H_3, J_2=2.5H_3), 4.15 (d, 2, J=8H_3), 5.3 (HOD), 4.9 (m), 5.71 (d, 1, J=2.5H_3).

[Reference to a 0.7H_2O form does not imply a stable state but only indicates the residual water content after dry for an extended period at oil pump pressure (>5mmHg) over P_2O_5 in an attempt to obtain a water free sample for analysis].

EXAMPLE 31.
PREPARATION OF CLAVULANIC ACID METHYL ESTER.

19.8 mg. of the sodium salt of clavulanic acid was dissolved in 0.5 ml. dry dimethylformamide and treated with 0.25 ml. methyl iodide. After standing at room temperature for 1.5 hours under anhydrous conditions, the solvents were removed *in vacuo*. The residue was purified by preparative layer chromatography on silica gel (Kieselgel 60F254 supplied by E. Merck, Darmstadt, Germany), eluting with ethyl acetate to give clavulanic acid methyl ester as a colourless oil (R_f 0.38; red colour with triphenyltetrazolium chloride spray) which had the following properties:
Analysis: Found C 50.49 H 5.43 N 6.29
$C_9H_{11}NO_5$ Requires C 50.70 H 5.20 N 6.57
λ max (methanol): no absorption >215 nm
ν max (Film): 3300—3600 (Broad), 1800, 1750, 1695 cm^{-1}
Approximate 1st order N.M.R. ($CDCl_3$): 2.49 (broad S, 1, exchanged with D_2O), 3.05 (d, 1, J=17.5 Hz), 3.54 (dd, 1, J=17.5 Hz, J_2=2.5 Hz), 3.84 (S, 3) 4.24 (d, 2, J=7 Hz), 4.93 (dt, 1, J=7 Hz, J_2=1.5 Hz), 5.07 (d, 1, J=1.5 Hz), 5.72 (d, 1, J=2.5 Hz)
Molecular weight (mass spectrum): 213.0635.
Calculated for $C_9H_{11}NO_5$: 213.0637

Thin layer chromatography of the methyl ester showed a single zone in each of the following solvent systems; butanol/ethanol/water 4:1:5 v/v top phase R_f 0.75; isopropanol/water, 7:3 v/v R_f 0.95; ethyl acetate/ethyl alcohol 8:2 v/v R_f 0.87. The zones were detected by bioauthography using *Klebsiella aerogenes* with added benzylpenicillin (synergism system).

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EXAMPLE 32.
HYDROYLSIS OF CLAVULANIC ACID METHYL ESTER TO CLAVULANIC ACID SODIUM SALT.

Clavulanic acid methyl ester (2.17 mg) was dissolved in 0.1 ml. methanol and treated with 0.208 ml. sodium hydroxide solution (0.0482N). After 1 hour at room temperature, the reaction mixture contained several products. T.L.C. analysis indicated that one of the major components had an R_f identical to that of the sodium salt of clavulanic acid; colour reactions and biological assay were consistent with this component being the sodium salt of clavulanic acid.

Slow conversion of the ester to the clavulanic acid salt was seen when 1 mg/ml. of the compound was incubated at 37°C in 0.05M phosphate buffer at pH 7. The reaction was followed by paper chromatography (bioautographic system). Using the butanol/ethanol/water system to follow the reaction over a period of 2 hours, the zone of the methyl ester at R_f 0.79 decreased in size as the zone of clavulanic acid at R_f 0.12 increased.

EXAMPLE 33.
ANTIBACTERIAL SPECTRUM OF CLAVULANIC ACID SODIUM SALT.

The antibacterial activity of clavulanic acid sodium salt against a range of bacteria was determined using the microtitre method. Serial dilutions of clavulanic acid sodium salt in Oxoid sensitivity test broth contained in a microtitre plastic tray were inoculated with an overnight broth culture of each organism so that the final dilution of the inoculum was 0.5×10^{-4}. The cultures were incubated overnight and the points of bacterial growth recorded next morning by observing the turbidity of the culture. The results, expressed as approximate MIC values (minimum inhibitory concentration, μg/ml.) are recorded in Table 3, which shows that the compound has a broad spectrum of antibacterial activity.

