PATENT SPECIFICATION (11) 1 573 718

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(21) Application No. 54001/76 (22) Filed 24 Dec. 1976
(31) Convention Application No. 50/155 646
(32) Filed 25 Dec. 1975
(31) Convention Application No. 51/088 770
(32) Filed 26 July 1976 in
(33) Japan (JP)
(44) Complete Specification published 28 Aug. 1980
(51) INT CL³ C07D 501/50; A61K 31/545
(52) Index at acceptance
C2C 1314 1440 1464 214 215 247 250 252 256 25Y 28X 306 30Y 321 32Y 33Y 342 34Y 351 352 364 366 367 368 36Y 373 37Y 614 638 648 650 672 801 802 80Y AA RP

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(54) 7-METHOXY CEPHALOSPORINS AND PROCESS OF PRODUCING THEM

(71) We, YAMANOUCHI PHARMACEUTICAL CO., LTD., a Company organised and existing under the laws of Japan, of No. 5—1, Nihonbashi-Honcho 2-chome, Chuo-ku, Tokyo, Japan, 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:—

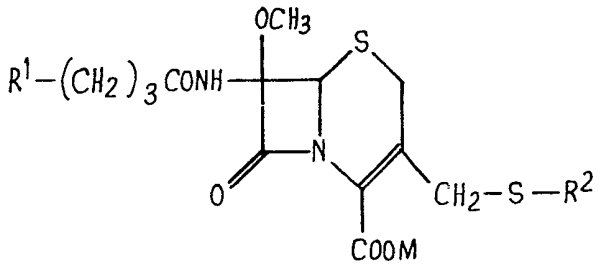
The present invention relates to cephalosporins having a methoxy group at the 7-position and also to a process of producing such cephalosporins by fermentation.

Some 7-methoxy-3-heterocyclothiomethyl cephalosporins are supposedly obtained by chemical syntheses in accordance with Patent Specification No. 1,321,412 (1970), but no practical physical and chemical properties of these compounds are disclosed in this specification.

Cephalosporins typically show excellent antimicrobial activity against gram-positive and gram-negative bacteria. Among such compounds, the cephalosporin derivatives having a methoxy group at the 7-position and a heterocyclic thiomethyl group at the 3-position exhibit particularly good results in the treatment of serious disease caused by infection with bacteria to which ordinary antibiotics are ineffective, such as *Pseudomonas* or *Proteus* species, or with bacteria which are not sensitive to conventional cephalosporins lacking a methoxy group at the 7-position. These compounds are usually produced by first preparing the corresponding compound having an acetoxymethyl or carbamoyloxymethyl group at the 3-position by fermentation and then reacting the product with a heterocyclic thiol compound.

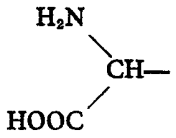
The present invention provides cephalosporins having a methoxy and a 5-amino-5-carboxyvaleramide or 4-carboxybutyramide group at the 7-position, and a heterocyclic thiomethyl group at the 3-position. It also provides a process of producing these cephalosporins by fermentation.

The compounds of this invention are represented by the following general formula (A)



(A)

wherein R^1 represents



or HOOC—,

R^2 represents a nitrogen-containing heterocyclic group, and

M represents a hydrogen atom or a cation forming a salt.

Examples of the nitrogen-containing heterocyclic group shown by R^2 of the above general formula are a 5-carboxymethylthio-1,3,4-thiadiazol-2-yl, 1-methyl-1H-tetrazol-5-yl, 5-methyl-1,3,4-thiadiazol-2-yl or 1,3,4-thiadiazol-2-yl group.

The cationic residue represented by M for forming a salt of the sephalosporin can be an inorganic or organic residue. Examples of an inorganic residue are an alkali metal such as sodium or potassium; an alkaline earth metal such as calcium, magnesium or barium; and a heavy metal such as iron, copper or zinc. Examples of the organic residue are bases forming quaternary salts or amine salts; such as triethylamine, diethanolamine, piperidine or morpholine.

Practical examples of compounds of this invention are:

7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio - 1,3,4-thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid);

7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (1 - methyl - 1H - tetrazol - 5-yl) - thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid;

7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4-thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid;

7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol-2 - yl) - thiomethyl - Δ^3 - cephem - 4 - carboxylic acid;

7 - (4 - carboxybutyramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol-2 - yl) - thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid;

7 - (4 - carboxybutyramido) - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl-7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid;

7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4 - thiadiazol-2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid;

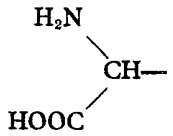
7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl)-thiomethyl - Δ^3 - cephem - 4 - carboxylic acid;

and salts of these compounds.

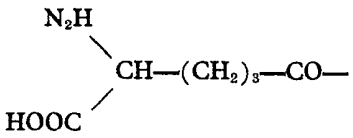
Compounds embodying the present invention, having a methoxy group at the 7-position and a heterocyclic thiomethyl group at the 3-position, can be obtained directly by a single fermentation step. This fermentation step involves cultivating a 7 - (5-amino - 5 - carboxyvaleramido) - 7 - methoxy - cephalosporin - producing microorganism belonging to the genus *Streptomyces* in a culture medium with a heterocyclic thiol represented by the formula

$$R^2SH$$

wherein R^2 has the same meaning as above, or with a salt of the heterocyclic thiol, or with a compound capable of being converted into said heterocyclic thiol or its salts during the cultivation. The compound thus obtained is a compound of general formula (A) wherein R^1 is



(hereinafter referred to as "Aa"). The compounds of formula A (R^1=Aa) exhibit good antimicrobial activity in themselves and furthermore the antimicrobial activity and antimicrobial spectra of the compounds can be increased or varied by replacing the acyl group side chain.



at the 7-position by another acyl group. Examples of the replacement acyl groups are: α-aminophenylacetyl, α-carboxyphenylacetyl, α-sulphophenylacetyl, α-hydroxyphenylacetyl, pyridylthioacetyl, thiadiazolylthioacetyl, triazolylacetyl, cyanomethylthioacetyl or trifluoromethylthioacetyl groups. Specific examples of compounds with the latter two acyl groups include, for example, 7β - cyanomethylthioacetamido - 7α-methoxy - 3 - (1 - methyltetrazol - 5 - ylthiomethyl - Δ^3 - cephem - 4 - carboxylic acid and 7α - methoxy - 3 - (1 - methyltetrazol - 5 - yl - thiomethyl - 7β - (trifluoromethylthioacetamido) - Δ^3 - cephem - 4 - carboxylic acid. The compounds of formula A(R^1=Aa) are thus also useful as intermediate compounds for producing these derivatives having such acyl groups.

The compounds of formula A(R^1=Aa) are amphoteric since they have an amino group and two carboxy groups in the molecule. Hence the isolation and purification of these compounds as produced by fermentation can be troublesome. However, when the group R^1 of the compounds is converted from Aa to HOOC— (hereinafter referred to as "Ab"), the compounds of formula A(R^1=Ab) merely show acidic properties. Since the compounds are acidic this facilitates the isolation and purification thereof, while the compounds become soluble in some organic solvents and this thus makes it worthwhile to perform the convertion reaction required. Therefore, the compounds are also useful as intermediate compounds for producing the derivatives having the aforesaid acyl groups.

An example of the microorganisms which can be used in the process according to the invention for producing 7 - methoxycephalosporins of formula R (R^1=Aa) and belonging to the species *Streptomyces* is a new strain, *Streptomyces organonensis* Y—G19Z. This strain has been recently isolated by the present inventors from soil at Ogano-Town, Chichibugun, Saitama Prefecture, Japan. This new strain has been deposited in the Institute of Microbial Industry, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan, under an accession No. FERM—P 2725 and also in American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA under ATCC No. 31167. The characteristic properties of the strain are as follows:

I. Morphological characteristics of *S. organonensis* Y—G19Z strain:

It grows both on natural and synthetic media with formation of well branched substrate mycelium, while formation of aerial mycelium is inadequate and hence the formation of spores is poor. Spore chains are straight, belong to the R (Rectus) or RF (Rectiflexibiles) type and bear 10—50 spores on each chain. Spores are elliptical, spherical or cylindrical in shape and 0.45—0.60 × 0.55—0.90 μ in size. Spore surface is smooth. Neither flagellate spore nor sporangium was observed.

II. Cultural characteristics of *S. organonensis* Y—G19Z strain:

Medium	Growth	Aerial mycelium	Soluble pigment
Czapek's agar	very poor, white	scant, white	none
Glucose Czapek's agar	good, cream yellow	fair, yellowish grey	none very slightly
Glucose asparagine agar	good, white	poor, white	none
Glycerol asparagine agar	good, white to yellowish white	poor, white to yellowish white	none
Inorganic salt starch agar	good, yellowish grey to pale yellowish brown	poor, yellowish grey	none
Tyrosine agar	good, pale yellow	poor, yellowish grey	slight, pale yellowish grey
Iron and yeast extract tyrosine agar	good, pale yellow to yellowish brown	good, brownish white to yellowish grey	very slight, light brownish grey
Nutrient agar	good, pale yellowish brown	good, powdery pale orange to pale brown	slight, yellowish brown
Bennett's agar	good, pale yellowish brown	good, brownish grey, pale orange to pale pink	very slight
Calcium malate agar	moderate, cream	none	none
Potato plug	good, pale yellowish brown	good, yellowish grey to pale brownish grey	brownish grey to dark yellowish brown
Blood agar	good, olive grey to dark olive grey	none	yellowish grey to dark reddish brown
Loeffler's serum medium	good	none	none

III. Physiological properties of *S. organonensis* Y—G19Z strain:

Tyrosinase formation	negative
Nitrate reduction	positive
Skim milk coagulation	positive, weakly
Skim milk peptonization	positive, weakly
Hydrolysis of starch	positive
Liquefaction of gelatin	positive, weakly
Cellulose decomposition	negative
Haemolysis	positive
Solubilization of calcium malate	positive

IV. Utilization of carbon compounds by *S. organonensis* Y—G19Z.

Carbon source	Utilization
Glucose	+
Arabinose	+
Sucrose	–
Xylose	+
Inositol	–
Mannitol	+
Fructose	+
Rhamnose	–
Rhaffinose	–

The characteristic features of *Streptomyces organonensis* Y—G19Z strain can be summarized as follows:

1. It belongs to a non-chromogenic *Streptomyces* strain.
2. Its aerial mycelium is straight without verticils (R or RF type).
3. Spores are spherical or elliptical.
4. Spore surface is smooth.
5. It gives pale yellowish grey to pale yellowish brown growth on various media.
6. Colour of aerial mycelium is brownish white, yellowish white and yellowish grey.
7. Antibiotic substance Y—G19ZD3, a 7-methoxy cephalosporin, is produced.

On searching for known strains having the above properties, the following species may be mentioned as giving the most closely related strains: *Streptomyces globisporus*, described in S. A. Waksman: the Actinomycetes *2*, 218 (1961) and International Journal of Systematic Bacteriology, *18*, (4) 324—325 (1968).

However, when compared with *S. globisporus*, disclosed in the above literature, strain Y—G19Z differs from it in the points shown in the following table.

TABLE

Characteristic	Y—G19Z	*S. globisporus*
Size of spore (μ)	0.45–0.60×0.55–0.90	1.2–1.4×1.8–2.0 or 0.9–1.4 spherical
Soluble pigment on glycerol asparagine medium	none	yes, yellow to greenish yellow
Rhamnose utilization	negative	positive
Starch hydrolysis	strong	weak
Skim milk coagulation	positive	negative
,, ,, peptonization	weak	strong
Production of cephalosporin antibiotics	positive	negative

As is clear from the differences shown in the above table, the strain used in the preferred process of this invention is a new strain differing from the aforesaid known strains.

Since Y—G19Z strain has been confirmed to be a new strain from the above observation results, it is designated "*Streptomyces organonensis*".

Streptomyces organonensis Y—G19Z strain is a strain producing 7-methoxy cephalosporins which can be used in the process for producing compounds of formula A(R^1=Aa). Other strains similarly belonging to the genus *Streptomyces* which are known to produce 7-methoxy cephalosporins can be used. Such similar strains include: *Streptomyces griseus, Streptomyces viridochromogenes, Streptomyces fimbriatus, Streptomyces halstedii, Streptomyces rochei, Streptomyces cinnamonensis, Streptomyces chartreusis* and *Streptomyces lactamdurans* (see Japanese Patent Application Laid Open No. 3286/71 and Belgian Patent No. 764,160); *Streptomyces lipmanii* (see U.S. Patent No. 3,719,563); *Streptomyces clavuligerus* (see Japanese Patent Publication No. 45594/74); *Streptomyces wadayamensis* (see Japanese Patent Application Laid Open No. 26488/74); *Streptomyces jumonjinensis* (see Japanese Patent Application Laid Open No. 42893/74); *Streptomyces heteromorphus* and *Streptomyces panayensis* (see Japanese Patent Application Laid Open No. 53594/75); and *Streptomyces chartreusis* SF—1623 (see Japanese Patent Application Laid Open Nos. 82291/75 and 121488/75).

However, the strains used in the process of this invention are not limited to the aforesaid strains; any strains which belong to the genus *Streptomyces* and which can produce 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy cephalosporins may be used.

Now, the production of the desired compounds A(R^1=Aa) can be carried out by cultivating in a conventional medium the aforesaid strain producing 7-methoxy cepharosporins together with a heterocyclic thiol corresponding to the desired heterocyclic thio group to be introduced at the 3-position.

Examples of the heterocyclic thiols added in accordance with this invention to the culture medium are those compounds based on ring systems comprising, for example, pyrrolethiol, imidazolethiol, dihydroimidazolethiol, pyrazolethiol, triazolethiol, tetrazolethiol, methyltetrazolethiol, pyridinethiol, diazinethiol, thiophenethiol, thiazolethiol, dihydrothiazolethiol, thiadiazolethiol, thiatriazolethio, furanthiol, pyranthiol, oxazolethiol, isoxazolethiol, oxadiazolethiol, indolethiol, benzimidazolethiol, benzoxazolethiol, benzothiazolethiol, triazolopyridinethiol, thianthrenethiol and purinethiol. These heterocyclic rings may have one or more substituents such as a halogen atom or an amino, nitro, alkyl, hydroxy, alkoxy, aryl, aralkyl, furyl, thienyl, oxazolyl, carboxy, carboxymethyl, carboxyalkylthio or carboxyalkyloxy group.

These heterocyclic thiols may be used as their salts. These salts can be inorganic salts such as alkali metal, alkaline earth metal or ammonium salts; or can be the

salts with organic bases such as triethylamine, triethanolamine, dicyclohexylamine, lysine, arginine, histidine, a basic water-soluble antibiotic e.g. kanamycin, an alkaloid or a basic protein. The salts having a high water solubility may be selectively used, if necessary although when the heterocyclic thiols have a strong toxicity to the antibiotic-producing strains, the salts sparingly soluble in water can be selected.

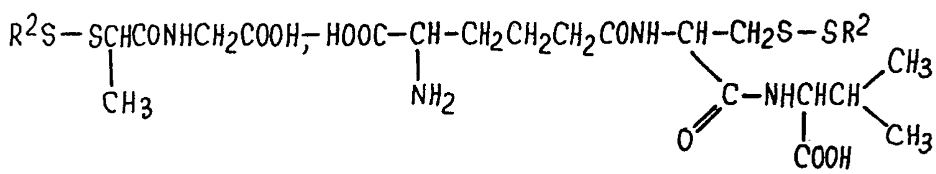
Compounds which can be converted into the aforesaid heterocyclic thiol during cultivation can be used. Illustrative examples are compounds which connect to an organic or inorganic SH compound by an S—S bond. Such convertable S—S compounds then form the aforesaid heterocyclic thiol in the culture medium or in the mycelium in the culture medium by chemical conversion and/or the enzymatic activity of the mycelium. Alternatively, the compound sometime may be utilized as it is as the precursor. Examples of these S—S compounds are as follows: $R^2S—SCH_3$, $R^2S—SC_2H_5$, $R^2S—SCH_2CH_2OH$,

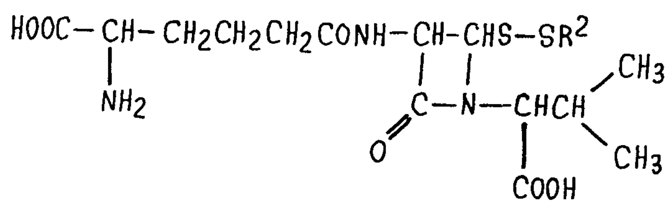
$$R^2S—SCH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH,$$

$R^2S—SG$ (where G here and hereinafter represents a glutathione residue),

$$R^2S—SCH_2CH_2NH_2,\ R^2S—SCoA$$

(wherein CoA represents coenzyme A residue),



and 

Also, two molecules of the same heterocyclic thiol bonded by an S—S bond can be used as a compound of formula $R^2S—SR^2$.

Furthermore, when the antibiotic-producing strain has the ability to reduce a substituted sulphuric acid residue, $R^2—SO_3H$, $R^2—SO_2H$, $R^2—SOH$, or the salts thereof may be used in place of R^2SH.

The production of the desired compounds of this invention is performed by cultivating in a culture medium with the addition of the above-described heterocyclic thiol or salt or derivative thereof strains belonging to the genus *Streptomyces* which produce 7-methoxy cephalosporins. The cultivation can be carried out according to conventional methods for typical microorganisms; submerged cultivation in a liquid culture medium is usually preferred. Any suitable culture media containing nutrients for the 7-methoxy cephalosporin-producing strains can be employed. That is, synthetic, semi-synthetic or natural culture media containing the aforesaid nutrients can be used in this invention. For the composition of the culture medium, glucose, sucrose, mannitol, glycerol, dextrin, starch, vegetable oil, or like source, can be used as the carbon source and meat extract, peptone, gluten meal, cotton seed meal, soybean meal, peanut meal, fish meal, corn steep liquor, dry yeast, yeast extract, ammonium sulphate, ammonium nitrate, urea, and other organic and inorganic nitrogen sources can be used as the nitrogen source. Also, if necessary, one or more metal salts such as sulphates, nitrates, chlorides, carbonates, phosphates of Na, K, Mg, Ca, Zn and Fe may be added to the culture medium. Still further and, if necessary, materials for promoting the formation of antibiotics such as methionine, cystein or cystine, or defoaming agents such as methyl oleate, lard oil, silicone oil or surface active agents may be suitably added to the culture medium.

The aforesaid heterocyclic thiol or salt or derivative thereof is usually added at a concentration of 0.1—5 mg./ml., preferably 0.5—2 mg./ml., calculated as the hetero-

cyclic thiol. It may be added to the culture medium at one fell swoop before cultivation or added thereto in several divided parts at the initial stages of the cultivation.

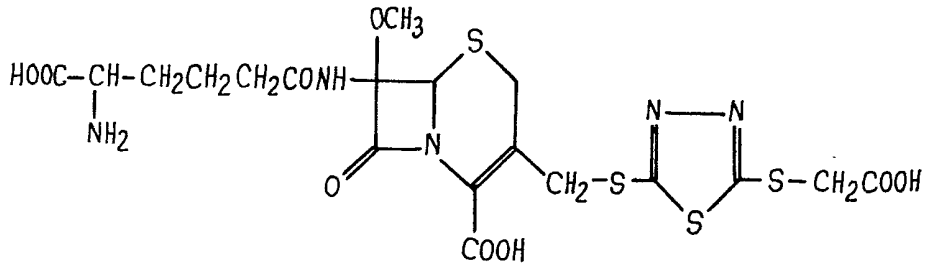
It is generally preferable to carry out the cultivation under aerobic conditions and moreover at a cultivation temperature of usually from about 18° C to about 35° C, preferably about 30° C. Moreover, desirable results are obtained when the pH of the culture medium is maintained at about 5—10, preferably about 6—8. The cultivation period depends upon the composition and temperature of the culture medium employed, but is generally from about 3 days to about 10 days. The desired material is selectively accumulated in the medium once the cultivation is finished.

For most purposes the compounds of formula A(R^1=Aa) will be isolated as the pure product or as a highly concentrated crude product.

The desired material of this invention can be isolated or recovered from the cultivated broth by an ordinary technique employed for isolating antibiotics from the cultivated broth of the mycelium. The desired cephalosporin of this invention is mainly contained in the culture broth and hence the mycelium is usually removed from the broth e.g. by centrifugal separation or filtration, and the effective product material extracted from the filtrate. That is, the desired material can be separated, recovered, and purified from the filtrate by means widely used for producing antibiotics, such as those utilizing differences in solubility in a suitable solvent, differences in adsorptive affinity to various adsorbents, or differences in partition between two liquid phases. These methods can, if necessary, be used individually or as a proper combination, or further may be repeatedly used.