TABLE 3

Antibacterial Spectrum of Clavulanic Acid Sodium Salt

Bacterial Strain	Minimum Inhibitory Concentration μg/ml.
Staphylococcus aureus (Oxford H)	7.5
Staphylococcus aureus (Russell)	7.5
Bacillus subtilis	62
Streptococcus faecalis	>500
Streptococcus pyogenes CN 10	125
Escherichia coli NCTC 10418	31
Klebsiella aerogenes	31–62
Klebsiella oxytocum	62
Enterobacter aerogenes T 624	31
Enterobacter cloacae	62
Acinetobacter anitratus	125
Providentia stuartii	125
Serratia marcescens	125
Proteus mirabilis C977	62
Proteus vulgaris W090	31
Salmonella typhimurium	31
Shigella sonnei	62
Pseudomonas aeruginosa A	500

EXAMPLE 34.
ANTIBACTERIAL SYNERGISM BETWEEN AMPICILLIN AND CLAVULANIC ACID SODIUM SALT.

The minimum inhibitory concentration (M.I.C. values) of ampicillin, clavulanic acid sodium salt and ampicillin in the presence of 1 μg/ml. clavulanic acid sodium salt were determined for a range of β-lactamase-producing bacteria. The organisms were inoculated into Oxoid sensitivity test broth located in small wells in a plastic tray and containing separate concentration gradients of ampicillin, clavulanic acid sodium salt or ampicillin plus 1 μg/ml. clavulanic acid sodium salt (microtitre method). The final dilution of the overnight broth inoculum was 0.5×10^{-2}. The tray was incubated at 37°C overnight and a record made next morning of the end points of bacterial growth. The M.I.C. values in μg/ml. are recorded in Table 4, which reveals that the synergist, at the low concentration of 1 μg/ml., markedly enhances the antibacterial activity of ampicillin against certain gram + ve and gram – ve bacteria. The mechanism of this synergism is likely to involve inhibition of ampicillin-destroying β-lactamase enzymes, but the existence of other mechanisms cannot be excluded.

Similar results to those shown in Table 4 were obtained when ampicillin was replaced by amoxycillin or by the phthalidyl ester of ampicillin.

TABLE 4

Antibacterial Synergism Between Ampicillin and Clavulanic Acid Sodium Salt

Bacterial strain	Minimum Inhibitory Concentrations μg/ml		
	Clavulanic acid sodium salt	Ampicillin	Ampicillin in presence of 1μg/ml clavulanic acid sodium salt
Escherichia coli NCTC 10481	31	1.8	<0.4
Escherichia coli B 11	62	>500	125
Klebsiella aerogenes A	31	125	<0.4
Klebsiella sp 62	31	125	<0.4
Enterobacter cloacae	62	250	62
Serratia marcescens	125	>500	62
Staphylococcus aureus (Russell)	15	500	<0.4
Staphylococcus aureus	62	250	7.5

EXAMPLE 35.
ANTIBACTERIAL SYNERGYSM BETWEEN CEPHALORIDINE AND CLAVULANIC ACID SODIUM SALT.

The minimum inhibitory concentrations of cephaloridine, clavulanic acid sodium salt and cephaloridine in the presence of 5 μg/ml clavulanic acid sodium salt were determined by the method described in Example 34. The results in Table 5 show that synergism can be obtained between clavulanic acid sodium salt and cephaloridine, particularly for the β-lactamase producing strain of *Staphylococcus aureus* (Russell).

TABLE 5

Antibacterial Synergism Between Cephaloridine and Clavulanic Acid Sodium Salt

Bacterial strain	Minimum Inhibitory Concentrations μg/ml.		
	Clavulanic acid sodium salt	Cephaloridine	Cephaloridine in presence of 5μg/ml clavulanic acid sodium salt
Proteus mirabilis 899	>500*	62	7.5
Staphylococcus aureus (Russell)	15	3.1	<0.03+
Staphylococcus aureus	62	15	3.7

* Tailing Point
+ Same value obtained when synergist added at 1 μg/ml. instead of 5 μg/ml.

EXAMPLE 36.

ANTIBACTERIAL SYNERGISM BETWEEN CLAVULANIC ACID SODIUM SALT AND VARIOUS PENICILLINS.

The results presented in Table 6 were obtained by the method described in Example 34.

TABLE 6

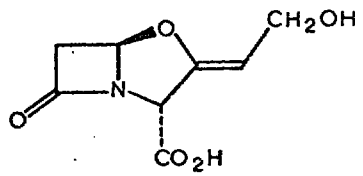
Antibacterial Synergism Between Clavulanic Acid Sodium Salt and Various Penicillins Against Strains of Klebsiella Aerogenes

Strain	MIC (μg/ml)					
	Amoxycillin		Carbenicillin*		Benzylpenicillin	
	Alone	+5μg/ml. synergist	Alone	+5μg/ml. synergist	Alone	+5μg/ml. synergist
A	500	0.97	500	7.8	250	7.8
E 70	500	3.9	500	15	500	15.6
62	250	15.6	125	7.8	250	15.6

* Similar results observed when carbenicillin replaced by carbenicillin phenyl α-ester or ticacillin.

WHAT WE CLAIM IS:—

1. Clavulanic acid, which is the compound of the formula (I):



(I)

or a salt thereof.

2. A salt of clavulanic acid as claimed in claim 1.

3. A pharmaceutically acceptable salt as claimed in claim 2.

4. An alkali metal or alkaline earth metal salt as claimed in any of claims 1 to 3.

5. An alkali metal salt as claimed in claim 4.

6. The lithium salt of clavulanic acid.

7. The sodium salt of clavulanic acid.

8. The potassium salt of clavulanic acid.

9. The calcium salt of clavulanic acid.

10. The magnesium salt of clavulanic acid.

11. The aluminium salt of clavulanic acid.

12. The silver salt of clavulanic acid.

13. The ammonium salt of clavulanic acid.

14. A di- or trialkylamine salt of clavulanic acid wherein the di- or trialkylamine contains up to 22 carbon atoms.

15. The trimethylamine salt of clavulanic acid.

16. The benzathine salt of clavulanic acid.

17. A polymeric anion exchange material salt of clavulanic acid.

18. A salt as claimed in any of claims 4 to 10 which is in crystalline form.

19. A salt as claimed in claim 18 when in the form of a crystalline hydrate.

20. Clavulanic acid.

21. A pharmaceutical composition which comprises clavulanic acid or a pharmaceutically acceptable salt thereof together with a pharmaceutically acceptable carrier.

EXAMPLE 36.

ANTIBACTERIAL SYNERGISM BETWEEN CLAVULANIC ACID SODIUM SALT AND VARIOUS PENICILLINS.

The results presented in Table 6 were obtained by the method described in Example 34.

TABLE 6

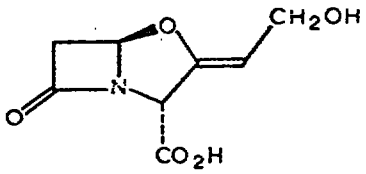
Antibacterial Synergism Between Clavulanic Acid Sodium Salt and Various Penicillins Against Strains of Klebsiella Aerogenes

Strain	MIC (μg/ml)					
	Amoxycillin		Carbenicillin*		Benzylpenicillin	
	Alone	+5μg/ml. synergist	Alone	+5μg/ml. synergist	Alone	+5μg/ml. synergist
A	500	0.97	500	7.8	250	7.8
E 70	500	3.9	500	15	500	15.6
62	250	15.6	125	7.8	250	15.6

* Similar results observed when carbenicillin replaced by carbenicillin phenyl α-ester or ticacillin.

WHAT WE CLAIM IS:—

1. Clavulanic acid, which is the compound of the formula (I):



(I)

or a salt thereof.