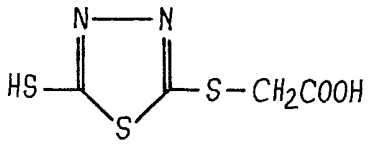
Several practical examples of the novel 7-methoxy cephalosporin compounds of this invention of formula A(R^1=a) are:

I. 7 - (5 - Amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio - 1,3,4-thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid;

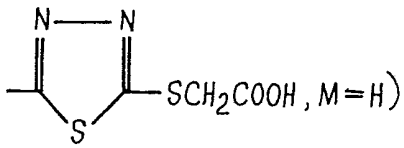


Heterocyclic thiol addition compound:

5 - mercapto - 1,3,4 - thiadiazol - 2 - thioacetic acid;



The physical and chemical properties of the product compound I of general formula A(R^1=Aa, R^2=



are as follows:

(1) White powder.
(2) Begins to melt from 156—160° C and becomes brown and decomposes from about 170° C.
(3) Easily soluble in water, sparingly soluble in methanol, and scarcely soluble in other organic solvents.
(4) Amphoteric material showing a positive ninhydrin reaction.

(5) Gives the ultraviolet absorption spectra as shown in Fig. 1 of the accompanying drawings when taken as a 1/100 *M* phosphate buffer solution having a pH of 6.4, and shows an absorption maximum at 287 mμ.

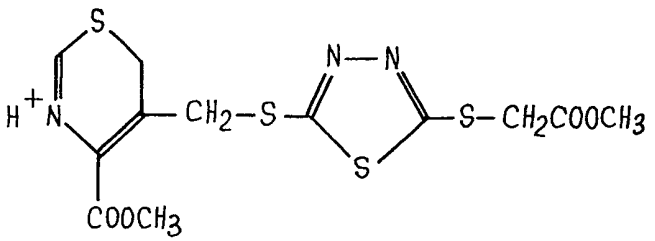
(6) Gives the infrared absorption spectra as shown in Fig. 2 when taken as a potassium bromide tablet, and shows absorptions at 3413 cm^{-1}, 2920 cm^{-1}, 1763 cm^{-1}, 1620 cm^{-1}, 1515 cm^{-1}, and 1380 cm^{-1}.

(7) Gives the following signals in the nuclear magnetic resonance spectrum when measured in heavy water using TMS as an external standard;
δ value (ppm):
2.35 (4H, multiplet), 2.96 (2H, multiplet),
4.00 (3H, singlet), 3.73—4.33 (2H, quartet, J=18 Hz),
4.25 (1H, multiplet), 4.44 (2H, singlet),
4.42—4.91 (2H, quartet, J=14 Hz),
5.63 (1H, singlet).

(8) The product material as obtained in its purest state at present has the following elemental analysis:
C: 35.95%, H: 3.87%, N: 10.85%, S: 18.33%.

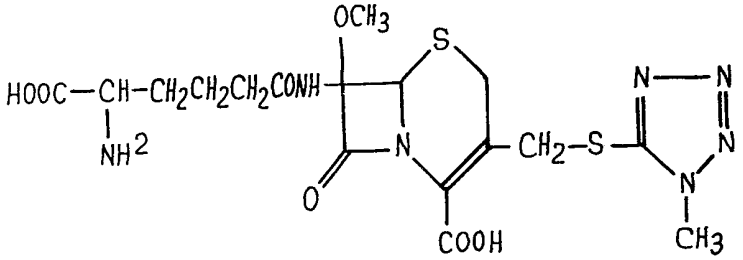
(9) Gives α-aminoadipic acid when hydrolyzed by 6 *N* hydrochloric acid.

(10) The mass spectrum of this compound after N-chloroacetylation and conversion of the product into the methyl ester gives the following fragment of m/e 392, i.e.,

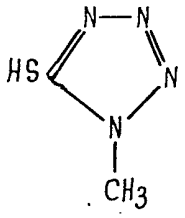


Considering the results shown above as a whole, it is clear that this compound is a 7-methoxycephalosporin compound since the compound (i) gives an absorption at 1763 cm^{-1} (cyclic lactam) in the infrared absorption spectra, (ii) has signals at 4.00 ppm (3H, singlet, 7—OCH_3), 5.63 ppm (1H, singlet, 6—CH), 3.73—4.33 (2H, quartet, J=18 Hz, 2—CH_2), and 4.42—4.91 (2H, quartet, J=14 Hz, 3-side chain CH_2) in the nuclear magnetic resonance spectra, and (iii) gives α-aminoadipic acid on acid hydrolysis. Further, from the facts that the compound gives the adsorption at 4.44 ppm (2H, singlet, CH_2 of —S—CH_2—COOH) in the nuclear magnetic resonance spectra and gives the fragment of m/e 392 in the mass spectra of the derivative, the compound has been assigned the aforesaid structure having the heterocyclic thiol introduced therein.

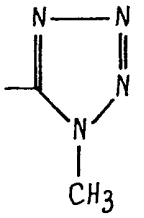
II. 7 - (5 - Amino - 5 - carboxyvaleramido) - 3 - (1 - methyl - 1H - tetrazol-5 - yl) - thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid:



Heterocyclic thiol addition compound:
5 - mercapto - 1 - methyl - 1H - tetrazole.



The physical and chemical properties of the product compound II of formula A(R^1=Aa, R^2=

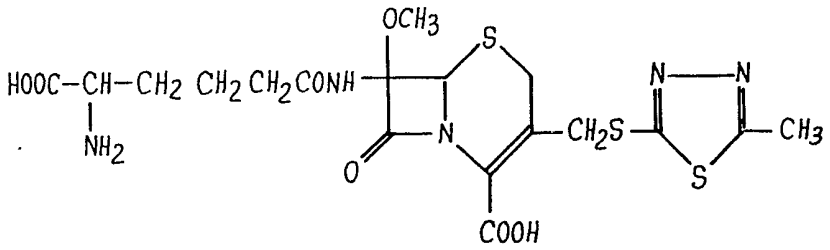


M=H) are as follows:

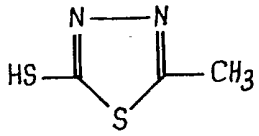
(1) White powder.
(2) Gives brown discolouration and decomposition at 160—170° C.
(3) Easily soluble in water, sparingly soluble in methanol, and scarcely soluble in other organic solvents.
(4) Amphoteric material showing a positive ninhydrin reaction.
(5) Gives the ultraviolet absorption spectra as shown in Fig. 3 when measured in a 1/100 *M* phosphate buffer solution having a pH of 6.4, and has an absorption maximum at 273 mμ.
(6) Gives the infrared absorption spectra as shown in Fig. 4 when measured as potassium bromide tablet, and shows absorptions at 3413 cm^{-1}, 2920 cm^{-1}, 1765 cm^{-1}, 1620 cm^{-1}, 1515 cm^{-1} and 1390 cm^{-1}.
(7) The nuclear magnetic resonance spectra measured in heavy water using TMS as the external standard shows the following signals:
δ value (ppm):
2.36 (4H, multiplet), 2.96 (2H, multiplet),
3.98 (3H, singlet), 4.38 (1H, multiplet),
4.50 (3H, singlet), 5.59 (1H, singlet).
(8) The product material as obtained in its purest state at present has the following elemental analysis:
C: 37.48%, H: 4.25%, N: 16.74%, S: 10.90%.
(9) Gives α-aminoadipic acid when hydrolyzed by 6 *N* hydrochloric acid, and gives 5 - mercapto - 1 - methyl - 1H - tetrazole when hydrolyzed in methanol by Dowex 50 W (H-type, trade mark).

Considering the results as a whole, it is clear that the compound is a 7 - methoxy-cephalosporin compound since the compound (i) gives the signals at 3.98 ppm (3H, singlet, 7—OCH_3) and 5.59 ppm (1H, singlet, 6—CH) in the nuclear magnetic resonance spectra, (ii) has the absorption at 1765 cm^{-1} (cyclic lactam) in the infrared absorption spectra, and (iii) gives α-aminoadipic acid by acid hydrolysis thereof. Further, from the facts that the absorption is present at 4.50 ppm (3H, singlet, tetrazole-N-methyl) in the nuclear magnetic resonance spectra and also 5-mercapto-1-methyl-1H-tetrazole is obtained by mild hydrolysis, the compound has been assigned the aforesaid structure having the heterocyclic thiol introduced therein.

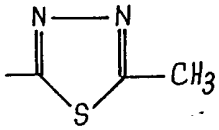
III. 7 - (5 - Amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (5 - methyl-1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.



Heterocyclic thiol addition compound:
2 - mercapto - 5 - methyl - 1,3,4 - thiadiazole:



The physical and chemical properties of the aimed compound III of formula A(R^1=Aa, R^2=

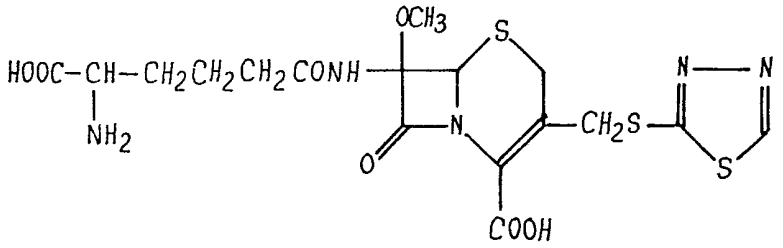


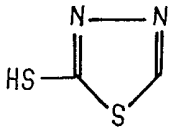
M=H) are as follows:

(1) Light yellow-white powder.
(2) Shows no distinct melting point and gives brown discolouration and decomposition at about 170° C.
(3) Easily soluble in water, slightly soluble in methanol, but insoluble in other organic solvents.
(4) Amphoteric material showing a positive ninhydrin reaction.
(5) Gives the ultraviolet absorption spectra as shown in Fig. 5 when measured in a 1/100 *M* phosphate buffer solution having a pH of 6.4, and has an absorption maximum at 272 mμ.
(6) Gives the infrared absorption spectra as shown in Fig. 6 when measured as potassium bromide tablet, and shows absorptions at 3420 cm^{-1}, 2930 cm^{-1}, 1765 cm^{-1}, 1610 cm^{-1}, 1515 cm^{-1} and 1385 cm^{-1}.
(7) The nuclear magnetic resonance spectra measured using TMS as the external standard in heavy water gives the following signals:
δ value (ppm):
2.34 (4H, multiplet), 2.95 (2H, multiplet),
3.19 (3H, singlet), 3.72—4.31 (2H, quartet, J=18 Hz),
3.99 (3H, singlet), 4.26 (1H, multiplet), 4.40—4.95 (2H, quartet, J=14 Hz), 5.63 (1H, singlet).
(8) The product compound as obtained in its purest state at present has the following elemental analysis:
C: 37.82%, H: 4.01%, N: 12.90%, S: 14.97%.
(9) Gives α-aminoadipic acid when hydrolyzed by 6 *N* hydrochloric acid, and gives 2-mercapto - 5 - methyl - 1,3,4 - thiadiazole when hydrolyzed in methanol by Dowex 50 W (H type, trade name).

Considering the results as a whole, it is clear that the compound is a 7-methoxycephalosporin compound since the compound (i) gives the absorption at 1765 cm^{-1} (cyclic lactam) in the infrared absorption spectra, (ii) has the signals at 3.99 ppm (3H, singlet, 7—OCH_3), 5.63 ppm (1H, singlet, 6—CH), 3.72—4.31 ppm (2H, quartet, J=18 Hz, 2—CH_2), 4.40—4.95 (2H, quartet, J=14 Hz, 3-side chain CH_2), and (iii) gives α-aminoadipic acid by the acid hydrolysis. Further from the facts that the absorption of 3.19 ppm (3H, singlet, thiodiazole C—CH_3) is present in the nuclear magnetic resonance spectra and also 2 - mercapto - 5 - methyl - 1,3,4-thiadiazole is obtained by mild hydrolysis, the compound has been assigned the aforesaid structure having the heterocyclic thiol introduced therein.

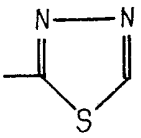
IV. 7 - (5 - Amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.





Heterocyclic thiol addition compound:
2 - mercapto - 1,3,4 - thiadiazole.

The physical and chemical properties of the product compound IV of formula A(R^1=Aa, R^2=



M=H) are as follows:

(1) Light yellow white powder.
(2) Shows no distinct melting point but gives brown discoloring and decomposition at about 175—180° C.
(3) Easily soluble in water, slightly soluble in methanol, but insoluble in other organic solvents.
(4) Amphoteric material showing a positive ninhydrin reaction.
(5) Gives the ultraviolet absorption spectra as shown in Fig. 7 when measured in a 1/100 *M* phosphate buffer solution of a pH of 6.4, and has a maximum absorption at 274 mμ.
(6) Gives the infrared absorption spectra as shown in Fig. 8 when measured as potassium bromide tablet, and shows absorptions at 3400 cm^{-1}, 2925 cm^{-1}, 1765 cm^{-1}, 1610 cm^{-1}, 1515 cm^{-1} and 1365 cm^{-1}.
(7) The magnetic resonance spectra measured in heavy water using TMS as the external standard gives the following signals:
δ value (ppm):
2.30 (4H, multiplet), 2.93 (2H, multiplet), 3.69—4.29 (2H, quartet, J=18 Hz),
3.97 (3H, singlet), 4.26 (1H, multiplet), 4.45—4.99 (2H, quartet, J=14 Hz),
5.56 (1H, singlet), 9.85 (1H, singlet).
(8) The product material as obtained in its purest state at present gives the following elemental analysis:
C: 37.53%, H: 4.36%, N: 12.77%, S: 16.42%.
(9) Gives α-aminoadipic acid when hydrolyzed by 6 *N* hydrochloric acid, and gives 2-mercapto-1,3,4-thiadiazole when hydrolyzed in methanol by Dowex 50 W (H type, trade name).

Considering the results as a whole, it is clear that the compound is a 7-methoxy-cephalosporin compound since the compound (i) gives the absorption at 1765 cm^{-1} in the infrared absorption spectra, (ii) gives the signals at 3.97 ppm (3H, singlet, 7—OCH_3), 5.56 ppm (1H, singlet, 6—CH), 3.69—4.29 ppm (2H, quartet, J=18 Hz, 2—CH_2), 4.45—4.99 (2H, quartet, J=14 Hz, 3-side chain CH_2), and (iii) gives α-aminoadipic acid by acid hydrolysis. Further, from the facts that the absorption

of 9.85 ppm (1H, singlet, thiazole CH) is present in the nuclear magnetic resonance spectra and 2-mercapto-1,3,4-thiadiazole is obtained by mild-hydrolysis, the compound of this invention has been assigned the aforesaid structure having the heterocyclic thiol introduced therein.

The results of various chromatographic analyses and of paper electrophoresis using the product compounds I, II, III and IV of this invention are shown below.

The Rf values of these compounds in thin layer chromatography using microcrystalline cellulose (Avicel SF, trade mark) are shown in the following table.

	Solvent system 1	Solvent System 2	Solvent System 3
Compound I	0.39	0.37	0.32
Compound II	0.39	0.34	0.31
Compound III	0.43	0.43	0.40
Compound IV	0.39	0.38	0.33
Cephalosporin C	0.37	0.36	0.31
7-Methoxy-cephalosporin C	0.41	0.36	0.32
Cephamycin C (a)	0.37	0.36	0.31
Y–G19Z–D3 (b)	0.26	0.26	0.22
Y–G19Z–D2 (c)	0.39	0.32	0.26

Developing solvent systems (volume ratio) used:

1. Isopropanol:*n*-butanol:acetic acid:water (21:3:7:9).
2. *n*-Butanol:acetic acid:water (4:1:2).
3. *n*-Butanol:acetic acid:water (6:1.5:2.5).

(a) The control compound cephamycin C is 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - carbamoyloxymethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

(b) and (c) The control compounds Y—G19Z—D3 and Y—G19Z—D2 used in the above comparison tests are the novel 7 - methoxycephalosporin compounds previously separated from the culture liquid of *Streptomyces organonensis* by the same inventors (see Japanese Patent Applications Nos. 109753/74 and 146593/75).

The Rf values obtained by paper partition chromatography using Wattman No. 1 filter paper and a developing solvent system of *n*-butanol:acetic acid:water (4:1:2 by volume ratio) are as follows:

	Rf value
Compound I	0.40
Compound II	0.39
Cephalosporin C	0.36
7-Methoxy-cephalosporin C	0.40
Cephamycin C	0.35
Y–G19Z–D3	0.24
Y–G19Z–D2	0.25

The compounds were analyzed using Hitachi 635 High Speed Liquid Chromatography Apparatus, the results under the following conditions being as follows in the table:

Column: 3 × 500 mm stainless steel column.
Resin: Hitachi 2610 (cationic exchange resin, trade name).
Developing solvent system: 0.2 *M* citrate buffer solution (pH 3.6).
Flow speed: 0.6 ml/min.
Chart speed: 1.0 cm/min.

	Retention time
Compound I	5 min. 33 sec.
Compound II	6 min. 00 sec.
Compound III	9 min. 35 sec.
Cephalosporin C	5 min. 18 sec.
7-Methoxy-cephalosporin C	5 min. 09 sec.
Cephamycin C	5 min. 18 sec.
Y–G19Z–D3	3 min. 42 sec.
Y–G19Z–D2	3 min. 42 sec.

The results obtained by analysis using the above apparatus under the following alternative conditions are shown in the following table.

Column: μ Bondapak C_{18} (made by Waters Ltd.) of 4 × 300 mm.
Developing solvent system: acetonitrile:0.1% acetic acid solution (pH 3.3) (1:9 by volume ratio).
Flow speed: 0.8 ml/min.
Chart speed: 1.0 cm/min.

	Retention time
Compound I	3 min. 14 sec.
Compound II	1 min. 52 sec.
Compound III	2 min. 55 sec.
Compound IV	1 min. 56 sec.

The results obtained by high-voltage paper electrophoresis are as follows:
Filter paper: Wattman No. 1.
Developing solvent: 10% acetic acid (pH 2.2).
Voltage: 42 volts/cm.
Running time: 1 hour.

	Migration distance
Compound I	– 3.6 cm
Compound II	– 3.3 cm
Compound III	– 3.6 cm
Compound IV	– 3.9 cm
Cephalosporin C	– 3.5 cm
7-Methoxy-cephalosporin C	– 3.4 cm
Cephamycin C	– 3.5 cm
Y–G19Z–D3	– 6.1 cm
Y–G19Z–D2	– 6.1 cm
Cysteic acid	– 7.5 cm
Glutathione	– 1.2 cm

The antibacterial activity of the product compounds I, II, III and IV of this invention is shown in the following table together with that of cephalosporin C as comparison. A heart infusion agar disc method (500 γ/ml. solution was used). The numerical values in the table show the diameter (mm) of the inhibition zone.

	Compound				
	I	II	III	IV	C
Sarcina lutea ATCC 9341	0	0	0	0	14.0
Staphylococcus aureus 209 P	0	0	0	0	12.8
Bacillus subtilis ATCC 6633	0	0	0	0	23.1
Escherichia coli NIHJ	19.2	18.7	14.2	13.0	10.4
Klebsiella pneumoniae	21.8	23.5	23.0	23.0	13.0
Salmonella gallinarum	24.0	25.2	23.5	25.1	23.5
Proteus vulgaris OX 19	22.6	20.2	20.0	21.0	17.5
Proteus mirabilis IMF OM–9	22.2	19.8	19.5	23.0	23.0

Key to compounds: I: Compound I of this invention.

II: Compound II of this invention.

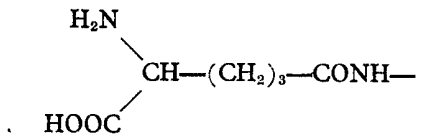
III: Compound III of this invention.

IV: Compound IV of this invention.

C: Cephalosporin C (comparison).

The compounds A(R^1=Aa) of this invention thus show good antimicrobial activity and may be used for the prophylaxis and treatment of diseases of men and animals.