2. A salt of clavulanic acid as claimed in claim 1.
3. A pharmaceutically acceptable salt as claimed in claim 2.
4. An alkali metal or alkaline earth metal salt as claimed in any of claims 1 to 3.
5. An alkali metal salt as claimed in claim 4.
6. The lithium salt of clavulanic acid.
7. The sodium salt of clavulanic acid.
8. The potassium salt of clavulanic acid.
9. The calcium salt of clavulanic acid.
10. The magnesium salt of clavulanic acid.
11. The aluminium salt of clavulanic acid.
12. The silver salt of clavulanic acid.
13. The ammonium salt of clavulanic acid.
14. A di- or trialkylamine salt of clavulanic acid wherein the di- or trialkylamine contains up to 22 carbon atoms.
15. The trimethylamine salt of clavulanic acid.
16. The benzathine salt of clavulanic acid.
17. A polymeric anion exchange material salt of clavulanic acid.
18. A salt as claimed in any of claims 4 to 10 which is in crystalline form.
19. A salt as claimed in claim 18 when in the form of a crystalline hydrate.
20. Clavulanic acid.
21. A pharmaceutical composition which comprises clavulanic acid or a pharmaceutically acceptable salt thereof together with a pharmaceutically acceptable carrier.

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22. A composition as claimed in claim 21 which comprises a compound as claimed in claim 3.

23. A composition as claimed in claim 22 which comprises an alkali metal or alkaline earth metal salt of clavulanic acid.

24. A composition as claimed in claim 22 which comprises an alkali metal salt of clavulanic acid.

25. A composition as claimed in claim 22 which comprises the sodium salt of clavulanic acid.

26. A composition as claimed in claim 22 which comprises the potassium salt of clavulanic acid.

27. A composition as claimed in claim 22 which comprises the calcium salt of clavulanic acid.

28. A composition as claimed in claim 22 which comprises the magnesium salt of clavulanic acid.

29. A composition as claimed in claim 22 which comprises the aluminium salt of clavulanic acid.

30. A composition as claimed in claim 22 which comprises the ammonium salt fo clavulanic acid.

31. A composition as claimed in claim 22 which comprises a di- or trialkylamine salt of clavulanic acid wherein the di- or trialkylamine contains up to 22 carbon atoms.

32. A composition as claimed in claim 22 which comprises the triethylamine salt of clavulanic acid.

33. A composition as claimed in claim 22 which comprises the benzathine salt of clavulanic acid.

34. A composition as claimed in any of claims 23—28 wherein the salt of clavulanic acid is in crystalline form.

35. A composition as claimed in claim 34 when the salt is in the form of a crystalline hydrate.

36. A composition as claimed in any of claims 21—35 which is adapted for oral administration.

37. A composition as claimed in any of claims 21—35 which is adapted for topical administration.

38. A composition as claimed in any of claims 22—26 which is adapted for administration by injection or infusion.

39. A composition as claimed in claim 38 wherein the injectable salt is the sodium salt of clavulanic acid.

40. A composition as claimed in claim 38 wherein the injectable salt is the potassium salt of clavulanic acid.

41. A composition as claimed in claim 38 which consists essentially of a sterile injectable salt of clavulanic acid.

42. A composition as claimed in any of claims 36—41 in unit-dosage form.

43. A composition as claimed in claim 36 which also comprises a buffering agent.

44. A composition as claimed in claim 36 which also comprises an enteric coating agent which prevents contact between the clavulanic acid or its salt and gastric juice after oral administration.

45. A pharmaceutical composition which comprises clavulanic acid or a pharmaceutically acceptable salt thereof, a pharmaceutically acceptable carrier and a penicillin or cephalosporin.

46. A composition as claimed in claim 45 which comprises a compound as claimed in claim 3.

47. A composition as claimed in claim 46 which comprises an alkali metal or alkaline earth metal salt of clavulanic acid.

48. A composition as claimed in claim 47 which comprises an alkali metal salt of clavulanic acid.

49. A composition as claimed in claim 47 which comprises the sodium salt of clavulanic acid.

50. A composition as claimed in claim 47 which comprises the potassium salt of clavulanic acid.

51. A composition as claimed in claim 47 which comprises the calcium salt of clavulanic acid.

52. A composition as claimed in claim 47 which comprises the magnesium salt of clavulanic acid.

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53. A composition as claimed in claim 45 which comprises the aluminium salt of clavulanic acid.