It is to be noted that when the substituent group



at the 7-position of the compound of formula A(R^1=Aa) is replaced by another acylamide group having no 5-amino substituent, the amphoteric properties disappear and the product thus modified becomes convenient for isolation as well as becoming insoluble in some organic solvents. The subsequent reaction can be carried out without difficulty and therefore the modification of the compound of formula A(R^1=Aa) into A(R^1=Ab) is preferred for industrial practice.

As the result of investigation with a view to achieving the aforesaid conversion, the inventors have further found that a compound of formula A(R^1=Aa) or a salt thereof can be converted into a compound of formula A(R^1=Ab) by the action of the mycelium of a strain belonging to the genus *Trigonopsis* which can produce an enzyme which oxidizes D-aminoacids or the treated mycelium thereof, or further the fermented broth containing them. As mentioned, the compound of formula A(R^1=Ab) can be easily dissolved in organic solvents, which simplifies greatly the separation and purification thereof.

In this process of the invention, the strain producing D-aminoacid oxidizing enzyme (oxidase) belonging to the genus *Trigonopsis* is suitably used. Such a strain

can be selected from the type culture preserved in strain preservation institutions or can be isolated from soil. Also, for increasing the activity of formation of the product material of formula A(R^1=Ab), mutants produced from the aforesaid strains by conventional means can be profitably used in this invention. An illustration of a microorganism having the aforesaid D-aminoacid oxidase activity is *Trigonopsis variabilis*. This strain is available from the Institute for Fermentation, Osaka, Japan, under the strain number IFO 0755 (ATCC 10679) and strain number IFO 0671.

For producing the desired material A(R^1=Ab) using the microorganism having such D-aminoacid oxidase or oxidizing enzyme activity, it is usually preferred that the microorganism is first cultivated and the mycelium or the treated mycelium thus obtained is reacted with the cephalosporin compounds of general formula A(R^1=Aa) under appropriate conditions.

As the method of producing mycelium by cultivation it is usually preferred to employ aerobic cultivation, and more preferred liquid cultivation with stirring under aeration. Conventional culture media are usually used in this process.

That is, synthetic, semi-synthetic or natural culture media can be used. The culture media can employ glucose, sucrose, mannitol, glycerol, dextrin, starch or vegetable oil as the carbon source and meat extract, peptone, gluten meal, cotton seed meal, soybean meal, peanut meal, fish meal, corn steep liquor, dry yeast, yeast extract, ammonium sulphate, ammonium nitrate, urea, and other organic and inorganic nitrogen compounds as the nitrogen sources.

Also, if necessary, one or more metal salts such as sulphates, nitrates, chlorides, carbonates or phosphates of Na, K, Mg, Ca, Zn, Fe, and other metals may be added to the culture medium. Furthermore, if necessary, methionine, cysteine, cystine, methyl oleate, lard oil, silicone oil, or surface active agent may be added to the culture medium as a formation promoter or a defoaming agent. Best results are obtained by maintaining the pH of the culture medium at about 4—10, preferably 5—6.

In particular, when the culture medium contains a D- (or DL-) amino-acid such as, for example, D- (or DL-) methionine, D- (or DL-) alanine or D- (or DL-) valine, good D-aminoacid oxidase activity is obtained. The cultivation temperature is usually 18—37° C, preferably about 30° C. The cultivation time will differ according to the cultivation conditions, in particular the cultivation apparatus, composition of cultivation medium, cultivation temperature and other factors, but it is preferable to complete the cultivation when the D-aminoacid oxidase activity reaches a maximum. Usually, 2—10 days of cultivation is appropriate.

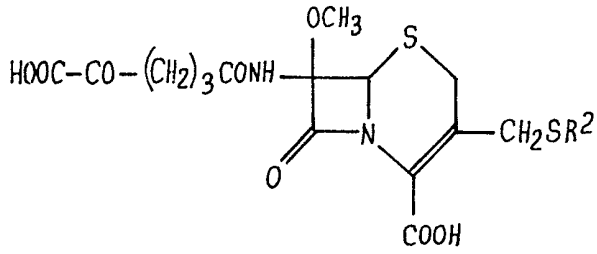
The mycelium thus obtained or the treated mycelium is used in the D-aminoacid oxidation reaction of the starting material of formula A (R^1=Aa). In this case, the treated mycelium means the mycelium which has been converted into an appropriate form for producing the product material A(R^1=Ab) as by increasing the D-aminoacid oxidase activity by the application of a suitable treatment thereto. For example, the D-aminoacid oxidase utilized in this invention usually exists in the mycelium and hence the treated mycelium means the cell-free extract obtained by application of physical and/or chemical processes to the mycelium collected from the cultivation product of the D-aminoacid oxidase producing strain; washed, or partially or completely purified D-aminoacid oxidase obtained from the cell-free extract by the application of a known enzyme separation and purification method; or further the activated mycelium obtained by anchoring or immobilizing the partially or wholly purified D-aminoacid oxidase on a water-insoluble polymer or an inorganic carrier by physical or chemical means to provide a solid D-aminoacid oxidase activator or mycelium and then subjecting the activator or mycelium to an activation treatment.

In the processes of this invention, preparation and recycling of the aforesaid soluble enzyme is of restricted practical use, but the use of the insoluble enzymes such as the activated mycelium is profitable for industrial applications as they can be readily recovered and reused.

The activation treatment of the mycelium referred to above can be performed by subjecting the mycelium to certain mild damage to an extent not sufficient to cause the collapse thereof. As examples of such activation treatment, illustrative methods are those wherein the mycelium is frozen at temperatures below −10° C at a pH of about 3—4 and then, defrozen; wherein the mycelium is treated in a bath by one or more organic solvents such as acetone, *n*-butanol, 2-phenyl ethanol, diethyl ether, cyclohexane, benzene or toluene; wherein the mycelium is treated by 0.1—10% surface active agent, for example, a cationic surface active agent such as cetyltrimethyl ammonium, cetylpyridinium or cetyldimethylbenzyl ammonium halide, an anionic surface active agent such as dodecyl sulphate, an alkali metal alkylarylsulphonate or sodium desoxycholate, or a nonionic surface active agent such as sorbitan monolaurate

or Triton X—100 (trade mark) in an aqueous solution thereof; wherein the mycelium is treated by a dilute aqueous solution of potassium or sodium hydroxide; or wherein the mycelium is suspended in a high osmotic pressure solution, for example 2 *M* cane sugar solution, and then the solution is quickly diluted with water. These activation treatments are influenced by various parameters such as temperature, processing period, pH value and the concentration of reagent, and hence it is necessary to select the appropriate activation condition.

When the action of catalase, which usually exists in a mycelium, is not inhibited, oxidative decarboxylation to the desired material A(R^1=Ab) becomes imperfect due to formation of the 7 - (5 - carboxy - 5 - oxovaleramido) - 7 - methoxycephalosporin derivative represented by the general formula B



(B)

Therefore, in order to obtain selectively the desired material A(R^1=Ab), it is preferred to inhibit the catalase activity. Examples of a suitable catalase inhibitor are ascorbic acid, 3-amino-1,2,4-triazole and alkali metal azide, sodium azide is particularly preferred. The inhibitor may be added to the reaction mixture during the conversion of the starting material A(R^1=Aa) to the product material A(R^1=Ab) or the mycelium may be pretreated by the inhibitor before the mycelium is used in the aforesaid conversion. The amount of sodium azide used for the purpose is about 1—100 mM. Furthermore, the catalase in the aforesaid mycelium can be inactivated by subjecting the mycelium to heat treatment before use in the aforesaid conversion step. That is, when the aforesaid mycelium is treated at 40—60° C, preferably at 50° C, for at least 3 hours, the catalase activity is remarkably decreased but the D-amino-acid oxidase activity remains substantially as it was. The heat treatment may be simply performed on the mycelium in an aqueous solution or a buffer suspension but it is particularly effective to subject the mycelium to a simultaneous aforesaid heat treatment and "activation" reagent treatment. For example, by subjecting the mycelium to treatment at 50° C for 4 hours using a solvent, toluene, the inhibition of the catalase activity and the activation of the mycelium can be attained simultaneously.

The reaction between the enzyme system of the aforesaid activated mycelium and the starting material A(R^1=Aa) is usually performed at a pH of 6—8. It is desirable that the reaction be carried out at 30—40° C. The reaction period mainly depends upon the potency of the enzyme but is usually 1—5 hours.

Since the aforesaid enzyme reaction is performed under an aerobic condition, it is preferred to perform the reaction under aeration with air or oxygen.

As stated above, it is usually difficult to extract the starting material of formula A(R^1=Aa) from the fermented broth due to the amphoteric structure, but according to a preferred embodiment of this invention, the recovery of the desired product of formula A(R^1=Ab) can be practiced under suitable conditions from the fermented broth of the starting material of formula A(R^1=Aa) after removing therefrom the mycelium. That is, the desired material A(R^1=Ab) formed can be easily recovered by solvent extraction or adsorption by ion-exchange resin. For example, the reaction product mixture is acidified to lower than pH 2.5 and then the product material is extracted from the reaction mixture with a suitable organic solvent such as ethyl acetate or *n*-butanol. In this case, the use of the combination of an ion-exchange resin and a solvent extraction give better results. A suitable ion-exchange resin is a liquid amine anionic exchange resin. Examples of the preferred solvent are ethyl acetate, butyl acetate or *n*-butanol. Also, the product can be separated using a solid ion-exchange resin. In this case, the appropriate solvent can be easily determined by preliminary experimentation.

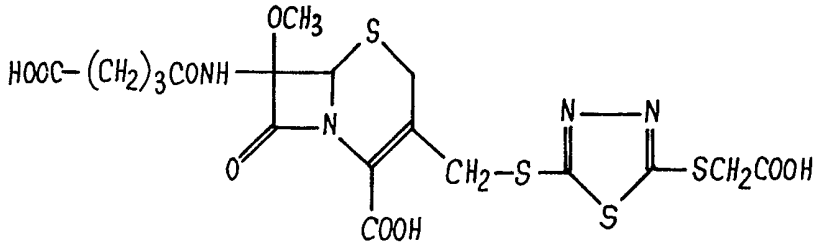
For obtaining the pure product, conventional methods usually used for the purification of antibiotics can be used. Compounds of formula A(R^1=Ab) thus produced show good antimicrobial activities.

The product material of formula A(R^1=Ab) can be recovered not only in a free

acid state, but also as an alkali metal salt, an alkaline earth metal salt, an organic amine salt or another salt thereof.

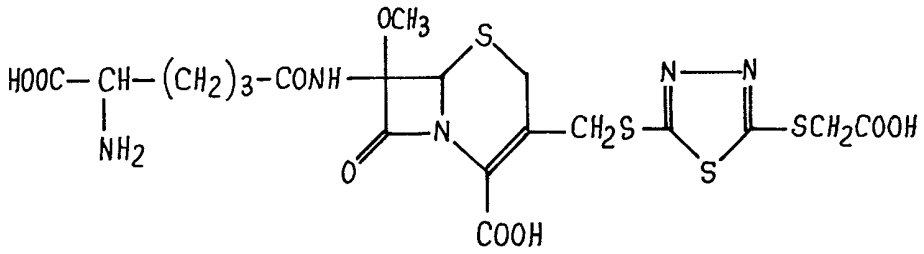
Several novel 7 - methoxycephalosporin derivatives of formula A(R^1=Ab) of this invention are illustrated below together with the properties thereof.

V. 7 - (4 - Carboxybutyramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid:

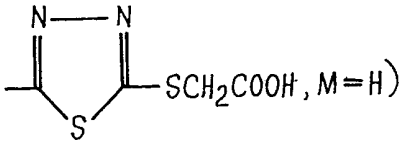


Starting material I:

7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethyl - thio-1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid;



The physical and chemical properties of the product compound V of formula A(R^1=Ab, R^2=



are as follows:

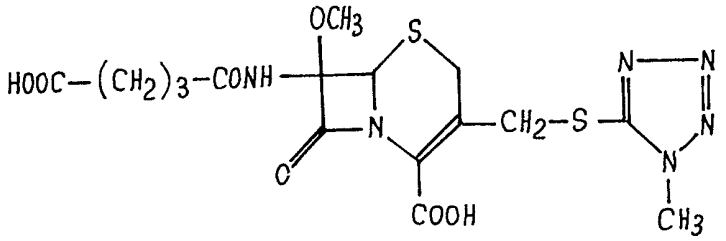
(1) White powder.
(2) Melting point 75—78° C.
(3) Easily soluble in methanol and ethanol, soluble in water, ethyl acetate, butyl acetate and butan, but scarcely soluble in other organic solvents.
(4) Acidic material showing a negative ninhydrin reaction.
(5) Gives the ultraviolet absorption spectra as shown in Fig. 9 when measured in a 1/100 *M* phosphate buffer solution having a pH of 6.5, and shows absorption maximum at 278 mμ.
(6) Gives the infrared absorption spectra as shown in Fig. 10 when measured as potassium bromide tablet, and shows the absorption at 3250 cm^{-1}, 2925 cm^{-1}, 1770 cm^{-1}, 1715 cm^{-1}, 1520 cm^{-1}, 1380 cm^{-1}.
(7) The nuclear magnetic resonance spectra measured in heavy methanol using TMS as the internal standard is shown in Fig. 11, and shows the following signals:
δ value (ppm):
1.93 (2H, multiplet), 2.41 (4H, multiplet),
3.51 (3H, singlet), 3.37—3.81 (2H, quartet, J=18 Hz),
4.11 (2H, singlet), 4.15—4.63 (2H, quartet, J=14 Hz),
5.05 (1H, singlet).
(8) Gives the following elemental analysis for $C_{18}H_{20}N_4O_9S_4 \cdot 2H_2O$

	C	H	N
Calculated:	35.99%	4.03%	9.33%
Found:	35.77%	3.81%	9.42%

(9) The mass spectra of the product material V measured after hydrolyzing the material in 6 *N* hydrochloric acid for 2.5 hours at 100° C, extracting the hydrolyzed product with ethyl ether, drying the product by evaporation, and then silylating it with BSA (bistrimethyl silyl acetamide) gave a fragment of m/e 276 $(CH_3)_3Si—OOC—CH_2—CH_2—CH_2—COO—Si(CH_3)_3$.

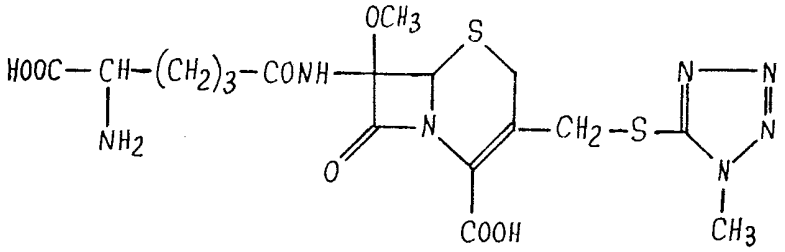
Considering the above results as a whole it is clear that the product compound is a 7-methoxycephalosporin compound from the presence of, in particular, the absorption at 1770 cm^{-1} (cyclic lactam) in the infrared absorption spectra, and the signals of 3.51 ppm (3H, singlet, 7—OCH_3), 5.05 ppm (1H, singlet, 6—CH), 3.37—3.81 ppm (2H, quartet, J=18 Hz, 2—CH_2), 4.15—4.6 ppm (2H, quartet, J=14 Hz, 3-side chain of CH_2), and further 4.11 ppm (2H, singlet, CH_2 of —S—CH_2—COOH). Further, from the facts that there are signals at 1.93 ppm (2H, multiplet, β—CH_2) and 2.41 ppm (4H, multiplet, α, γ—CH_2) showing the existence of 4-carboxybutyramido in the nuclear magnetic resonance spectra and that the mass spectra of the derivative of the product material which was hydrolyzed by hydrochloric acid and silylated gives the fragment of m/e 276, the product compound V of this invention has been determined to have the aforesaid structure wherein the 5 - amino - 5 - carboxyvaleramido group at the 7-position of the starting material has been oxidatively deaminated to a 4-carboxybutyramido group.

VI. 7 - (4 - Carboxybutyramido) - 7 - methoxy - 3 - (1 - methyl - 1H-tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid:

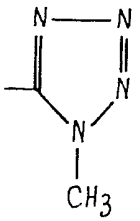


Starting material II;

7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl - 1H-tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.



The physical and chemical properties of the product compound VI of formula A(R^1=Ab, R^2=



M=H) are as follows:

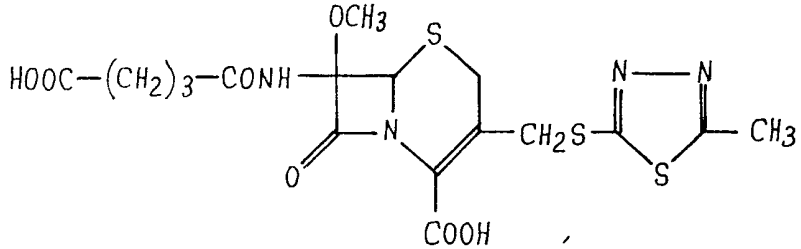
(1) White powder.
(2) Melting point 80—83° C.
(3) Easily soluble in methanol and ethanol, soluble in water, ethyl acetate, butyl acetate, and butanol but scarcely soluble in other organic solvents. Sodium salt is easily soluble in water.
(4) Acidic material having a negative ninhydrin reaction.
(5) Gives the ultraviolet absorption spectra as shown in Fig. 12 when measured in 1/100 *M* phosphate buffer solution having a pH of 6.5, and has absorption maximum at 269 mμ.
(6) Gives the infrared absorption spectra as shown in Fig. 13 when measured as potassium bromide tablet, and shows the absorptions at 3420 cm^{-1}, 2940 cm^{-1}, 1765 cm^{-1}, 1680 cm^{-1}, 1610 cm^{-1}, 1525 cm^{-1}, 1390 cm^{-1}.
(7) The nuclear magnetic resonance spectra measured using TMS as the internal standard in heavy methanol gives, as shown in Fig. 14, the following signals:
δ value (ppm):
1.94 (2H, multiplet), 2.40 (4H, multiplet),
3.51 (3H, singlet), 3.40—3.83 (2H, quartet, J=18 Hz),
3.99 (3H, singlet), 4.17—4.50 (2H, quartet, J=14 Hz),
5.02 (1H, singlet).
(8 Gives the following elemental analysis value for $C_{16}H_{20}N_6O_7S_2 \cdot 2H_2O$.

	C	H	N
Calculated:	37.79%	4.76%	16.53%
Found:	37.78%	4.75%	16.41%

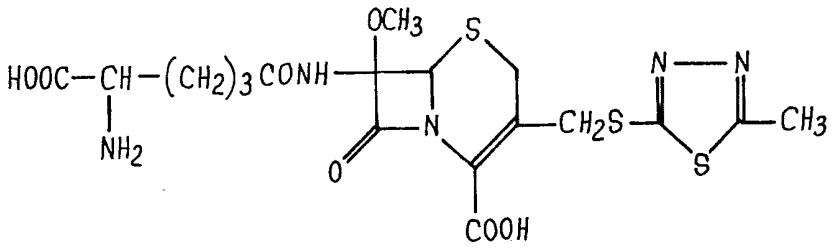
(9) The mass spectra of the desired material VI measured after hydrolyzing the material in 6 *N* hydrochloric acid for 2.5 hours at 100° C, extracting the hydrolyzed product with ethyl ether, drying it by evaporation, and silylaing it by BSA (bistrimethylsilyl acetamide) gives a fragment of m/e 276 $(CH_3)_3Si—OOC—CH_2CH_2CH_2—COOSi(CH_3)_3$.

Considering the above results as a whole, it is clear that the product compound is a 7-methoxycephalosporin compound from, in particular, the existence of the absorption at 1765 cm^{-1} (cyclic lactam) in the infrared absorption spectra and the signals at 3.51 ppm (3H, singlet, 7—OCH_3), 5.02 ppm (1H, singlet, 6—CH), 3.99 ppm (3H, singlet, N—CH_3 of tetrazole), 3.40—3.83 ppm (2H, quartet, J=18 Hz, 2—CH_2), 4.17—4.50 ppm (2H, quartet, J=14 Hz, 3-side chain CH_2). Further, from the facts that there are 1.94 ppm (2H, multiplet, β—CH_2) and 2.40 ppm (4H, multiplet, α, γ—CH_2) showing the existence of 4-carboxybutyramido in the nuclear magnetic resonance spectra and that the mass spectra of the derivative of the product material hydrolyzed with hydrochloric acid and silylated gives the fragment of m/e 276, the compound VI of this invention has been assigned the aforesaid structure wherein the 5-amino-5-carboxyvaleramido group at the 7-position of the starting material has been oxidatively deaminated into a 4-carboxybutyramido group.