54. A composition as claimed in claim 45 which comprises the ammonium salt of clavulanic acid.

55. A composition as claimed in claim 45 which comprises a di- or trialkylamine salt of clavulanic acid wherein the di- or trialkylamine contains up to 22 carbon atoms.

56. A composition as claimed in claim 45 which comprises the trimethylamine salt of clavulanic acid.

57. A composition as claimed in claim 45 which comprises the benzathine salt of clavulanic acid.

58. A composition as claimed in any of claims 47—52 wherein the salt of clavulanic acid is in crystalline form.

59. A composition as claimed in claim 58 when the salt is in the form of a crystalline hydrate.

60. A composition as claimed in any of claims 45—59 which is adapted for oral administration.

61. A composition as claimed in any of claims 45—50 which is adapted for administration by injection or infusion.

62. A composition as claimed in claim 60 which comprises benzylpenicillin, phenoxymethylpenicillin, propicillin, amoxycillin, ampicillin, epicillin, cyclacillin or a pharmaceutically acceptable salt or *in-vivo* hydrolysable ester or aldehyde or ketone adduct of those penicillins containing a 6-α-aminoacylamido side chain and pharmaceutically acceptable salts thereof.

63. A composition as claimed in claim 62 which comprises the acetoxymethyl, pivaloyloxymethyl, α-ethoxycarbonyloxyethyl or phthalidyl esters of ampicillin or amoxycillin or a pharmaceutically acceptable salt thereof.

64. A composition as claimed in claim 62 which comprises the phenyl, tolyl or indanyl α-ester of carbenicillin or ticarcillin or a pharmaceutically acceptable salt thereof.

65. A composition as claimed in claim 62 which comprises the formaldehyde or acetone adduct of ampicillin or amoxycillin or a pharmaceutically acceptable salt thereof.

66. A composition as claimed in claim 61 which comprises a pharmaceutically acceptable salt of benzylpenicillin, phenoxymethylpenicillin, carbenicillin, propicillin, ampicillin, amoxycillin, epicillin, ticarcillin or cyclacillin.

67. A composition as claimed in claim 60 which comprises cephalexin, cephradine, cephaloglycine or their pharmaceutically acceptable salts or *in-vivo* hydrolysable esters or aldehyde or ketone adducts of those cephalosporins containing a 7-α-aminoacylamido side chain and pharmaceutically acceptable salts thereof.

68. A composition as claimed in claim 61 which comprises a pharmaceutically acceptable salt of cephaloridine, cephalothin, cefazolin, cephalexin, cephacetrile, cephamandole, cephapirin, cephradine or cephaloglycine.

69. A composition as claimed in any of claims 45—68 wherein the weight ratio of clavulanic acid or its salt to penicillin or cephalosporin is from 10:1 to 1:10.

70. A composition as claimed in claim 69 wherein the ratio is from 3:1 to 1:3.

71. A composition as claimed in any of claims 45—70 in unit dosage form.

72. A composition as claimed in any of claims 60, 62—65 or 67 which also comprises a buffering agent.

73. A composition as claimed in any of claims 60, 62—65 or 67 which also comprises an enteric coating agent which prevents contact between the clavulanic acid or its salt and gastric juice after oral administration.

74. A composition as claimed in any of claims 21—73 which comprises 50 to 500 mg of clavulanic acid or its salt.

75. A composition as claimed in claim 74 which contains 50 to 250 mg of clavulanic acid or its salt.

76. A pharmaceutical composition which comprises from 150 to 1000 mg of amoxycillin, ampicillin or an *in-vivo* hydrolysable ester or aldehyde or ketone adduct thereof or a pharmaceutically acceptable salt thereof and from 50 to 500 mg of clavulanic acid or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier.

77. A composition as claimed in claim 76 which comprises from 200 to 500 mg of amoxycillin or a salt thereof or ampicillin or a salt thereof.