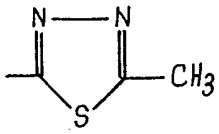
VII. 7 - (4 - Carboxy butyramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4-thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid:



Starting material III:



The physical and chemical properties of the desired compound VII of this invention having formula A(R^1=Ab, R^2=



M=H) are as follows:

(1) White powder.

(2) Melting point 95—99° C.

(3) Easily soluble in methanol and ethanol, soluble in water, ethyl acetate, butyl acetate, and butanol, but scarcely soluble in other organic solvents.

(4) Acidic material showing a negative ninhydrin reaction.

(5) Gives the ultraviolet absorption spectra as shown in Fig. 15 when measured in a 1/100 *M* phosphate buffer solution having a pH of 6.5, and has an absorption maximum at 273 mμ.

(6) Gives the infrared absorption spectra as shown in Fig. 16 when measured as potassium bromide tablet, and shows the absorptions at 3260 cm^{-1}, 2925 cm^{-1}, 1773 cm^{-1}, 1515 cm^{-1} and 1375 cm^{-1}.

(7) The nuclear magnetic resonance spectra measured using TMS as the internal standard in heavy methanol gives, as shown in Fig. 17, the following signals; δ value (ppm):

1.92 (2H, multiplet), 2.40 (4H, multiplet),
2.71 (3H, singlet), 3.38—3.80 (2H, quartet, J=18 Hz),
3.51 (3H, singlet), 4.17—4.64 (2H, quartet, J=14 Hz),
5.03 (1H, singlet).

(8) Shows the following elemental analysis value for $C_{17}H_{20}N_4O_7S_3$

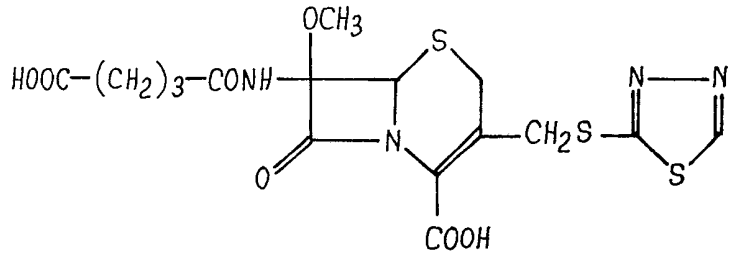
	C	H	N
Calculated:	41.79%	4.13%	11.47%
Found:	41.89%	4.27%	11.17%

(9) The mass spectra of the product material VII measured after hydrolyzing the material in 6 *N* hydrochloric acid for 2.5 hours at 100° C, extracting the product with ethyl ether, drying the extract by evaporation, and silylating it with BSA (bistrimethylsilylacetamide) gives a fragment of m/e 276

$$(CH_3)_3Si—OOC—CH_2CH_2CH_2—CO—OSi(CH_3)_3.$$

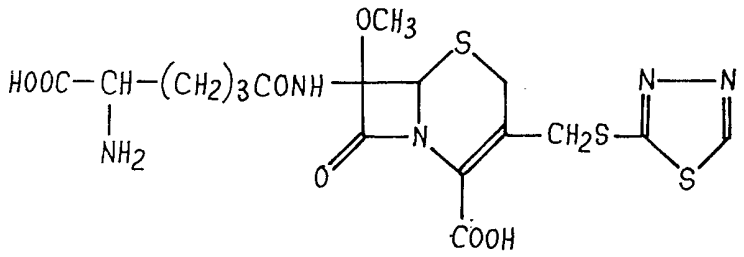
Considering the results as a whole, it is clear that the product compound is a 7-methoxycephalosporin compound from, in particular, the existence of the absorption at 1773 cm^{-1} (cyclic lactam) in the infrared absorption spectra, and the signals at 3.51 ppm (3H, singlet, 7—OCH_3), 5.03 ppm (1H, singlet, 6—CH), 2.71 ppm (3H, singlet, C—CH_3 of thiadiazole), 3.38—3.80 ppm (2H, quartet, J=18 Hz, 2—CH_2) and 4.17—4.64 ppm (2H, quartet, J=14 Hz, 3-side chain CH_2). Further, from the facts that there are 1.92 ppm (2H, multiplet, β—CH_2) and 2.40 ppm (4H, multiplet, α, γ—CH_2) showing the existence of 4-carboxybutyramido in the nuclear magnetic resonance spectra and that the mass spectra of the derivative prepared by hydrolyzing the compound with hydrochloric acid and silylating the product gives the fragment of m/e 276, the product compound of formula VII has been determined to have the aforesaid structure wherein the 5 - amino - 5 - carboxyvaleramido group at the 7-position of the starting material has been oxidatively deaminated into the 4-carboxybutyramido group.

VIII. 7 - (4 - Carboxybutyramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol-2 - yl) - thiomethyl - Δ^3 - cephem - 4 - carboxylic acid:

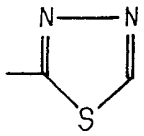


Starting material IV

7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol-2 - yl) - thiomethyl - Δ^3 - cephem - 4 - carboxylic acid:



The physical and chemical properties of the product compound VII of formula A(R^1=Ab, R^2=



M=H are as follows:

(1) White powder.

(2) Melting point 88—92° C.

(3) Easily soluble in methanol and ethanol, soluble in water, ethyl acetate, butyl acetate, and butanol, but scarcely soluble in other organic solvents.

(4) Acidic material showing a negative ninhydrin reaction.

(5) Gives the ultraviolet absorption spectra as shown in Fig. 18 when measured in a 1/100 *M* phosphate buffer solution having a pH of 6.5, and has the absorption maximum at 274 mμ.

(6) Gives the infrared absorption spectra as shown in Fig. 19 when measured as potassium bromide tablet, and shows the absorptions at 3250 cm^{-1}, 2925 cm^{-1}, 1770 cm^{-1}, 1515 cm^{-1} and 1365 cm^{-1}.

(7) The nuclear magnetic resonance spectra measured using TMS as the internal standard in heavy methanol gives, as shown in Fig. 20, the following signals: δ value (ppm):
1.92 (2H, multiplet), 2.40 (4H, multiplet), 3.39—3.83 (2H, quartet, J=18 Hz),
3.51 (3H, singlet), 4.24—4.73 (2H, quartet, J=14 Hz),
5.03 (1H, singlet) and 9.35 (1H, singlet).

(8) Shows the following elemental analysis value for $C_{16}H_{18}N_4O_7S_3 \cdot 1/2H_2O$

	C	H	N
Calculated:	39.74%	3.96%	11.59%
Found:	39.69%	3.87%	11.32%

(9) The mass spectra of the product compound VIII measured after hydrolyzing the compound in 6 *N* hydrochloric acid for 2.5 hours at 100° C, extracting the product with ethyl ether, drying the extract by evaporation, and silylating it with BAS (bistrimethylsilylacetamide) gives a fragment of m/e 276 $(CH_3)_3Si—OOC—CH_2CH_2CH_2—CO—OSi(CH_3)_3$.

Considering the above results as a whole, it is clear that the product compound of this invention is a 7 - methoxycephalosporin compound from the existence of the absorption at 1770 cm^{-1} (cyclic lactam) in the infrared absorption spectra, and the signals at 3.51 ppm (3H, singlet, 7—OCH_3), 5.03 ppm (1H, singlet, 6—CH), 9.35 ppm (1H, singlet, CH of thiadiazole), 3.39—3.83 ppm (2H, quartet, J=18 Hz, 2—CH_2) and 4.24—4.73 ppm (2H, quartet, J=14 Hz, 3-side chain CH_2). Further, from the facts that there are 1.92 ppm (2H, multiplet, β—CH_2) and 2.40 ppm (4H, multiplet, α, γ—CH_2) showing the existence of 4-carboxybutyramido in the nuclear magnetic resonance spectra, and that the mass spectra of the derivative obtained by hydrolyzing the product compound with hydrochloric acid and silylating the product gives the fragment of m/e 276, the product compound VIII has been assigned the aforesaid structure wherein the 5 - amino - 5 - carboxylvaleramido group at the 7-position of the starting material has been oxidatively deaminated into the 4 - carboxybutyramido group.

The results of analyzing the product compounds V—VII of this invention by thin layer chromatography and high speed liquid chromatography are shown in the following table together with the results of the starting materials I—IV also of this invention.

The Rf values by thin layer chromatography using microcrystalline cellulose (Avicel, trade mark) are as follows:

TABLE 1

	Solvent system		Ninhydrin colouring
	1	2	
Starting material I	0.44	0.37	+
Product compound V	0.79	0.72	–
Starting material II	0.44	0.34	+
Product compound VI	0.81	0.64	–
Starting material III	0.42	0.44	+
Product compound VII	0.82	0.77	–
Starting material IV	0.42	0.36	+
Product compound VIII	0.81	0.65	–
7-(5-Amino-5-carboxyvaleramido)-3-[(3-p-hydroxyphenyl-2-methoxypropenoyl)oxymethyl]-7-methoxy-Δ^3-cephem-4-carboxylic acid	0.66	0.67	+
7-(4-Carboxybutyramido)-3-[(3-p-hydroxyphenyl-2-methoxypropenoyl)oxymethyl]-7-methoxy-Δ^3-cephem-4-carboxylic acid	0.67	0.67	–

Developing solvent system:
1. Isopropanol: *n*-butanol:acetic acid:water (21:3:7:9 by volume ratio).
2. *n*-Butanol:acetic acid:water (4:1:2) by volume ratio).

Detection: Ninhydrin reaction or ultraviolet absorption (Manasulu Light, trade name, 2536 Å) or bioautography (*Proteus mirabilis* used). All the compounds showed positive in the latter two tests.

The results from high speed liqudid chromatography are shown in the following table.

TABLE 2

	Retention time
Starting material I	3 min. 14 sec.
Product compound V	13 min. 24 sec.
Starting material II	1 min. 53 sec.
Product compound VI	4 min. 54 sec.
Starting material III	2 min. 55 sec.
Product compound VII	11 min. 18 sec.
Starting material IV	1 min. 56 sec.
Product compound VIII	5 min. 28 sec.

Column used: Waters Ltd. μ Bondapak C_{18}
Solvent system: Acetonitrile: 0.1% acetic acid (pH 3.3) (1:9 by volume ratio)

The retention times of the two control cephalosporin compounds by high speed liquid chromatography are as follows:

	Retention time
7-(5-Amino-5-carboxyvaleramido)-3-[(3-p-hydroxyphenyl-2-methoxy-propenoyl)oxymethyl]-7-methoxy-Δ^3-cephem-4-carboxylic acid	5 min. 18 sec.
7-(4-Carboxybutyramido)-3-[(3-p-hydroxyphenyl-2-methoxy-propenoyl)oxymethyl]-7-methoxy-Δ^3-cephem-4-carboxylic acid	5 min. 55 sec.

Column used: μ Bondapak C 18 (Waters) Ltd.)
Solvent system: Acetonitrile: 0.1% acetic acid (pH 3.3) (2:8 by volume ratio)

The *in vivo* effect of the compounds II, III, VI and VII are shown below:

5 healthy male mice of ddY strain were injected intraperitoneally with 10^6 cells of *E. coli* NIHJ, and after 2 hours a test compound was given subcutaneously. The survival % after 5 days are shown in the following table. Similar experiments were carried out with 10^5 cells of *Proteus mirabilis* 1287.

The control group each consists of 10 mice.

Test Compound \ Dosage (mg/mouse)	*E. coli* NIHJ				*Proteus mirabilis* 1287			
	3	1	0.5	0	3	1	0.5	0
II	100	100	100	0	40	20	0	0
VI	100	100	0	0	60	20	0	0
III	80	80	40	0	100	20	20	0
VII	80	60	0	0	40	20	20	0

It has thus been demonstrated that compounds of formula A(R^1=Aa or Ab) possess antibacterial activity, and they are thus expected to be useful in the prophylaxis and treatment of disease.

Compounds of this invention of formula A can be administered in various forms either alone or in combination with other medicaments. That is, the compounds of this invention can be administered orally, by intramuscular injection, by intravenous injection, or by other techniques in the appropriate forms of capsules, tablets, powders, granules, solutions, or suspensions. Various carriers can be added in formulating the preparations, for example, mannitol, sucrose, glucose, sterilized distilled water, saline solution, or a vegetable oil such as peanut oil, sesame oil. Furthermore, other ingredients such as a stabilizer, binding agent, antioxidant, preservative, lubricant used when preparing tablets, suspending agent, viscosity agent or perfume may be added. The salts of the compounds of formula A or inorganic or organic bases which are pharmacologically non-toxic or useful can be used.

The dosage of the medicaments mainly depends upon the condition and the weight of the parent and also depends on the manner of administration, e.g. oral administration or parenteral administration. In general, 50 mk/kg is administered once or over a few times per day.

The examples of this invention will be illustrated below in detail.

Example 1.

Preparation of 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethyl thio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem-4 - carboxylic acid I:

A culture medium containing 1% starch, 1% glucose, 1.5% soybean flour 0.5% yeast extract, 0.1% dipotassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml. Sakaguchi flasks at 100 ml. each and sterilized at 120° C. for 20 minutes. Each medium was inoculated with Streptomyces organonensis Y—G19Z strain and cultivated for 48 hours at 30° C.

The aforesaid another culture medium was placed in 2,000 ml. Sakaguchi flasks at 400 ml. each, sterilized at 120° C. for 20 minutes, and inoculated with the above inoculum at 2—3% concentration followed by cultivation for 24 hours at 30° C to provide a seed culture.

Furthermore, 60 liters of a culture medium containing 7% starch, 2% glutenmeal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid, 0.01% ferric sulfate, and 55 g. of sodium hydroxide was charged in each of two 100 liter fermentors together with 10 ml. of Adecanol (trade mark) as a defoaming agent and after sterilizing for 30 minutes at 120° C., each medium was inoculated with 800 ml. of the seed culture followed by cultivation for 24 hours at 30° C. In an aqueous sodium hydroxide solution, 5 - mercapto - 1,3,4 - thiadiazole - 2 - thioacetic acid was dissolved and the solution thus formed was sterilized at high pressure and added to each fermentor up to 0.05% of the inoculum and cultivated for further 90 hours.

After the cultivation was completed, the cultured broth was adjusted to pH 2.0 and then mixed with Radiolite (trade mark). The mixture was filtered using filter press and the filtrates were combined with each other to provide about 100 liters of a filtrate mixture. The filtrate was adjusted to pH 3.0 with an aqueous sodium

hydroxide solution, passed through a 12 liter Amberlite XAD—2 (trade mark) column, and the column was washed with 30 liters of water, then eluted with 30 liters of an aqueous 50% acetone. The eluate thus collected was concentrated up to 5.5 liters and after removing impurities formed, water was added to the residue to make 10 liters of solution. The solution thus prepared was adjusted to pH 3.5 with a diluted aqueous hydrochloric acid solution and then passed through a 3 liter Amberlite IRA—68 (Cl-type) (trade name) column. After washing the column with 6 liters of water, elution was carried out using an aqueous solution (pH 7.2) containing 1 M of sodium nitrate and 0.1 M of sodium acetate to provide about 5 liters solution containing antimicrobially active material. The solution was adjusted to pH 3.0, passed through a one liter Amberlite XAD—2 (trade mark) column, and after washing the column with water, the column was eluted with an aqueous solution of 50% acetone to provide about 400 ml. aqueous solution containing antimicrobially active material. By lyopholizing the solution, about 18 g. of the crude powder of the aimed compound I was obtained.

Then, 18 g. of the crude powder was subjected to a column chromatography using about 800 ml. of DEAE-Sephadex A—25 (acetic acid-type) (trade mark) filled with a small amount of 0.5 M ammonium bromide-acetic acid buffer solution and fractionated effective components. The antimicrobially active fractions obtained were collected, passed through a 500 ml. Amberlite XAD—2 (trade mark) column, and after washing the column with water, the column was eluted with an aqueous solution of 25% acetone, and the eluate was evaporated to dryness.

Then, using a solvent mixture of isopropanol:water (7:3 by volume ratio), the product residue obtained was subjected to a column chromatography using microcrystalline cellulose (Avicel) prepared by a solvent mixture having the same composition as above. The antimicrobially active fraction obtained was spotted onto a thin layer plate of Avicel SF (trade mark), developed by a mixture of isopropanol:n-butanol: acetic acid:water (21:3:7:9 by volume ratio), and then a pyridine solution of 0.20% ninhydrin was sprayed onto it followed by heating to cause coloring. Then, the fractions showing a Rf value of 0.39 were collected. The fraction was vacuum evaporated to dryness at 45—50° C. and then subjected to a column chromatography of microcrystalline cellulose (Avicel) prepared by a solvent mixture of isopropanol:n-butanol: acetic acid:water (21:3:7:9 by volume ratio). The antimicrobially active fraction thus obtained was then subjected to a thin layer chromatography of Avicel SF with the solvent mixture as above and by following the same procedure as above, the fractions showing 0.39 Rf value were collected and vacuum evaporated to dryness.

The product residue was further purified by a microcrystalline cellulose column chromatography using a solvent mixture of n-butanol:acetic acid:water (6:1.5:2.5 by volume ratio). The purified active fraction was evaporated to dryness and then dissolved in a small amount of distilled water. The solution was developed on a column of Sephadex G—10 (trade mark) using distilled water. The fractions showing an antimicrobial activity were collected and subjected to a thin layer chromatography as stated above using a solvent mixture of n-butanol:acetic acid:water (6:1.5:2.5 by volume ratio). The fractions showing Rf 0.32 were collected, concentrated, and then subjected to lyophilization to provide about 80 mg. of white 7 - (5 - amino - 5-carboxy - valeramido) - 3 - (5 - carboxymethyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

Example 2.

Preparation of 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (1 - methyl - 1H-tetrazol - 5 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid II.

A culture medium containing 1% starch, 1% glucose, 1.5% soybean flour, 0.5% yeast extract, 0.1% dipotassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml. Sakaguchi flasks at 100 ml. each and sterilized for 20 minutes at 120° C. Then, each medium was inoculated with the Streptomyces organonensis Y—G19Z strain followed by cultivation for 48 hours at 30° C. Furthermore, the aforesaid culture medium was placed in two liter Sakaguchi flasks at 400 ml. each and after sterilizing for 20 minutes at 120° C., each medium was inoculated with up to 2—3% the cultivated broth prepared above followed by cultivation for further 24 hours at 30° C. to provide a inoculum.

Also, 60 liters of a culture medium containing 7% starch, 2% glutene meal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid, 0.01% ferric sulfate, and 55 g. of sodium hydroxide was placed in two 100 liter fermentors each together with 10 ml. of *Adecanol* (trade mark) as a defoaming agent and after sterilizing for 30 minutes at 120° C., each medium was inoculated with 800 ml. of the inoculum prepared

above followed by cultivation for 24 hours at 30° C. Then, 1 - methyl - 5 - mercapto-1H - tetrazole was dissolved in an aqueous sodium hydroxide solution and the solution was sterilized at high pressure and added to the cultivated broth to make the concentration thereof to 0.05%, and the mixture was further cultivated for 90 hours.

After the cultivation was completed, the cultivated broth thus formed was adjusted to pH 2.0 and then mixed with Radiolite (trade mark) with stirring. The mixture was filtered using a filter press and the filtrates obtained were combined to provide about 100 liters of a filtrate mixture.

The filtrate was adjusted to pH 3.0 with an aqueous sodium hydroxide solution, passed through a 12 liter Amberlite XAD—2 (trade mark column, and after washing the column with 30 liters of water, and the column was eluted with 30 liters of an aqueous solution of 50% acetone. The eluate was concentrated up to 5:5 liters and the concentrate was adjusted to pH 3.5 with a diluted aqueous hydrochloric acid solution and passed through a three liter Amberlite IRA—68 (Cl-type) (trade mark) column.

The column was washed with 6 liters of water and fractionated with an aqueous solution (pH 7.2) containing 1 M sodium nitrate and 0.1 M of sodium acetate to provide about 5 liters of a solution containing an antimicrobially active material. The solution was adjusted to pH 3.0, passed through a one liter Amberlite XAD—2 (trade mark) column, washed with water, and eluted with an aqueous solution of 50% acetone to provide about 400 ml. of aqueous solution containing the antimicrobially active material. By lyophilizing the solution, about 54 g. of the crude powder of the aimed compound II was obtained. The crude powder was subjected to a column chromatography with about 800 ml. of DEAE Sephadex A—25 (acetic acid-type) (trade mark) filled with a small amount of 0.5 M ammonium bromide acetic acid buffer solution to fractionate active components. The antimicrobially active fractions were collected, passed through a 500 ml. Amberlite XAD—2 (trade mark) column, and the column was washed with water and eluted with an aqueous solution of 25% acetone. The antimicrobially active fractions were collected and then vacuum evaporated to dryness.

The residue formed was subjected to a column chromatography using microcrystalline cellulose (Avicel) (trade mark) filled with a mixed solvent of isopropanol: water (7:3 by volume ratio) with the solvent mixture having the same composition as above. The antimicrobially active fraction obtained were collected, spotted onto a thin layer plate of Avicel SF (trade mark), developed by a mixed solvent of n-butanol: acetic acid:water (6:1.5:2.5 by volume ratio, and a solution of 0.25% ninhydrin-pyridine was sprayed onto it followed by heating to cause coloring. Then, the fractions showing the Rf 0.31 were collected. The fractions were concentrated under reduced pressure and dried and then the residue formed was subjected to a column chromatography of microcrystalline cellulose (Avicel) prepared by a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio). The antimicrobially active fractions obtained were subjected to a thin layer chromatography of Avicel SF (trade mark) with the solution mixture having the same composition as above and then by following the same procedure as above, the fractions showing the Rf 0.39 were collected and vacuum evaporated to dryness.

The residue thus formed was further subjected to a microcrystalline cellulose column chromatography using a solvent mixture of n-butanol:acetic acid:water (6:1.5:2.5 by volume ratio) to purify the effective component. The active fraction thus purified was vacuum evaporated to dryness, dissolved in a small amount of distilled water, and developed on a column of Sephadex G 10 (trade mark) using distilled water. The fractions having antimicrobial activity were collected and subjected to a thin layer chromatography using a solvent mixture of n-butanol:acetic acid: water (6:1.5:2.5 by volume ratio) as stated above. Then, the fractions having the Rf 0.31 were collected, concentrated, and then lyophilized to provide about 60 mg. of white 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (1 - methyl - 1H - tetrazol-5 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

Example 3.

Preparation of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (5-methyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid III.

A culture medium containing 1% starch, 1% glucose, 1.5% soybean flour, 0.5% yeast extract, 0.1% dipotassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml. Sakaguchi flasks at 100 ml. each and sterilized for 20 minutes at 120° C. Each medium was then inoculated by Streptomyces oganonensis Y—G19Z strain and cultivated for 48 hours at 30° C. Another

culture medium as described above was placed in two liter Sakaguchi flasks at 400 ml. each and sterilized for 20 minutes at 120° C. Each medium was inocubated with the cultivated broth prepared above at 2—3% concentration and then cultivated for 24 hours at 30° C. to provide a seed culture.

Separately, 60 liters of the culture medium containing 7% starch, 2% gluten meal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid, 0.01% ferric sulfate, and 55 g. of sodium hydroxide was placed in two 100 liter fermentors together with 10 ml. of Adecanol (trade mark) as a defoaming agent, sterilized for 30 minutes at 120° C., and inoculated by 800 ml. of seed culture followed by cultivation for 24 hours at 30° C. Then, 2 - mercapto - 5 - methyl - 1,3,4 - thiadiazole was dissolved in aqueous sodium hydroxide solution, sterilized at high pressure, and added to the cultivated broth so that the concentration of it became 0.05% of the broth followed by further cultivation for 90 hours.

After the cultivation was completed, the cultivated broth was adjusted to pH 2.0 and mixed with Radiolite (trade mark) with stirring. The mixture was filtered using filter press and the filtrates were combined to provide about 100 liters of a filtrate mixture.

The filtrate was adjusted to pH 3.0 by the addition of an aqueous sodium hydroxide solution, passed through a 12 liters Amberlite XAD—2 (trade mark) column, and the column was washed with 30 liters of water, and eluted with 30 liters of aqueous solution of 50% acetone. The eluate was concentrated to 5.5 liters and the concentrate was adjusted to pH 3.5 with a diluted aqueous hydrochloric acid solution and passed through a 3 liters Amberlite IRA—68 (Cl-type) (trade mark) column. The column was washed with 6 liters of water and eluted with an aqueous solution (pH 7.2) containing 1 M sodium nitrate and 0.1 M sodium acetate to provide a solution containing about 5 liters of an antimicrobially active material. The solution was adjusted to pH 3.0, passed through a one liter Amberlite XAD—2 (trade mark) column, and after washing the column with water, and the column was eluted with an aqueous solution of 50% acetone to provide about 400 ml. of an aqueous solution containing the antimicrobially active material. The product was lyophlized.

Using a solvent mixture of n-butanol:acetic acid: water (4:1:2 by volume ratio), the residue formed was subjected to a column chromatography using microcrystalline cellulose (Avicel) (trade mark) filled with the solvent mixture having the same composition as above. Then, the antimicrobially active fractions obtained were fractionated and spotted onto a thin layer plate of Avicel SF (trade mark), developed by a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio), and a pyridine solution of 0.25% ninhydrin was sprayed onto it to cause coloring under heating. Thus, the fractions showing the Rf 0.43 were collected, vacuum evaporated to dryness at 45—50° C., and then subjected to a column chromatography of microcrystalline cellulose (Avicel) prepared by a solvent mixture of acetonitrile:water (7:3 by volume ratio). The antimicrobially active fraction obtained was subjected to a thin layer chromatography of Avicel SF (trade mark) as in the above procedure and then the fractions showing the Rf of 0.43 were collected and evaporated to dryness to give 0.78 g of crude powder.

The powder was dissolved in a small amount of distilled water and developed on a column of Sephadex G 10 (trade mark) using distilled water. The antimicrobially active fractions were fractionated and subjected to thin layer chromatography using a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) as stated above and the fractions showing the Rf 0.43 were collected, concentrated and lyophilized to provide 82 mg. of white 7 - (5 - amino - 5 - carboxyvaleramido) - 7-methoxy - 3 - (5 - methyl - 1,3,4 - thiadiazol - 2 - yl) - thiomethyl - Δ^3 - cephem-4 - carboxylic acid.

Example 4.

By following the same procedure as in Example 1 using, in this example, a solution of bis(5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)disufide prepared by dissolving the disulfide in water-containing methanol and sterilized by filtration using Millipore filter in place of the solution of 5 - mercapto - 1,3,4 - thiadiazol - 2 - thioacetic acid prepared in water using an aqueous sodium hydroxide solution and sterilizing at high pressure, 23 g. of the crude powder of the aimed compound I was prepared and by purifying the product as in Example 1, about 45 mg. of 7 - (5-amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol-2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid (Aimed compound I) was obtained.

Example 5.

By following the same procedure as in Example 2 using, in this example, a solution of bis(1 - methyl - 1H - tetrazol - 5 - yl)disulfide prepared by dissolving the disulfide in water containing methanol and sterilizing by filtration using Millipore filter in place of a solution of 1 - methyl - 5 - mercapto - 1H - tetrazole prepared by dissolving the tetrazole in water using an aqueous solution of sodium hydroxide and sterilizing at high pressure, about 26 g. of the crude powder of the aimed compound II was obtained and by purifying the product as in Example 2, about 37 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (1 - methyl - 1H - tetrazol - 5-yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid (aimed compound II) was obtained.

Example 6.

By following the same procedure as in Example 3 using, in this example, a solution of bis(5 - methyl - 1,3,4 - thiadiazol - 2 - yl)disulfide prepared by dissolving the disulfide in water-containing methanol and sterilizing by filtration using Millipore filter in place of the solution of 2 - mercapto - 5 - methyl - 1,3,4 - thiadiazole prepared in water using an aqueous sodium hydroxide solution and sterilizing at high pressure, about 19 g. of the crude powder of the aimed compound III was obtained and by purifying the product as in Example 3, about 50 mg. of 7 - (5 - amino - 5-carboxyvaleramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4 - thiadiazol - 2 - yl)-thiomethyl - Δ^3 - cephem - 4 - carboxylic acid (Aimed compound III) was obtained.

Example 7.

Preparation of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3-(1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid IV.

A culture medium containing 1% starch, 1% glucose, 1.5% soybean flour, 0.5% yeast extract, 0.1% dipotassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml. Sakaguchi flasks at 100 ml. each, sterilized for 20 minutes at 120° C., and inoculated by the Streptomyces oganonensis Y—G19Z strain followed by cultivation for 48 hours at 30° C. Another aforesaid culture medium was also placed in two liters Sakaguchi flasks at 400 ml. each, sterilized for 20 minutes at 120° C., and then inoculated by 2—3% the cultured broth prepared in the above procedure followed by further cultivation for 24 hours at 30° C. to provide a seed culture.

Separately, 60 liters of a culture medium containing 7% starch, 2% gluten meal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid, 0.01% ferric sulfate, and 55 g. of sodium hydroxide was placed in two 100 liter fermentors together with 10 ml. of Adecanol (trade mark) as a defoaming agent, sterilized for 30 minutes at 120° C., and inoculated by 800 ml. of the seed culture followed by cultivation for 24 hours at 30° C. Then, a solution of 2 - mercapto - 1,3,4 - thiadiazole prepared by dissolving the thiadiazole in water using an aqueous solution of sodium hydroxide and sterilizing at high pressure was added to the cultured broth so that the concentration of the thiadiazole became 0.05% and then the system was further cultivated for 90 hours.

After the cultivation was completed, the cultured broth was adjusted to pH 2.0 and mixed with Radiolite (trade mark) with stirring. The mixture was filtered using a filter press and the filtrates were combined to provide about 100 liters of the filtrate mixture.

The filtrate was adjusted to pH 3.0, passed through a 12 liter Amberlite XAD—2 (trade mark) column, and the column was washed with 30 liters of water, and eluted by 30 liters of an aqueous solution of 50% acetone. The eluate was concentrated up to 5.5 liters. The concentrate was adjusted to pH 3.5 and passed through a 3 liter Amberlite IRA—68 (Cl-type) (trade mark) column. The column was washed with 6 liters of water and eluted with an aqueous solution (pH 7.2) containing 1 M of sodium nitrate and 0.1 M of sodium acetate to provide about 5 liters of a solution containing an antimicrobially active material. The solution was adjusted to pH 3.0, passed through a one liter Amberlite XAD—2 (trade mark) column, washed with water, and eluted by an aqueous solution of 50% acetone to provide about 400 ml. of an aqueous solution containing the antimicrobially active material. The solution was lyophilized.

Using a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio), the residue formed was subjected to a column chromatography using microcrystalline cellulose (Avicel, trade name) prepared with the solvent mixture having the same composition as above. The antimicrobially active fractions were fractionated spotted onto a thin layer plate of Avicel (trade mark), developed using a solvent mixture

of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio), and a pyridine solution of 0.25% ninhydrin was sprayed onto it to cause coloring under heating. Then, the fractions showing the Rf of 0.39 were collected and vacuum evaporated to dryness at 45—50° C. The product residue was subjected to a column chromatography of microcrystalline cellulose (Avicel) prepared by a solvent mixture of acetonitrile: water (7:3 by volume ratio). The antimicrobically active fractions were also subjected to a thin layer chromatography of Avicel SF as in the above procedure and then the fractions showing the Rf 0.39 were collected, vacuum evaporated to dryness provide 0.92 g. of a crude powder.

The crude powder was dissolved in a small amount of distilled water and subjected to a column chromatography using Amberlite CG—50 (H-type) (trade mark) with distilled water and the antimicrobially active fractions were collected, concentrated, and lyophilized. The residue was dissolved in a small amount of distilled water and developed on a column of Sephadex G 10 (trade mark) using distilled water. The antimicrobial activity of each fraction was checked and the effective fractions were subjected to a thin layer chromatography using a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) as described above. Then, the fractions showing the Rf 0.38 were collected, concentrated, and lyophilized to provide 75 mg. of white 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol - 2-yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

Example 8.

a). A culture medium of pH 6.0 consisting of 20 g. of glucose, 4 g. of potassium dihydrogen phosphate, 1 g of magnesium sulfate, 2 g. of ammonium sulfate, 0.5 g. of calcium chloride, 0.1 g. of boric acid, 0.04 g. of ammonium molybdate, 0.04 g. of manganese sulfate, 0.04 g. of zinc sulfate, 0.045 g. of copper sulfate, 0.025 g. of ferrous sulfate, 20 mcg. of biotin, 2 mg. of thiamine hydrochloride, 1 g. of DL-methionine, and 1000 ml. of water was placed in 500 ml. Erlenmyer flasks at 100 ml. each and after sterilizing by conventional way, each medium was inoculated with Trigonopsis variabilis IFO 0755 strain followed by shaking cultivation for 72 hours at 30° C.

After the cultivation was completed, about 1000 ml. of the cultured broth was collected and by subjecting the broth to centrifugation at 2,000 rpm. for 30 minutes at 4° C., the mycelium was collected and suspended in 500 ml. of a 0.1 M pyrophosphoate buffer solution having pH 8.1 to provide a mycelium suspension. To the mycelium suspension was added 5 ml. of Triton X—100 (trade mark) and the mixture was shaked for 20—40 minutes at 37° C. to activate the mycelium. By subjecting then the shaked mixture to a centrifugation at 2000 r.p.m. for 30 minutes at 4° C., the activated mycelium was collected, washed twice with a pyrophosphate buffer solution of pH 7—8, and resuspended in 100—200 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 to provide a suspension of activated mycelium.

b). In 10 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 containing 0.026% sodium azide was dissolved 54.5 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3-cephem - 4 - carboxylic acid and after adding thereto 0.5 ml. of the activated mycelium suspension and the mixture was stirred under aeration in a water bath at 33° C. The reaction system was checked every 30 minutes by means of a Hitachi High Speed liquid chromatographic apparatus (using μ Bondapak C_{18} made by Waters Co., Solvent system:acetonitrile:0.1% acetic acid (1:9 volume ratio) to determine the completion of the reaction. That is, the retention time of the starting material, 7 - (5 - amino - 5-carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid shows 1 minute 53 seconds, while the retension time of 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (1 - methyl - 1H-tetrazol - 5 - yl) - thiomethyl - Δ^3 - cephem - 4 - carboxylic acid thus converted by the D-aminoacid oxidation shows 4 minutes 54 seconds.

After the reaction was over, the mycelium was removed by centrifugation and the supernatant was separated and recovered, adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution, and then extracted 4 times each with equal volume of ethyl acetate. The ethyl acetate extracts were recovered and re-extracted with phosphate buffer solution of pH 6.0. The phosphate buffer solution was then adjusted to pH 1.5—2.0 with a diluted hydrochloric acid solution and further extracted 4 times each with equal volume of ethyl acetate. The ethyl acetate extracts were combined, dehydrated with anhydrous sodium sulfate, and evaporated to dryness. The product was developed using a column filled with a micro crystalline cellulose (Avicel, trade mark) and a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume

ratio) with the solvent mixture having the same composition as above and fractionated. The antimicrobial activity of each fraction to Proteus mirabilis was checked and the fractions having the antimicrobial activity were selected, spotted onto a thin layer plate of Avicel SF (trade mark), and developed by a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio) and a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio). Then, the fractions showing the ultraviolet absorption to a Manasulu Light 2536 Å (made by Manasulu Kagaku Kogyo K. K.) and showing Rf 0.81 and Rf 0.64 respectively were collected, concentrated, and lyophilized to provide 35 mg. of pure 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid. This material showed antimicrobial activity to Proteus mirabilis, Salmonella gallinarum, and Escherichia coli.

Example 9.

In 10 ml. of a 0.1 M pyrophosphate solution of pH 8.1 containing 0.026% sodium azide was dissolved 50 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid and to the solution was added 0.5ml. of the activated mycelium suspension of Trigonopsis variabilis IFO 0755 obtained by the same manner as in Example 8. The mixture was stirred under aeration in a water bath at 33° C. to perform the D-amino acid oxidation and the completion of the reaction was determined by the same high speed liquid chromatography as in Example 8. The retention time of the starting material, 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid was 2 minutes 55 seconds and the retention time of 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4 - thadiazol - 2 - yl) - thiomethyl - Δ^3 - cephem - 4 - carboxylic acid formed by the D-aminoacid oxidation was 11 minutes 18 seconds.

After the reaction was over, the mycelium was removed at 4° C. and the supernatant was recovered, adjusted to pH 1.5—2.0 with a diluted hydrochloric acid solution, and extracted four times each with equal volume of ethyl acetate. The ethyl acetate extracts were combined and extracted with a phosphate buffer solution of pH 6. The phosphate solution was adjusted to pH 1.5—2.0 and extracted again four times each with equal volume of ethyl acetate. The ethyl acetate extracts were collected, dehydrated with anhydrous sodium sulfate, and vacuum evaporated to dryness. Using a column filled with microcrystalline cellulose (Avicel, trade mark) by a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio), the residue product was developed using the solvent mixture having the same composition as above. The fractions showing an antimicrobial activity to Proteus mirabilis were selected, spotted onto a thin layer plate of Avicel SF, and developed with a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio) and a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) respectively. Then, the fractions showing the ultraviolet absorption to Manasulu Light 2536 Å (made by Manasulu Kagaku Kogyo K. K.) and also showing Rf 0.82 and Rf 0.77 respectively were collected, concentrated, and lyophilized to provide 32 mg. of pure 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid. This material shows an antimicrobial activity to Proteus mirabilis, Salmonella gallinarum and Escherichia coli.

Example 10.

In 10 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 containing 0.026% sodium azide was dissolved 50 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl) - thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid and after adding to the solution 0.5 ml. of the activated mycelium suspension prepared by the same manner as in Example 8a the mixture was stirred by aeration in a water bath at 33° C. to perform the D-amino acid oxidation. The completion of the reaction was checked every 30 minutes by means of the Hitachi High Speed Liquid Chromatographyic Apparatus as in Example 8. That is, the retention time of the starting material 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid showed 3 minutes 14 seconds and the retention time of 7 - (4 - carboxybutyramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl) - thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid formed by the action of D-amino acid oxidative enzyme showed 13 minutes 24 seconds.

After the reaction was over, the mycelium was removed by centrifugation at

4° C., the supernatant was recovered and then adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution followed by extraction four times each with equal volume of ethyl acetate. The ethyl acetate extracts thus recovered were combined and re-extracted with a phosphate buffer solution at pH 6.0. The phosphate buffer solution was adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution and extracted again four times each with equal volume of ethyl acetate.

The ethyl acetate extract was collected, dehydrated with anhydrous sodium sulfate, and vacuum evaporated to dryness.

The residue was developed by a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) using a column filled with microcrystalline cellulose (Avicel, trade name) by using the solvent mixture having the same composition as above. The antimicrobial activity of each fraction was checked and the fractions having an antimicrobial activity to Proteus mirabilis were selected, spotted onto a thin layer plate of Avicel SF, and developed with a solvent mixture of isopropanol:n-butanol: acetic acid:water (21:3:7:9 by volume ratio) and a solvent mixture of n-butanol: acetic acid:water (4:1:2 by volume ratio) respectively. Then, the fractions showing the ultraviolet absorption to the Manasulu Light 2536 Å (made by Manasulu Kagaku Kogyo K. K.) and showing Rf 0.79 and 0.72 respectively were collected, concentrated and lyophilized to give 30 mg. of pure 7 - (4 - carboxybutyramido) - 3 - (5-carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3-cephem - 4 - carboxylic acid. This material shows an antimicrobial activity to Proteus mirabilis, Salmonella gallinarum, and Escherichia coli.

Example 11.

a). A culture medium containing 1% starch, 1% glucose, 1.5% soybean flour, 0.5% yeast extract, 0.1% di potassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml Sakaguchi flasks at 100 ml. each and sterilized for 20 minutes at 120° C. Each medium was inoculated with Streptomyces oganonensis Y—G19Z strain followed by cultivation for 48 hours at 30° C. Another culture medium was placed in two liter Sakaguchi flasks at 400 ml. each, sterilized for 20 minutes at 120° C. and inoculated with 2—3% the cultured broth prepared above followed by cultivation for 24 hours at 30° C. to give seed culture.