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78. A composition as claimed in claims 76 or 77 which comprises from 50 to 250 mg of clavulanic acid or a salt thereof.

79. A composition as claimed in any of claims 76—78 which comprises a salt as referred to in any of claims 47—59.

80. A process for the preparation of clavulanic acid or a salt thereof which process comprises cultivating a strain of *Streptomyces clavuligerus* and recovering clavulanic acid or a salt thereof from the culture medium.

81. A process as claimed in claim 80 wherein the strain of *Streptomyces clavuligerus* is ATCC 27064 or a high yielding mutant thereof.

82. A process as claimed in claims 80 or 81 wherein the cultivation is performed at 20—35°C.

83. A process as claimed in claim 82 wherein the cultivation is performed at 25—30°C.

84. A process as claimed in any of claims 80—83 wherein the cultivation is performed at a pH of between 6 and 7.5.

85. A process as claimed in any of claims 80—84 wherein the cells of the *Streptomyces clavuligerus* are removed from the culture medium and the clavulanic acid or its salt thereafter extracted from the clarified culture medium.

86. A process as claimed in any of claims 80—85 which comprises the extraction into an organic solvent of clavulanic acid from cold clarified culture medium adjusted to an acid pH value.

87. A process as claimed in any of claims 80—85 which utilizes the anionic nature of clavulanic acid at neutral pH for the recovery.

88. A process as claimed in any of claims 80—86 for the recovery of clavulanic acid which process comprises lowering the pH of the medium to pH 2—3 while mixing with a water-immiscible organic solvent.

89. A process as claimed in claim 88 wherein the pH is lowered by the addition of hydrochloric, sulphuric, nitric or phosphoric acid.

90. A process as claimed in claims 88 or 89 wherein the organic solvent is n-butanol, ethyl acetate, n-butyl acetate or methyl isobutyl ketone.

91. A process as claimed in claims 88 or 89 wherein the organic solvent is n-butanol.

92. A process for the extraction of a salt of clavulanic acid which comprises forming a solution of clavulanic acid in an organic solvent by a process as claimed in any of claims 88—91 and back-extracting the β-lactamase-inhibiting metabolite into an aqueous phase.

93. A process as claimed in claim 92 wherein the back extraction is into an aqueous solution or suspension of an alkali metal or alkaline earth metal base or into water while maintaining the pH at approximate neutrality.

94. A process as claimed in claim 93 wherein the base is sodium bicarbonate.

95. A process as claimed in claim 93 wherein the base is potassium hydrogen phosphate buffer.

96. A process as claimed in claim 93 wherien the base is calcium carbonate.

97. A process as claimed in any of claims 88—96 wherein the aqueous extract is concentrated under reduced pressure.

98. A process as claimed in any of claims 88—97 wherein the water is removed by freeze-drying.

99. A process for the recovery of a salt of clavulanic acid from a clarified culture medium which comprises contacting the medium with an organic phase which contains an acid addition salt of a lipophilic di- or trialkylamine and thereafter separating the organic phase from the aqueous phase.

100. A process as claimed in claim 99 wherein the lipophilic di- or trialkylamine is one which contains one alkyl group of 12—16 carbon atoms and one tertiary alkyl group.

101. A process as claimed in claims 99 or 100 wherein the clavulanic acid is back-extracted into an aqueous solution of an electrolyte.

102. A process for the recovery of a salt of clavulanic acid from a clarified culture medium which comprises contacting the medium at pH 5.5—7.5 with a bed of a polymeric anion exchange material until the exchange material is substantially saturated and thereafter removing a salt of clavulanic acid from the bed of anion exchange material by passing therethrough a solution of an electrolyte.

103. A process as claimed in claim 102 wherein the pH of the culture medium is 6—7.

104. A process as claimed in claims 102 or 103 wherein the anion exchange material is a weak base anion exchange resin.

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105. A process as claimed in claim 104 wherein the resin has a cross-linked polystyrene-divinylbenzene matrix and polyamine active groups.