Separately, 60 liters of a culture medium containing 7% starch, 2% gluten meal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid. 0.01% ferric sulfate, and 55 g. of sodium hydroxide was placed in two 100 liter fermentors together with each 10 ml. of Adecanol (trade mark), sterilized for 30 minutes at 120° C., and inoculated with 800 ml. of the seed culture prepared in the above procedure followed by cultivation for 24 hours at 30° C. Then, a solution of 5 - mercapto - 1 - methyl-1H - tetrazole prepared by an aqueous sodium hydroxide solution and sterilizing at high pressure was added to the cultured broth to that the concentration of the tetrazole became 0.05%. The cultivation was further carried out for 90 hours.

After the cultivation was completed, the cultured broth was adjusted to pH 2.0 and mixed with Radiolite (trade mark) with stirring. The mixture was filtered with a filter press and the filtrates were combined to provide 100 liters of a filtrate mixture containing 100 mcg./ml. of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy-3 - (1 - methyl - 1H - tetrazol - 5 - yl) - thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

b. The filtrate was adjusted to pH 3.0, passed through a 12 liter Amberlite XAD—2 (trade mark) column, and the column was washed with 30 liters of water, and eluted with 30 liters of an aqueous 50% acetone. The eluate was concentrated up to 5.5 liters and the concentrate was adjusted to pH 7.5 using an aqueous sodium hydroxide solution. After removing the insolubles formed, 320 ml. of the activated mycelium suspension of the Trigonopsis variabilis IFO 0755 strain prepared in Example 8a was added to the solution. The mixture was stirred under aeration for 4 hours, adjusted to pH 1.5—2.0 with an aqueous hydrochloric acid solution, and extracted four times each using equal volume of ethyl acetate. The ethyl acetate extracts were collected and 20 liters of the ethyl acetate extract thus obtained was then re-extracted with 2 liters of a phosphate buffer solution of pH 6.0. The phosphate solution was further adjusted to pH 1.5—2.0 with aqueous hydrochloric acid and extracted again four times each with equal volume of ethyl acetate. The ethyl acetate filtrates were combined and 8 liters of the extract thus obtained was evaporated in vacuum to dryness to provide about 15 g. of a crude material. The material was subjected to column chromatography using cellulose powder by the same manner as in Example 8b to provide 6.1 g of white 7 - (4 - carboxybutyramido) - 7 - methoxy-

3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

Example 12.

A culture medium comprising 50 g. of glucose, 10 g. of peptone, 1 g. of potassium dihydrogen phosphate, 0.5 g. of magnesium sulfate, 10 g. of malt extract, 1 g. of DL-methionine, and 1,000 ml. of water having pH 6.0 was inoculated with the Trigonopsis variabilis IFO 0755 strain followed by cultivation as in Example 8a and 1,000 ml. of the cultured broth thus formed was collected. By subjecting 1,000 ml. of the cultured broth to a centrifugation at 2,000 r.p.m. at 4° C., and mycelium formed was collected. The mycelium was suspended in 200 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 and the suspension of the mycelium was placed in 500 ml. Erlenmeyer flasks at 50 ml. each. Then, after adding to the suspension 5 ml. of toluene, the activation was performed for one hour at 37° C. Thereafter, the activated mycelium was collected by a centrifugation for 30 minutes at 2,000 r.p.m. and then centrifugally washed with 200 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1. The activated mycelium was suspended again in 200 ml. of a 0.1 M pyrophosphate buffer solution at pH 8.1 and the suspension was stirred in a water bath at 50° C. to inactivate the catalase activity. Then, 5 ml. of the activated mycelium suspension was added to a solution of 100 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid in 20 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 and after stirring the mixture under aeration for 5 hours at 33° C., the mixture was treated as in Example 8-b to provide 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

Example 13.

The mycelium obtained from the Trigonopsis variabilis by the same manner as in Example 8a was frozen at temperatures below −20° C. for more than one hour, then allowed to stand at room temperature to melt it, and suspended in 200 ml. of a 0.1 M pyrophosphate solution of pH 8.1. Then, 5 ml. of the suspension was added to a solution of 100 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid in 20 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 containing 0.026% sodium azide and after stirring the mixture under aeration for 5 hours at 33° C., the mixture was treated by the same manner as in Example 8b to provide 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)-thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

Example 14.

a). Preparation of 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (1 - methyl - 1H-tetrazol - 5 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

A culture medium containing 1% starch, 1% glucose, 1.5% soybean flour, 0.5% yeast extract, 0.1% di potassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml. Sakaguchi flasks at 100 ml. each and each medium was sterilized for 20 minutes at 120° C. The sterile culture medium was inoculated with the streptomyces oganonensis Y—G19Z strain followed by cultivation for 48 hours at 30° C. Another culture medium prepared as above was placed in two liter Sakaguchi flasks at 400 ml. each and after sterilizing the medium for 20 minutes at 120° C., the culture medium was inoculated with the cultured broth formed in the above procedure at 2—3% in amount and cultivated for 24 hours at 30° C. to provide seed culture.

Separately, 60 liters of a culture medium containing 7% starch, 2% gluten meal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid, 0.01% ferric sulfate, and 55 g. of sodium hydroxide was placed in two 100 liter fermentors together with each 10 ml. of Adecanol (trade mark) as a defoaming agent. Each culture medium was sterilized for 30 minutes at 120° C. and inoculated with 800 ml. of the seed culture prepared in the above procedure. Then, to each fermentator was added a solution of 1 - methyl - 5 - mercapto - 1H - tetrazole prepared in an aqueous solution of sodium hydroxide and sterilized at high pressure so that the content became 0.05% and then the cultured broth was further cultivated for 90 hours.

After the cultivation was completed, the cultured broth was adjusted to pH 2.0 and mixed with Radiolite (trade mark) with stirring. The mixture was filtrated by

filter press and the filtrates were combined to provide about 100 liters of a filtrate mixture.

The mixture was adjusted to pH 3.0 by the addition of an aqueous sodium hydroxide solution, passed through a 12 liter Amberlite XAD—2 (trade mark) column, and the column was washed with 30 liters of water, and eluted with 30 liters of aqueous 50% acetone. The eluate was concentrated up to 5.5 liters and the concentrate was adjusted to pH 3.5 with a diluted aqueous hydrochloric acid solution, passed through a 3 liter Amberlite IRA—68 (Cl-type) (trade mark) column, and the column was washed with 6 liters of water, and eluted with an aqueous solution (pH 7.2) containing 1 N sodium nitrate and 0.1 M sodium acetate to provide about 5 liter of solution containing an antimicrobially active material. The solution was adjusted to pH 3.0, passed through a one liter Amberlite XAD—2 (trade mark) column, and the column was washed with water, and eluted with an aqueous 50% acetone to provide about 400 ml. of an aqueous solution containing the antimicrobially active material, which was lyophilized to give about 54 g. of a crude powder. The crude powder was subjected to column chromatography with about 800 ml. of DEAE Sephadex A—25 (acetic acid-type) (trade mark) filled with a small amount of 0.5 M ammonium bromide acetic acid buffer solution to fractionate effective fractions. The antimicrobially active fractions thus collected were passed through 500 ml. of Amberlite XAD—2 (trade mark) column, and the column was washed with water, and eluted with an aqueous 25% acetone. The eluate was then evaporated in vacuo to dryness.

The dried product was subjected to a column chromatography with a solvent mixture of isopropanol:water (7:3 by volume ratio) using microcrystal line cellulose (Avicel, trade mark) filled with the solvent mixture having the same composition as above. Then, the fraction showing antimicrobial activity was spotted onto a thin layer plate of Avicel SF (trade mark), developed by a solvent mixture of n-butanol: acetic acid:water (6:1.5:2.5 by volume ratio), and a pyridine solution of 0.25% ninhydrin was sprayed onto it to cause coloring under heating. Thus, the fractions showing Rf 0.31 were collected, evaporated in vacuo to dryness at 45—50° C., and subjected to a column chromatography of microcrystal line cellulose (Avicel, trade mark) prepared with a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume retio). The antimicrobially active fractions were then selected and subjected to a thin layer chromatography of Avicel SF (trade name) and by following the same procedure as above, the fractions showing Rf 0.39 were collected and evaporated in vacuo to dryness.

The dried product was further subjected to a microcrystalline cellulose column chromatography using a solvent mixture of n-butanol:acetic acid:water (6:1.5:2.5 by volume ratio) to perform the purification of the effective components. The purified active fractions were dried by concentration, dissolved in a small amount of distilled water, and developed on a column of Sephadex G 10 (trade mark) using distilled water. The antimicrobial activity of each fraction was checked and the effective fractions were selected, subjected to a thin layer chromatography as stated above using a solvent mixture of n-butanol:acetic acid:water (6:1.5:2.5 by volume ratio), and the fractions showing Rf 0.31 were collected, concentrated and lyophilized to provide about 60 mg. of white 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (1 - methyl - 1H-tetrazol - 5 - yl) - thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

b). Preparation of 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (1 - methyl-1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

In 10 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 containing 0.02% of sodium azido was dissolved 54.5 mg. of 7 - (5 - amino - 5 - carboxyvaleramido)-7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4-carboxylic acid prepared in the step a) and after adding to the solution 0.5 ml. of the activated mycelium suspension prepared by the same manner as in Example 8a, the mixture was stirred under aeration in a water bath at 33° C.

The completion of the reaction was confirmed by checking the reaction system every 30 minutes by means of a Hitachi high speed chromatography apparatus (using μ Bandapak C_{18} made by Waters Ltd., solvent system: acetonitrile:0.1% acetic acid solution of 1:9 by volume ratio). That is, the retention time of the starting material, 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl - 1H-tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid was 1 minute 53 seconds and the retention time of 7 - (4 - carboxybutyramido) - 7 - methoxy - 3-(1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid produced by D-amino acid oxidation was 4 minutes 54 seconds. After the reaction was over, the mycelium was removed by centrifugation at 4° C. The supernatant was

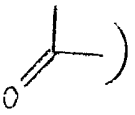
recovered, adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution, and extracted four times each with equal volume of ethyl acetate. The ethyl acetate extracts were combined and then re-extracted with a phosphate buffer solution of pH 6.0. The phosphate buffer solution was then adjusted to pH 1.5—2.0 with a diluted hydrochloric acid solution and extracted again four times each with equal volume of ethyl acetate. The ethyl acetate extracts were combined, dehydrated with anhydrous sodium sulfate, and evaporated in vacuo to dryness. The dried product was developed with a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) using a column filled with microcrystalline cellulose (Avicel, trade mark) using the solvent mixture having the same composition as above. The antimicrobial activity of each fraction to against Proteus mirabilis was checked and the fractions having the antimicrobial activity were selected, spotted onto a thin layer plate of Avicel SF (trade mark), and developed with a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio) and a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) to collect the fractions showing the ultraviolet absorption to Manasgulu Light 2536 Å (made by Manasulu Kagaku Kogyo K. K.) and showing Rf 0.81 and Rf 0.64 respectively. The fractions were combined then concentrated and lyophilized to provide 35 mg. of pure 7 - (4-carboxybutyramido) - 7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid. This material thus produced showed an antimicrobial activity to Proteus mirabilis, Salmonella gallinarum, and Escherichia coli.

In addition, the methyl ester compound derived from the product obtained in this example by the process shown in following Reference example A coincided completely in structure with the corresponding compound produced by the synthetic process of Reference example B shown below.

Reference example A.

In 10 ml. of chloroform was suspended 100 mg. of 7 - (4 - carboxybutyramido)-7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl) - thiomethyl - Δ^3 - cephem-4 - carboxylic acid and after adding to the suspension 4 ml. of a 1% diazomethane ether solution, the mixture was stirred for 30 minutes at room temperature. The reaction mixture obtained was washed with diluted acetic acid and water, dried with anhydrous magnesium sulfate, and concentrated under reduced pressure. The residue obtained was subjected to a column chromatography using a silica gel column and eluted with a solvent mixture of benzene:ethyl acetate (1:3 by volume ratio). The aimed fractions were collected and concentrated under reduced pressure to provide 80 mg. of methyl 7 - methoxy - 7 - (4 - methoxycarbonylbutyramido) - 3 - (1-methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylate.

Infrared absorption spectra:

ν_{max}^{KBr} cm^{-1}: 1780 (lactam 

1725 (ester, carbonyl).

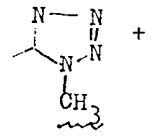
Nuclear magnetic resonance spectra ($CDCl_3$):

δ: 2.03 (2H, $-CO-CH_2-CH_2-CH_2-CO-$),

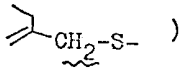
2.39 (4H, $-CO-CH_2-CH_2-CH_2-CO-$),

3.51 (3H, 7-position, $-OCH_3$),

3.64 (3H, $CH_3-O-CO-(CH_2)_3-$),

3.87 3H, 

3.92 3H,

4.23, 4.53 (2H, )

5.04 (1H, 6-position, -H).

Reference example B.

a). In a mixture of 30 ml. of ethyl acetate and 50 ml. of methanol were dissolved 1.0 g. of diphenylmethyl 7β - (3,5 - di - tert - butyl - 4 - hydroxybenzylideneamino) - 7α - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3-cephem - 4 - carboxylate and 1.8 g. of a Gilard reagent and the mixture was stirred for 30 minutes at room temperature. After the reaction was over, the reaction mixture was concentrated under reduced pressure and the residue formed was dissolved in 50 ml. of ethyl acetate and washed three times each with 20 ml. of water. The organic solvent layer formed was recovered, dried with anhydrous magnesium sulfate, and then the solvent was distilled away under reduced pressure to provide about 0.6 g. of crude diphenylmethyl 7β - amino - 7α - methoxy - 3 - (1 - methyl - 1H - tetrazol-5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylate. The product was dissolved in 10 ml. of chloroform, and after cooling the solution to temperature of from −20° C. to −30° C., 0.6 g. of methyl 4 - chloroformyl butyrate was added dropwise to the solution with stirring. The mixture was then further stirred for one hour at the same temperature. The reaction mixture was then mixed with 20 ml. of chloroform and the mixture was washed with 10 ml. of 1 N hydrochloric acid and then 10 ml. of water. The organic solvent layer formed was recovered and dried with anhydrous magnesium sulfate. The solvent was distilled away under the reduced pressure and the residue formed was subjected to a silica gel chromatography. Then, the column was eluted with a solvent mixture of benzene:ethyl acetate (3:1 by volume ratio) and then a solvent mixture of benzene:ethyl acetate (1:3 by volume ratio) to provide 500 mg. of pure 7α - methoxy - 7β - (4 - methoxycarbonylbutyramido) - 3 - (1 - methyl-1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylate having the following properties.

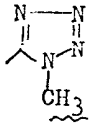
Nuclear magnetic resonance spectra ($CDCl_3$):

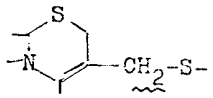
δ: 2.00 (2H, $-CO-CH_2-CH_2-CH_2-CO-$),

2.36 (4H, $-CO-CH_2-CH_2-CH_2-CO-$),

3,50 (3H, 7-position, $-OCH_3$),

3.63 (3H, $CH_3-O-CO-(CH_2)_3-$),

3.78 (3H, ),

4.19, 4.44 (2H, ),

5.04 (1H, 6-position, H),

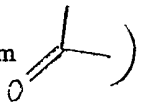
6.91 (1H, $-CH(C_6H_5)_2$),

7.32 (10H, $-CH(C_6H_5)_2$).

b. In 4 ml. of a solvent mixture of trifluoroacetic acid:anisole (4:1 by volume ratio) was dissolved 400 mg. of diphenylmethyl 7α - methoxy - 7β - (4 - methoxycarbonylbutyramido) - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3-cephem - 4 - carboxylate at temperatures of from −10° C. to −20° C. and the mixture was stirred for 30 minutes at the same temperatures. After the reaction was over, the solvent was distilled away under reduced pressure and then ether was added to the residue, whereby crude 7α - methoxy - 7β - (4 - methoxycarbonylbutyramido)-3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid precipitated, which was recovered by filtration and suspended in 10 ml. of chloroform. The suspension was mixed with 0.4 ml. of a 1% diazomethane ether solution at 10—20° C. and the mixture was stirred for 30 minutes at room temperature. The reaction mixture formed was washed with a diluted acetic acid solution

and then water and dried with anhydrous magnesium sulfate. Then, the solvent was distilled away under reduced pressure and the residue formed was subjected to a silica gel column chromatography. The column was eluted with a solvent mixture of benzene:ethyl acetate (1:3 by volume ratio) and the aimed fractions were collected and concentrated under reduced pressure to provide 150 mg. of methyl 7α - methoxy-7β - (4 - methoxycarbonylbutyramido) - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)-thiomethyl - Δ^3 - cephem - 4 - carboxylate having the following properties:

Infrared absorption spectra:

ν_{max}^{KBr} cm^{-1}: 1780 (lactam )

1725 (ester, carbonyl).

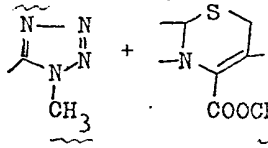
Nuclear magnetic resonance spectra ($CDCl_3$):

δ: 2.03 (2H, $-CO-CH_2-CH_2-CH_2-CO-$),

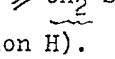
2.39 (4H, $-CO-CH_2-CH_2-CH_2-CO-$),

3.51 (3H, 7-position $-OCH_3$),

3.64 (3H, $CH_3OCO(CH_2)_3-$),

3.87 (3H, 

3.92 (3H,

4.23, 4.53 (2H, ),

5.04 (1H, 6-position H).

Example 15.

a. Preparation of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3-(5 - methyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

A culture medium containing 1% starch, 1% glucose, 1.5% soybean flour, 0.5% yeast extract, 0.1% dipotassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml. Sakaguchi flasks at 100 ml. each and each medium was sterilized for 20 minutes at 120° C. The culture medium was then inoculated with the Streptomyces oganonensis Y—G19Z strain followed by cultivation for 48 hours at 30° C.

Another aforesaid culture medium was placed in 2 liter Sakaguchi flasks at 400 ml. each, sterilized for 20 minutes at 120° C., and then inoculated by 2—3% cultured broth prepared in the above procedure followed by cultivation for 24 hours at 30° C. to provide a seed culture.

Separately, 60 liters of a culture medium containing 7% starch, 2% gluten meal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid, 0.01% ferric sulfate, and 55 g. of sodium hydroxide was placed in two 100 liter fermentors together with 10 ml. of Adecanol (trade mark), sterilized for 30 min. at 120° C., and then inoculated with 800 ml. of the seed culture prepared in the above procedure followed by cultivation for 24 hours at 30° C. To each fermentor was added a solution of 2 - mercapto - 5-methyl - 1,3,4 - thiadiazole prepared by aqueous sodium hydroxide solution and sterilizing at high pressure so that the content thereof became 0.05% of the culture broth and the cultivation was further carried out for 90 hours.

After the cultivation was completed, the cultured broth was adjusted to pH 2.0 and Radiolite (trade mark) was added thereto with stirring. The mixture was filtrated using a filter press and the filtrates were combined to provide about 100 liters of a filtrate mixture.

The filtrate was adjusted to pH 3.0 by the addition of an aqueous sodium hydroxide solution, absorbed in a 12 liter Amberlite XAD—2 (trade mark) column, and the column was washed with 30 liters of water, and eluted with 30 liters of an aqueous 50% acetone. The eluate was concentrated up to 5.5 liters, adjusted to pH

3.5 with a diluted aqueous hydrochloric asid solution, and absorbed in a 3 liter Amberlite IRA—68 (Cl-type) (trade mark) column. The column was washed with 6 liters of water and eluted with an aqueous solution (pH 7.2) containing 1M of sodium nitrate and 0.1 M of sodium acetate to provide about 5 liters of a solution containing an antimicrobially active material. The solution obtained was adjusted to pH 3.0, absorbed in one liter Amberlite XAD—2 (trade mark) column, and the column was washed with water, and eluted with an aqueous 50% acetone to provide about 400 ml. of an aqueous solution containing the antimicrobial material, which was lyophilized.

The product was subjected to a column chromatography with a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) using microcrystalline cellulose (Avicel, trade mark) filled with the solvent mixture having the same composition as above and the antimicrobially active fractions obtained were spotted onto a thin layer plate of Avicel SF (trade mark), developed by a solvent mixture of isopropanol: n-butanol:acetic acid:water (21:3:7:9 by volume ratio), and then a pyridine solution of 0.25% ninhydrin was sprayed to cause coloring under heating. Then, the fractions showing Rf 0.43 were collected evaporated to dryness under reduced pressure at 45—50° C., and the product obtained was subjected to column chromatography of microcrystalline cellulose Avicel) prepared by a solvent mixture of acetonitrile:water (7:3 by volume ratio). The antimicrobially active fractions thus obtained were also subjected to thin layer chromatography of Avicel SF as in the above procedure and the fractions showing Rf 0.43 were collected and evaporated to dryness under reduced pressure to provide 0.78 g. of a crude powder.