106. A process as claimed in claims 102 or 103 wherein the anion exchange material is a strong base anion exchange resin.

107. A process as claimed in claim 106 wherein the resin has a cross-linked polystyrene-divinylbenzene matrix and quaternary ammonium active groups.

108. A process as claimed in any of claims 102—107 wherein the electrolyte is an alkali or alkaline earth metal salt.

109. A process as claimed in any of claims 102—108 wherein the eluate containing the salt of clavulanic acid and the electrolyte is desalted.

110. A process as claimed in any of claims 102—109 wherein the solvent is removed from the eluate containing the salt of clavulanic acid by evaporation under reduced pressure.

111. A process as claimed in any of claims 102—109 wherein the solvent is removed from the eluate containing the salt of clavulanic acid by freeze-drying.

112. A process for the purification of a salt of clavulanic acid which process comprises ion-exchange chromatography.

113. A process as claimed in claim 112 wherein a solution of a salt of clavulanic acid is applied to a bed of polymeric anion-exchange material from which it is thereafter eluted with a solution of an electrolyte.

114. A process as claimed in claims 112 or 113 wherein the ion-exchange material is a weak base anion exchange resin.

115. A process as claimed in claim 114 wherein the resin has a cross-linked polystyrene-divinylbenzene matrix and polyamine active groups.

116. A process as claimed in claims 112 or 113 wherein the anion-exchange material is a strong base anion exchange resin.

117. A process as claimed in claim 116 wherein the resin has a cross-linked polystyrene-divinylbenzene matrix and quaternary ammonium active groups.

118. A process as claimed in claims 112 or 113 wherein the anion-exchange material is β-diethylaminoethyl cellulose.

119. A process as claimed in any of claims 113—119 wherein the solution of the electrolyte gradiently increases in concentration during the elution.

120. A process as claimed in any of claims 113—119 wherein the electrolyte is an alkali metal or alkaline earth metal salt.

121. A process as claimed in any of claims 113—120 wherein the eluate containing the salt of clavulanic acid and the electrolyte is desalted.

122. A process as claimed in any of claims 113—121 wherein the solvent is removed from the eluate containing the salt of clavulanic acid by evaporation under reduced pressure.

123. A process as claimed in any of claims 113—121 wherein the solvent is removed from the eluate containing the salt of clavulanic acid by freeze-drying.

124. A process for recovering a pure form of clavulanic acid or its salt which comprises isolating from a clarified culture medium an impure form of clavulanic acid or salt thereof, forming an ester of clavulanic acid, purifying the ester and thereafter regenerating pure clavulanic acid or salt thereof from the ester.

125. A process as claimed in claim 124 which comprises esterifying a salt of clavulanic acid with a reactive chloride, bromide or iodide.

126. A process as claimed in claim 125 wherein the salt is an alkali metal salt of clavulanic acid.

127. A process as claimed in claim 126 wherien the salt is the sodium salt of clavulanic acid.

128. A process as claimed in claim 126 wherein the salt is the potassium salt of clavulanic acid.

129. A process as claimed in claim 125 wherein the salt is a polymeric anion-exchange material salt of clavulanic acid.

130. A process as claimed in any of claims 125—129 carried out in the presence of sodium iodide.

131. A process as claimed in any of claims 124—130 in which the ester is purified chromatographically.

132. A process as claimed in claim 131 wherein the ester is dissolved in ethyl acetate, methylene chloride or chloroform.

133. A process as claimed in claims 131 or 132 wherein the solid phase is silica gel or a hydroxypropyl derivative of a cross-linked polydextran gel.

134. A process as claimed in any of claims 124—133 wherein a salt of clavulanic acid is regenerated from the ester by hydrolysis.

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135. A process as claimed in any of claims 124—133 wherein the clavulanic acid or its salt is regenerated by hydrogenolysis.

136. A process for the preparation of a salt of clavulanic acid which comprises the mild base hydrolysis of an ester of clavulanic acid of up to 6 carbon atoms optionally substituted by one chlorine, bromine or iodine atom or one methoxy or hydroxyl group.