The product was dissolved in a small amount of distilled water and developed on a column of Sephadex G 10 (trade mark) using distilled water. The antimicrobial activity of each fraction was checked and the effective fractions were subjected to a thin layer chromatography as stated above using a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio). Then, the fractions showing Rf 0.43 were collected, concentrated, and lyophilized to provide 82 mg. of white 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (5 - methyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

b). Preparation of 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (5 - methyl-1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid:

In 10 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 containing 0.026% sodium azide was dissolved 50 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 7-methoxy - 3 - (5 - methyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4-carboxylic acid and after adding to the solution 0.5 ml of the activated mycelium suspension prepared using the Trigonopsis variabilis IFO 0755 strain as in Example 8a, the mixture was stirred under aeration in a water bath at 33° C. to perform the D-amino acid oxidation. The completion of the reaction was determined by the high speed liquid chromatography as in Example 8b. The retention time of the starting material, 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (5 - methyl-1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid was 2 minutes 55 seconds and that of 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (5-methyl - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid prepared by the D-amino acid oxidation was 11 minutes 18 seconds.

After the reaction was over, the mycelium was removed at 4° C. and the supernatant was recovered, adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution and extracted four times each with equal volume of ethyl acetate. The ethyl acetate extracts were combined and re-extracted with a phosphate buffer solution of pH 6.0. The phosphate solution was then adjusted to pH 1.5—2.0 and then extracted again four times each with equal volume of ethyl acetate. The ethyl acetate extracts were combined, dehydrated with anhydrous sodium sulfate, and evaporated to dryness. The product was developed with a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) using a column filled with microcrystal cellulose (Avicel, trade mark) by using the solvent mixture having the same composition as above. Then, the fractions showing the antimicrobial activity to Proteus mirabilis were selected, spotted onto a thin layer plate of Avicel SF (trade mark) and developed with a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio) and a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) to select the fractions showing the ultaviolet absorption to Manasulu light 2536 Å (made by Manasulu Kagaku Kogyo K. K.) and showing Rf 0.82 and Rf 0.77 respectively. The fractions thus collected were concentrated and then lyophilized to provide 32 mg. of pure 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (5 - methyl-1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid. This

material showed an antimicrobial activity to Proteus mirabilis, Salmonella gallinarum, and Escherichia coli.

Example 16.

a). Preparation of 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4-carboxylic acid.

A culture medium contining 1% starch, 1% glucose, 1.5% soybean flour, 0.5% yeast extract, 0.1%, dipotassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml. Sakaguchi flasks at 100 ml. each and sterilized for 20 minutes at 120° C. Eeach culture medium was then inoculated with the Streptomyces oganonensis Y—G19Z strain followed by cultivation for 48 hours at 30° C. Another aforesaid culture medium was placed in 2,000 ml Sakaguchi flasks at 400 ml. each and each culture medium was sterilized for 20 minutes at 120° C. and inoculated by 2—3% the cultured broth prepared in the above procedure followed by cultivation for 24 hours at 30° C. to provide seed culture.

Separately, 60 liters of a culture medium containing 7% starch, 2% gluten meal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid, 0.01% ferric sulfate, and 55 g. of sodium hydroxide was placed in two 100 liter fermentors together with 10 ml. of Adecanol (trade mark) as a defoaming agent. Each medium was sterilized for 30 minutes at 120° C. and inoculated by 800 ml. of the seed culture prepared in the aforesaid procedure followed by cultivation for 24 hours at 30° C. Then, to each fermentator was added a solution of bis(5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)disulfide prepared by dissolving the disulfide in water-containing methanol and sterilizing by filtration using a Millipore filter so that the content became 0.05% of the culture broth and then the cultivation was carried out for 90 hours.

After the cultivation was completed, the cultured broth was adjusted to pH 2.0 and mixed with Radiolite (trade mark). The mixture was filtrated using a filter press and the filtrates were combined to provide about 100 liters of a filtrate mixture. The filtrate was adjusted to pH 3.0 by the addition of an aqueous sodium hydroxide solution, absorbed in a 12 liter Amberlite XAD—2 (trade mark) column, and the column was washed with 30 liters of water, and eluted with 30 liters of an aqueous 50% acetone. The eluate was concentrated up to 5.5 liters and after removing insoluble matters formed, water was added to the concentrate to make 10 liters of the solution. The solution was adjusted to pH 3.5 with a diluted aqueous hydrochloric acid solution, passed through a 3 liter Amberlite IRA—68 (Cl-type) (trade mark) column, and the column was washed with 6 liters of water, and eluted with an aqueous solution (pH 7.2) containing 1 M of sodium nitrate and 0.1 M of sodium acetate to provide about 5 liters of a solution containing an antimicrobially active material. The solution was adjusted to pH 3.0, absorbed in 1 liter Amberlite XAD—2 (trade mark) column, and the column was washed with water, and eluted with an aqueous 50% acetone to provide about 400 ml. of an aqueous solution containing the antimicrobially active material. By lyophilizing the aqueous solution, about 23 g. of a crude powder was obtained.

Then, 23 g. of the crude powder was subjected to a column chromatography using about 800 ml. of DEAE-Sephadex A—25 (acetic acid-type) (trade mark) filled with a small amount of 0.5 M ammonium bromide acetic acid buffer solution to select the effective components. The antimicrobially active fractions were collected, absorbed in 500 ml. of Amberlite XAD—2 (trade mark) column, and the column was washed with water, and eluted with an aqueous 25% acetone. The eluate was evaporated to dryness in vacuo.

The product was subjected to a column chromatography with a solvent mixture of isopropanol:water (7:3 by volume ratio) using microcrystalline cellulose (Avicel, trade mark) filled using the solvent mixture having the same composition as above to fractionate antimicrobially active fractions. The fractions thus were spotted onto at thin layer plate of Avicel SF (trade mark), developed with a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio), and then a pyridine solution of 0.25% nihydrin was sprayed to cause coloring under heating. Then, the fractions showing Rf 0.39 were collected, evaporated to dryness under reduced pressure and then subjected to a column chromatography of microcrystalline cellulose (Avicel) prepared using a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio). The antimicrobially active fractions were subjected to thin layer chromatography of Avicel SF with the solution mixture having the same composition as above and by following the same procedure as described

above, the fractions showing Rf 0.39 were collected and evaporated in vacuo to dryness.

The concentrate was further purified by a microcrystalline cellulose column chromatography using a solvent mixture of n-butanol:acetic acid:water (6:1.5:2.5 by volume ratio). The purified active fractions were evaporated in vacuo to dryness, dissolved in a small amount of distilled water, and developed on a column of Sephadex G—10 (trade mark) using distilled water. The antimicrobial activity of each fraction was checked and the effective fractions were subjected to a thin layer chromatography as stated above using a solvent mixture of n-butanol:acetic acid:water (6:1.5:2.5 by volume ratio). The fractions showing Rf 0.32 were collected, concentrated, and lyophilized to provide about 45 mg. of white 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7-methoxy - Δ^3 - cephem - 4 - carboxylic acid.

b). Preparation of 7 - (4 - carboxybutyramido) - 3 - (5 - carboxymethylthio-1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

In 5 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 containing 0.026% sodium azide was dissolved 25 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 3-(5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3-cephem - 4 - carboxylic acid prepared in above process a) and after adding to the solution 0.5 ml. of the activated mycelium suspension prepared in Example 8a, the mixture was stirred under aeration in water at 33° C. to carry out the D-aminoacid oxidation. The completion of the reaction was checked every 30 minutes using the Hitachi high speed chromatography apparatus by the same manner as in Example 8a to determine the completion of the reaction. That is, the retention time of the starting material, 7 - (5 - amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4-carboxy acid was 3 minutes 14 seconds and that of 7 - (4 - carboxybutyramido) - 3-(5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3-cephem - 4 - carboxylic acid prepared by the D-amino acid oxidation was 13 minutes 24 seconds.

After the reaction was over, the mycelium was removed by centrifugation at 4° C. and the supernatant was recovered, adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution and extracted four times each with equal volume of ethyl acetate. The ethyl acetate extracts were combined and re-extracted with a phosphate buffer solution of pH 6.0. The phosphate solution was adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution and extracted four times each with equal volume of ethyl acetate. The ethyl acetate extracts were collected, dried over anhydrous sodium sulfate, and evaporated to dryness in vacuo.

The concentrate was developed with a solvent mixture of n-butanol:acetic acid: water (4:1:2 by volume ratio) using a column filled with microcrystalline cellulose (Avicel, trade mark) using the aforesaid solvent mixture.

The antimicrobial activity of each fraction was checked and the fractions showing the antimicrobial activity to Proteus mirabilis were spotted onto a thin layer plate of Avicel SF, and developed by a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio) and a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) respectively to collect the fractions showing the ultraviolet absorption to Manasulu light 2536 Å (made by Manasulu Kagaku Kogyo K. K.) and showing Rf 0.79 and Rf 0.72 respectively. The fractions were concentrated and lyophilized to provide 16 mg. of pure 7 - (4 - carboxybutyramido)-3 - (5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy-Δ^3 - cephem - 4 - carboxylic acid. This material showed an antimicrobial activity to Proteus mitabilis, Salmonella gallinarum, and Escherichia coli.

Example 17.

In 10 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 containing 0.026% sodium azide was dissolved 50 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 7-methoxy - 3 - (1,3,4 - diadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid and after adding thereto 0.5 ml. of the activated mycelium suspension prepared using the Trigonopsis variabilis as in Example 8a, and the mixture was stirred under aeration in a water bath of 33° C. to perform the D-amino acid oxidation. The completion of the reaction was determined by the same high speed liquid chromatography as in Example 8b. The retention time of the starting material, 7 - (5 - amino - 5-carboxyvaleramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3-cephem - 4 - carboxylic acid was 1 minute and 56 seconds and that of 7 - (4 - carb-

oxybutyramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl) - thiomethyl - Δ^3-cephem - 4 - carboxylic acid formed by the D-amino acid oxidation was 5 minutes 28 seconds.

After the reaction was over, the mycelium was removed at 4° C. and the supernatant was recovered, adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution, and extracted four times each with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and re-extracted with a phosphate buffer solution of pH 6.0. The phosphate solution was extracted again four times each with equal volume of ethyl acetate and the ethyl acetate extracts were collected, dried over anhydrous sodium sulfate, and evaporated to dryness in vacuo.

The product obtained was developed with a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) using a column filled with microcrystalline cellulose (Avicel, trade mark) using the solvent mixture having the same composition as above to select the fractions showing the antimicrobial activity to Proteus mirabilis. The fractions thus selected were spotted onto a thin layer plate of Avicel SF and developed using a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:2 by volume ratio) and a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) respectively to collect the fractions showing the ultraviolet absorption to Manasulu Light 2536 Å (made by Manasulu Kagaku Kogyo K. K.) and showing Rf 0.81 and Rf 0.65 respectively. The fractions were concentrated and lyophilized to provide 40 mg. of pure 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (1,3,4-thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid. This material showed an antimicrobial activity to Proteus mirabilis, Salmonella gellinarum, and Escherichia coli.

Example 18.

a). Preparation of 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3-(1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

A culture medium containing 1% starch, 1% glucose, 1.5% soybean flour, 0.5% yeast extract, 0.1% dipotassium hydrogen phosphate, 0.05% magnesium sulfate, and 0.3% sodium chloride was placed in 500 ml. Sakaguchi flasks at 100 ml. each sterilized for 20 minutes at 120° C. Then, each culture medium was inoculated with the Streptomyces oganonensis Y—G19Z followed by cultivation for 48 hours at 30° C. Another aforesaid culture medium was placed in 2 liter Sakaguchi flasks at 400 ml. each and sterilized for 20 minutes at 120° C. Each culture medium was inoculated by the culture broth prepared in the above procedure and cultivated for 24 hours at 30° C. to provide a seed culture.

Separately, 60 liters of a culture medium containing 7% starch, 2% gluten meal, 2% soybean flour, 0.8% glycerol, 0.1% Casamino acid, 0.01% ferric sulfate, and 55 g. of sodium hydroxide was placed in two 100 liter fermentors together with 10 ml. of Adecanol (trade mark) as a defoaming agent and sterilized for 30 minutes at 120° C. Each culture medium was then inoculated by 800 ml. of the seed culture and cultivated for 24 hours at 30° C. Then, a solution of 2 - mercapto - 1,3,4 - thiadiazole prepared in aqueous sodium hydroxide solution and sterilizing at high pressure was added to each fermentor so that the content became 0.05% of the culture broth and then the cultivation was further carried out for 90 hours.

After the cultivation was completed, the cultured broth was adjusted to pH 2.0 and mixed with Radiolite (trade mark) with stirring. The mixture was filtrated using a filter press and the filtrates were combined to provide about 100 liters of the filtrate mixture.

The filtrate was adjusted to pH 3.0 by adding an aqueous sodium hydroxide solution, charged into a 12 liter Amberlite XAD—2 (trade mark) column, and the column was washed with 30 liters of water, and eluted with 30 liters of an aqueous 50% acetone. The eluate was concentrated up to 5.5 liters, adjusted to pH 3.5 with a diluted aqueous hydrochloric acid solution and charged into a 3 liter Amberlite IRA—68 (Cl-type) (trade mark). The column was washed with 6 liters of water and eluted with an aqueous solution (pH 7.2) containing 1 M sodium nitrite and 0.1 M sodium acetate to provide 5 liters of a solution containing an antimicrobially active material. The solution was adjusted to pH 3.0, charged into 1 liter of Amberlite XAD—2 (trade mark) column, and the column was washed with water, and eluted with an aqueous 50% acetone to provide about 400 ml. of an aqueous solution containing the antimicrobially active material, which was lyophilized.

The product obtained was subjected to a column chromatography with a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) using microcrystalline cellulose (Avicel, trade mark) filled in the column by using the solvent mixture having

the same composition as above to select antimicrobially active fractions. The fractions were spotted onto a thin layer plate of Avicel SF (trade mark), developed with a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9), and a pyridine solution of 0.25% ninhydrin was sprayed to cause coloring under heating. Then, the fractions showing Rf 0.39 was collected, evaporated to dryness under reduced pressure at 45—50° C., and then subjected to a column chromatography of microcrystalline cellulose (Avicel) prepared by a solvent mixture of acetonitrile:water (7:3 by volume ratio). Then, the antimicrobially active fractions thus selected was subjected to a thin layer chromatography of Avicel SF by the manner as described above to collect the fractions showing Rf 0.39. The fractions were evaporated to dryness to provide 0.92 g. of a crude powder. The powder was dissolved in a small amount of distilled water and subjected to a column chromatography with distilled water using Amberlite CG—50 (H-type) to select antimicrobially active fractions. The fractions were then concentrated and lyophilized. The product was further dissolved in a small amount of distilled water and developed on a column of Sephadex G—10 (trade mark) using distilled water. The antimicrobial activity of each fraction was checked and the effective fractions were subjected to a thin layer chromatography by the manner as stated above using a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) to collect the fractions showing Rf 0.38. The fractions were concentrated and lyophilized to provide 75 mg. of white 7 - (5 - amino - 5 - carboxyvaleramido) - 7-methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl) - thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

b). In 10 ml. of a 0.1 M pyrophosphate buffer solution of pH 8.1 containing 0.026% sodium azide was dissolved 50 mg. of 7 - (5 - amino - 5 - carboxyvaleramido)-7 - methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid and after adding to the solution 0.5 ml. of the activated mycelium suspension prepared using the Trigonopsis variabilis as in Example 8a, the mixture was stirred under aeration in a water bath at 33° C. to perform the D-amino acid oxidation. The completion of the reaction was determined by the same high speed liquid chromatography as in Example 8b. The retention time of the starting material, 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol-2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid was 1 minute 56 seconds and that of 7 - (4 - carboxybutyramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl)-thiomethyl - Δ^3 - cephem - 4 - carboxylic acid formed by the D-amino acid oxidation was 5 minutes 28 seconds.

After the reaction was over, the mycelium was removed at 4° C. and the supernatant was recovered, adjusted to pH 1.5—2.0 with a diluted aqueous hydrochloric acid solution and extracted four times each with equal volume of ethyl acetate. The ethyl acetate fractions were combined and re-extracted with phosphate buffer solution of pH 6.0. The phosphate solution was then adjusted to pH 1.5—2.0 and extracted again four times each with equal volume of ethyl acetate. The ethyl acetate extracts were combined dehydrated over anhydrous sodium sulfate, and evaporated to dryness in vacuo. The product was developed with a solvent mixture of n-butanol:acetic acid: water (4:1:2 by volume ratio) using a column filled with microcrystalline cellulose (Avicel, trade mark) by using the solvent mixture having the same composition as above to select the fractions showing an antimicrobial activity to Proteus mirabilis. The fractions were spotted onto a thin layer plate of Avicel SF and developed with a solvent mixture of isopropanol:n-butanol:acetic acid:water (21:3:7:9 by volume ratio) and a solvent mixture of n-butanol:acetic acid:water (4:1:2 by volume ratio) respectively to collect the fractions showing the ultraviolet absorption to Manasulu Light 2536 Å (made by Manasulu Kagaku Kogyo K. K.). The fractions were concentrated and lyophilized to provide 40 mg. of pure 7 - (4 - carboxybutyramido)-7 - methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid. This material showed an antimicrobial activity against Proteus mirabilis, Salmonella gallinarum, and Escherichia coli.

Example 19.

Dry filled capsule containing 120 mg. of 7 - (5 - amino - 5 - carboxyvaleramido)-7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4-carboxylic acid.

	Per capsule
7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl-1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid	120 mg
Lactose	20 mg

Magnesium stearate	5 mg
Capsule No. 3	145 mg.

The 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl-1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid is reduced to a No. 60 powder and then lactose and magnesium stearate are passed through a No. 60 bolting cloth onto the powder and the combined ingredients admixed for ten minutes and then filled into No. 3 dry gelatin capsules.

Example 20.

Tablet containing 150 mg. of 7 - (5 - amino - 5 - carboxyvaleramido) - 7-methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4-carboxylic acid.

7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl-1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid	150 mg
Dicalcium phosphate J.P.	115 mg.
Magnesium stearate	3 mg.
Lactose J.P.	39 mg.

The active component is blended with the dicalcium phosphate and lactose. The mixture is granulated with 15% corn-stearch paste (4 mg.) and rough-screened. It is dried at 40° C. and screened again through a No. 16 screen. The magnesium stearate is added and the mixture is compressed into tablets approximately 0.3 inch in diameter.

Example 21.

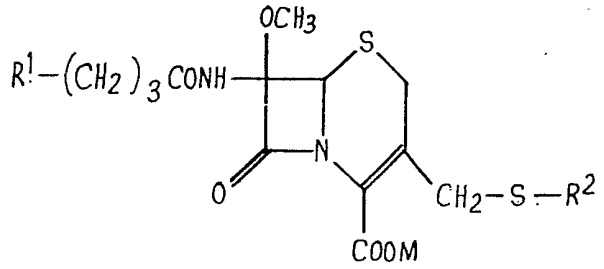
Parenteral solution containing 500 mg. of 7 - (5 - amino - 5 - carboxyvaleramido)-7 - methoxy - 3 - (1 - methyl - 1H - tetrazol - 5 - yl) - thiomethyl - Δ^3 - cephem - 4-carboxylic acid.

	Per ampoule
7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxy - 3 - (1 - methyl-1H - tetrazol - 5 - yl)thiomethyl - Δ^3 - cephem - 4 - carboxylic acid	500 mg

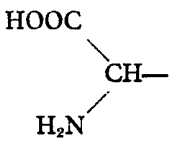
The active compound (50.0 g.) is added in 150 ml. of sterile water for injection and the obtained solution was adjusted to pH 8.0 by adding dilute sodium hydroxide and the volume of the solution was adjusted to 200 ml. The solution was divided into 100 ampoules, lyophilized and sealed.

WHAT WE CLAIM IS:—

1. A 7 - methoxy - 3 - heterocyclic thiomethylcephalosporin derivative represented by the formula



wherein R^1 represents

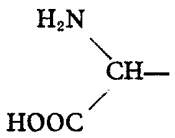


or HOOC—,

R^2 represents a nitrogen-containing heterocyclic group, and

M represents a hydrogen atom or a cationic residue forming a salt.