137. A process for the preparation of clavulanic acid or a salt thereof which process comprises the hydrogenolysis of a hydrogenolysable ester of clavulanic acid in the presence of a transition metal catalyst and optionally in the presence of a base.

138. A process as claimed in claim 137 wherein the ester of clavulanic acid is one containing a $CO.O.CHR^1R^2$ moiety wherein R^1 is a hydrogen atom or an optionally substituted phenyl group and R^2 is an optionally substituted phenyl group.

139. A process as claimed in claim 137 wherein R^1 is a hydrogen atom or a phenyl, tolyl, chlorophenyl or methoxyphenyl group.

140. A process as claimed in claims 138 or 139 wherein R^2 is a phenyl, tolyl, chlorophenyl or methoxyphenyl group.

141. A process as claimed in any of claims 138—140 wherein R^1 is a hydrogen atom.

142. A process as claimed in any of claims 138—141 wherein R^2 is a phenyl group.

143. A process as claimed in claim 137 wherein the benzyl ester of clavulanic acid is the hydrogenolysable ester.

144. A process as claimed in any of claims 137—143 wherein the transition metal is palladium.

145. A process as claimed in claim 144 wherein the catalyst is palladium on charcoal.

146. A process as claimed in claim 145 wherein the catalyst is 10% palladium on charcoal.

147. A process as claimed in claim 146 wherein the weight of catalyst is 1/3 the weight of ester.

148. A process as claimed in any of claims 137—147 which employs a slightly super-atmospheric pressure of hydrogen.

149. A process as claimed in any of claims 137—148 carried out at 12—20°C.

150. A process as claimed in any of claims 137—149 carried out in solution in an optionally aqueous alkanol of 1—4 carbon atoms, tetrahydrofuran or dioxane.

151. A process as claimed in any of claims 137—148 for the preparation of clavulanic acid, said process being carried out in the absence of base.

152. A process as claimed in any of claims 137—148 for the preparation of a salt of clavulanic acid, said process being carried out in the presence of a base.

153. A process for the further purification of a salt of clavulanic acid of already good purity which comprises chromatography over cellulose using butanol/ethanol/water 4/1/5 v/v top phase as solvent and thereafter recovering the salt from the obtained solution.

154. A process for the preparation of a compound as claimed in claim 18 which comprises concentrating a solution of the substantially pure salt of clavulanic acid in aqueous ethanol.

155. A process for the preparation of a compound as claimed in claim 18 which comprises trituration under or crystallisation or recrystallisation from moist acetone.

156. A compound as claimed in any of claims 1—20 when prepared by a process as claimed in any of claims 80—155.

157. A compound as claimed in any of claims 4—10 when prepared by a process as claimed in any of claims 102—123.

158. A compound as claimed in any of claims 1—10 when prepared by a process as claimed in any of claims 124—152.

159. A compound as claimed in claim 18 when prepared by a process as claimed in claims 154 or 155.

160. A compound as claimed in claim 2 substantially as described with reference to any one of Examples 14—22 or 29—30 herein.

161. A process for the preparation of a compound as claimed in claim 2 substantially as described with reference to any one of Examples 14—22 or 29—30 herein.

162. A compound as claimed in claim 2 whenever prepared by a process

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substantially as described with reference to any one of Examples 14—22 or 29—30 herein.

163. A compound as claimed in claim 18 whenever prepared by a process substantially as described with reference to any one of Examples 19, 29 or 30 herein.

164. A compound as claimed in claim 19 whenever prepared by a process substantially as described with reference to any one of Examples 19, 29 or 30 herein.

165. A method of treating bacterial infections in mammals other than humans which comprises the administration of a composition as claimed in any of claims 21—79.

166. A method as claimed in claims 165 for the treatment of mastitis in cattle.

167. A process for the preparation of a composition as claimed in any of claims 21—79 which comprises bringing together the components thereof in known manner.

168. A process as claimed in any of claims 102—123 adapted to the preparation of a compound as claimed in any of claims 6—10.

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Agent for the Applicants.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1978.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

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