2. A derivative according to claim 1 wherein R^1 represents



3. A derivative according to claim 1 wherein R^1 represents HOOC—.

4. A derivative according to claim 1, 2 or 3 wherein R^2 is a 5 - carboxymethylthio - 1,3,4 - thiadiazol - 2 - yl, a 1 - methyl - 1H - tetrazol - 5 - yl, a 5 - methyl-1,3,4 - thiadiazol - 2 - yl or a 1,3,4 - thiadiazol - 2 - yl group.

5. A derivative according to any one of claims 1 to 4 wherein M is hydrogen.

6. A derivative according to any one of claims 1 to 4 wherein M is an inorganic or organic residue.

7. A derivative according to claim 6 wherein M is an alkali, alkaline earth or heavy metal.

8. A derivative according to claim 6 wherein M is a quaternary or amine salt.

9. 7 - (5 - Amino - 5 - carboxyvaleramido) - 3 - (5 - carboxymethylthio-1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

10. 7 - (5 - Amino - 5 - carboxyvaleramido) - 3 - (1 - methyl - 1H - tetrazol)-5 - yl) - thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

11. 7 - (5 - Amino - 5 - carboxyvaleramido) - 3 - (5 - methyl - 1,3,4 - thiadiazol-2 - yl)thiomethyl - 7 - methoxy - Δ^3 -cephem - 4 - carboxylic acid.

12. 7 - (5 - Amino - 5 - carboxyvaleramido) - 3 - (1,3,4 - thiadiazol - 2 - yl)-thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

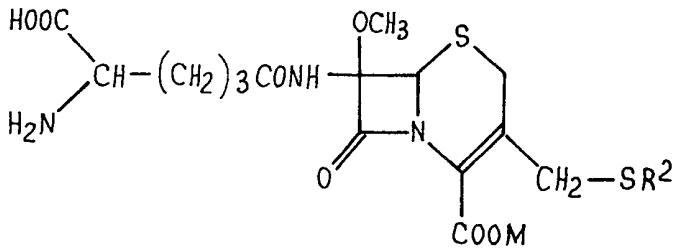
13. 7 - (4 - Carboxybutyramido) - 3 - (5 - carboxymethylthio) - 1,3,4 - thiadiazol - 2 - yl)thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

14. 7 - (4 - Carboxybutyramido) - 3 - (1 - methyl - 1H - tetrazol - 5 - yl)-thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

15. 7 - (4 - Carboxybutyramido) - 3 - (5 - methyl - 1,3,4 - thiadiazol - 2 - yl)-thiomethyl - 7 - methoxy - Δ^3 - cephem - 4 - carboxylic acid.

16. 7 - (4 - Carboxybutyramido) - 7 - methoxy - 3 - (1,3,4 - thiadiazol - 2 - yl)-thiomethyl - Δ^3 - cephem - 4 - carboxylic acid.

17. A process of producing a 7 - methoxycephalosporin derivative represented by the formula



wherein R^2 and M have the same meaning as in claim 1, which comprises cultivating a 7 - (5 - amino - 5 - carboxyvaleramido) - 7 - methoxycephalosporin - producing microorganism belonging to the genus *Streptomyces* in a culture medium with a heterocyclic thiol represented by the formula

R^2SH

wherein R^2 has the same meaning as above, or with a salt of the heterocyclic thiol, or with a compound capable of being converted into said heterocyclic thiol or its salt during the cultivation.

18. A process according to claim 17 wherein the microorganism is *Streptomyces* oganonensis Y—G19Z.

19. A process according to claim 17 wherein the microorganism is *Streptomyces griseus, Streptomyces viridochromogenes, Streptomyces fimbriatus, Streptomyces halstedii, Streptomyces rochei, Streptomyces cinnamonensis, Streptomyces chartreusis, Streptomyces lactamdurans, Streptomyces lipmanii, Streptomyces clavuligerus, Strep-*

tomyces wadayamensis, Streptomyces jumonjinensis, Streptomyces heteromorphus and *Streptomyces panayensis* or *Streptomyces chartreusis* SF—1623.

20. A process according to claim 17, 18 or 19 wherein the heterocyclic thiol added to the culture medium is optionally substituted pyrrolethiol, imidazolethiol, dihydroimidazolethiol, pyrazolethiol, triazolethiol, tetrazolethiol, methyltetrazolethiol, pyridinethiol, diazinethiol, thiophenethiol, thiazolethiol, dihydrothiazolethiol, thiadiazolethiol, thiatriazolethiol, furanthiol, pyranthiol, oxazolethiol, isoxazolethiol, oxadiazolethiol, indolethiol, benzimidazolethiol, benzoxazolethiol, benzothiazolethiol, triazolopyridinethiol, thianthrenethiol or purinethiol.

21. A process according to claim 20 wherein the heterocyclic thiol is substituted with one or more halogen atoms or amino, nitro, alkyl, hydroxy, alkoxy, aryl, aralkyl, furyl, thienyl, oxazolyl, carboxy, carboxymethyl, carboxyalkylthio or carboxyalkyloxy groups.

22. A process according to any one of claims 17 to 21 wherein the heterocyclic thiol is used as its salt.

23. A process according to claim 22 wherein the salt is an alkali metal, alkaline earth metal or ammonium salt.

24. A process according to claim 22 wherein the salt is formed with triethylamine, triethanolamine, dicyclohexylamine, lysine, arginine, histidine, a basic water-soluble antibiotic kanamycin, an alkaloid or a basic protein.

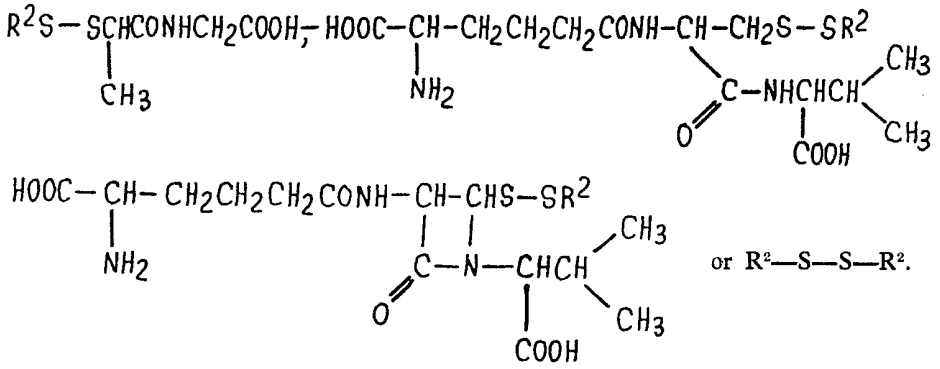
25. A process according to any one of claims 17 to 21 wherein the heterocyclic thiol is used as a compound which can be converted into the thiol during cultivation.

26. A process according to claim 25 wherein the convertible compound comprises the thiol connected to an organic or inorganic SH compound by an S—S bond.

27. A process according to claim 26 wherein the convertible compound is

$R^2S—SCH_3$, $R^2S—SC_2H_5$, $R^2S—SCH_2CH_2OH$, $R^2S—SCH_2\underset{\underset{NH_2}{|}}{C}HCOOH$, $R^2S—SG$

(wherein G represents a glutathione residue), $R^2S—SCH_2CH_2NH_2$, $R^2S—SCoA$ (wherein CoA represents coenzyme A residue),



or $R^2—S—S—R^2$.

28. A process according to claim 25 wherein the microorganism can reduce sulphuric acid derivatives and the convertible compound is $R^2—SO_3H$, $R^2—SO_2H$, $R^2—SOH$ or a salt thereof.

29. A process according to any one of claims 17 to 28 wherein the heterocyclic thiol or salt or derivative thereof is added at a concentration of 0.1 to 5 mg./ml. calculated as the heterocyclic thiol.

30. A process according to any one of claims 17 to 29 wherein M is hydrogen.

31. A process according to any one of claims 17 to 29 wherein M is a cationic residue.

32. A process according to claim 30 or 31 wherein R^2 is a 5 - carboxymethylthio-1,3,4 - thiadiazol - 2 - yl group.

33. A process according to claim 30 or 31 wherein R^2 is a 1 - methyl - 1H-tetrazol - 5 - yl group.

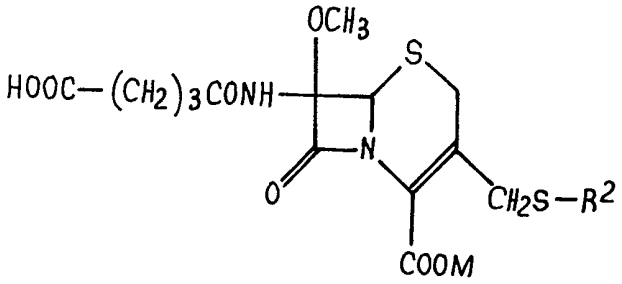
34. A process according to claim 30 or 31 wherein R^2 is a 5 - methyl - 1,3,4-thiadiazol - 2 - yl group.

35. A process according to claim 30 or 31 wherein R^2 is a 1,3,4 - thiadiazol-2 - yl group.

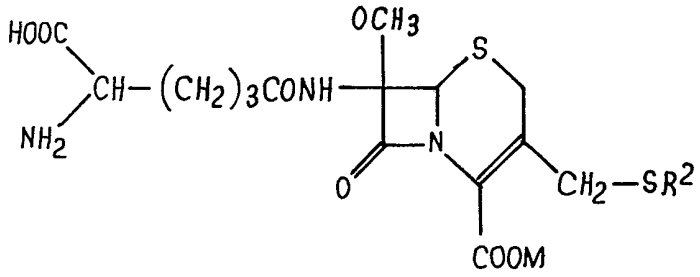
36. A process according to any one of claims 17 to 35 wherein the derivative is at least partially isolated from the culture medium.

37. A process according to claim 36 wherein the derivative is isolated and purified.

38. A process of producing a 7 - (4 - carboxybutyramido) - 7 - methoxy-cephalosporin - derivative represented by the formula



wherein R^2 and M have the same meaning as in claim 1, which comprises aerobically treating a compound



with a D-amino acid oxidase of the medium of a microorganism belonging to the genus *Trigonopsis*.

39. A process according to claim 38 wherein the microorganism is *Trigonopsis variabilis*.

40. A process according to claim 38 or 39 wherein the microorganism is first cultivated and the mycelium or treated mycelium thus obtained is used as the oxidase source.

41. A process according to any one of claims 38 to 40 wherein a cell-free extract of the mycelium is used as the oxidase source.

42. A process according to claim 41 wherein partially or completely purified D-amino acid oxidase is used.

43. A process according to claim 42 wherein the oxidase is immobilized on a water-insoluble polymer or an inorganic carrier to provide an activatable oxidase.

44. A process according to claim 43 wherein the oxidase is subjected to an activation treatment prior to the aerobic treatment.

45. A process according to any one of claims 38 to 44 wherein catalase activity in the mycelium is inhibited.

46. A process according to claims 44 and 45 wherein the mycelium is subjected to simultaneous heat treatment to inhibit catalase activity and activation to enhance oxidase activity.

47. A process according to any one of claims 38 to 46 wherein M is hydrogen.

48. A process according to any one of claims 38 to 46 wherein M is a cationic residue.

49. A process according to claim 47 or 48 wherein R^2 is a 5 - carboxymethylthio-1,3,4 - thiadiazol - 2 - yl group.

50. A process according to claim 47 or 48 wherein R^2 is a 1-methyl-1H-tetrazol-5 - yl group.

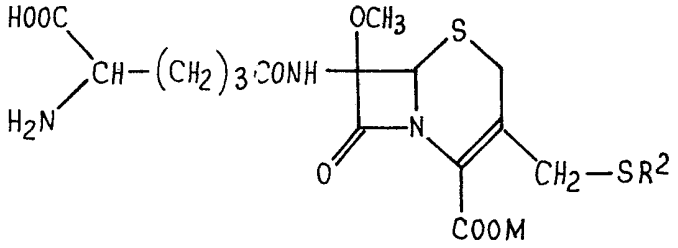
51. A process according to claim 47 or 48 wherein R^2 is a 5 - methyl - 1,3,4-thiadiazol - 2 - yl group.

52. A process according to claim 47 or 48 wherein R^2 is a 1,3,4 - thiadiazol-2 - yl group.

53. A process according to any one of claims 38 to 52 wherein the derivative is at least partially isolated from the culture medium.

54. A process according to claim 53 wherein the derivative is isolated and purified.

55. A process according to any one of claims 38 to 54 wherein the compound represented by the formula



is the product from a process according to any one of claims 17 to 37.

56. A process for producing a 7 - methoxycephalosporin derivative as defined in claim 1 conducted substantially as described in any one of the Examples.

57. A process according to claim 17, 38 or 55 substantially as hereinbefore described.

58. A 7 - methoxycephalosporin derivative as defined in claim 1 produced by a process according to any one of claims 17 to 57.

59. A pharmaceutical composition containing a 7 - methoxycephalosporin derivative according to any one of claims 1 to 16 or 58 together with a carrier or diluent.

60. A composition according to claim 59 in the form of capsules, tablets, powders, granules, a solution, or a suspension.

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Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1980.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

Fig. 1

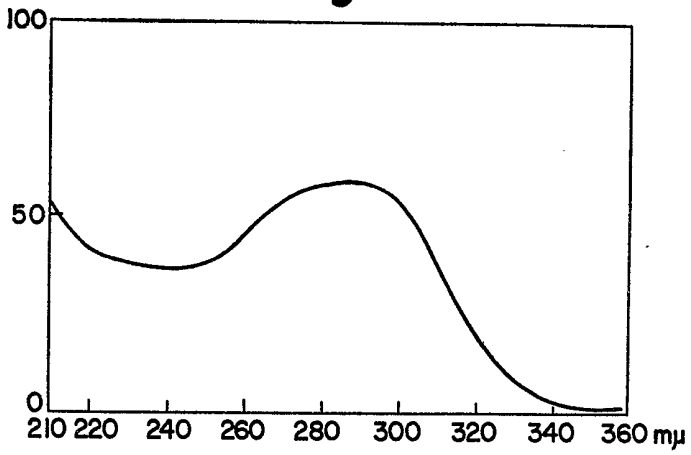
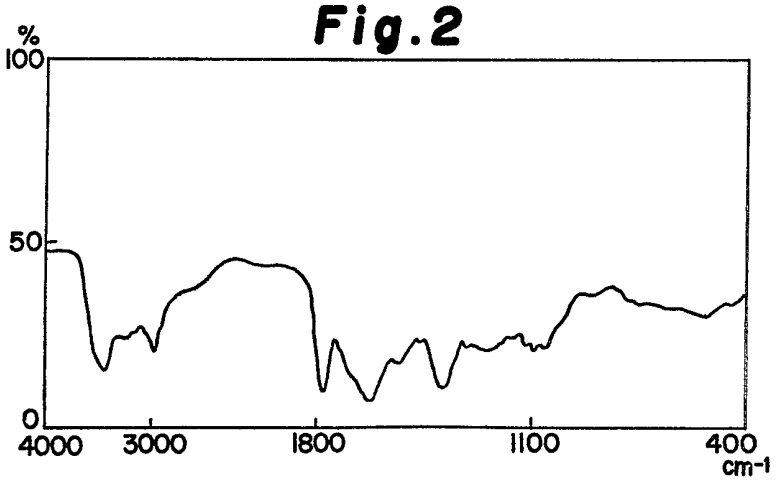

100
50
0
210 220 240 260 280 300 320 340 360 mμ

Fig. 2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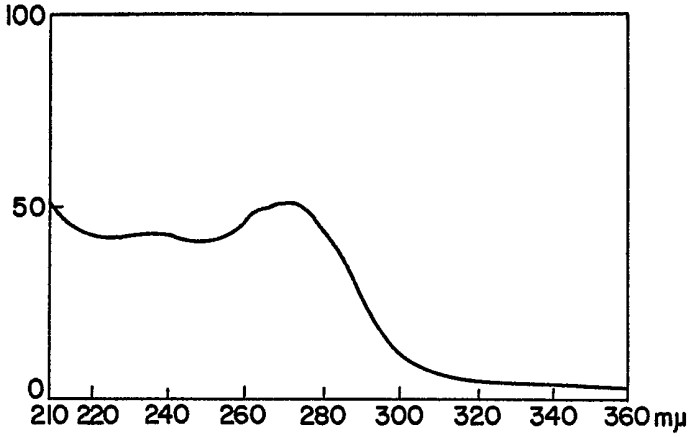
50
0
4000 3000 1800 1100 400
cm-1

Fig.3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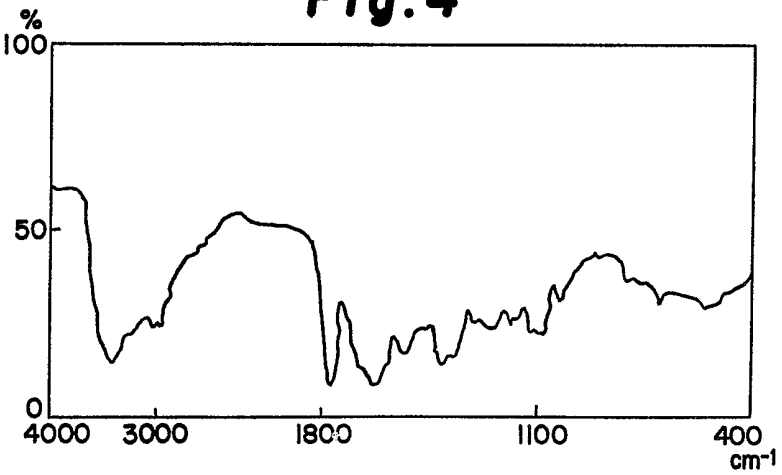
0
210 220 240 260 280 300 320 340 360 mμ

Fig.4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50
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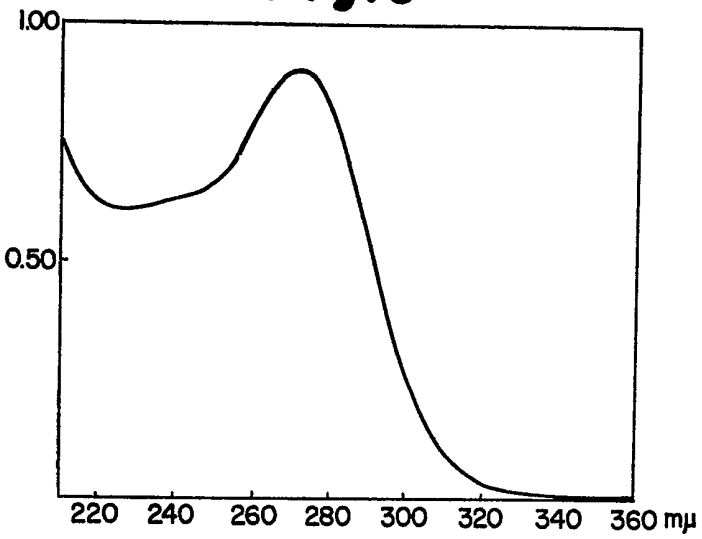
4000 3000 1800 1100 400
cm-1

Fig.5



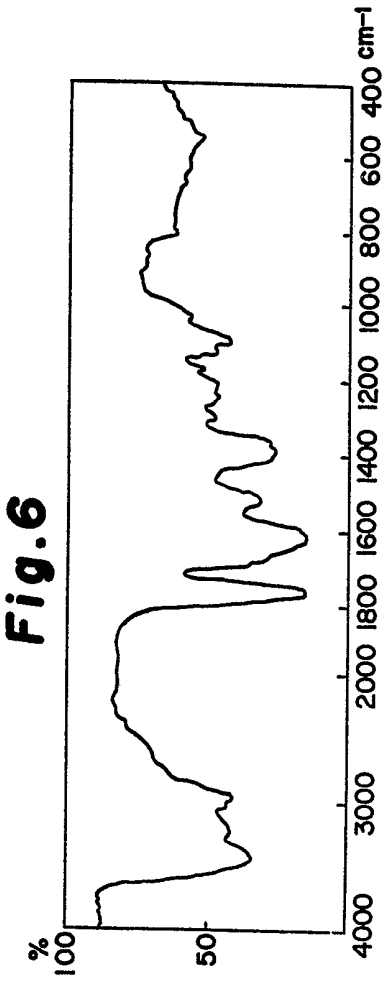
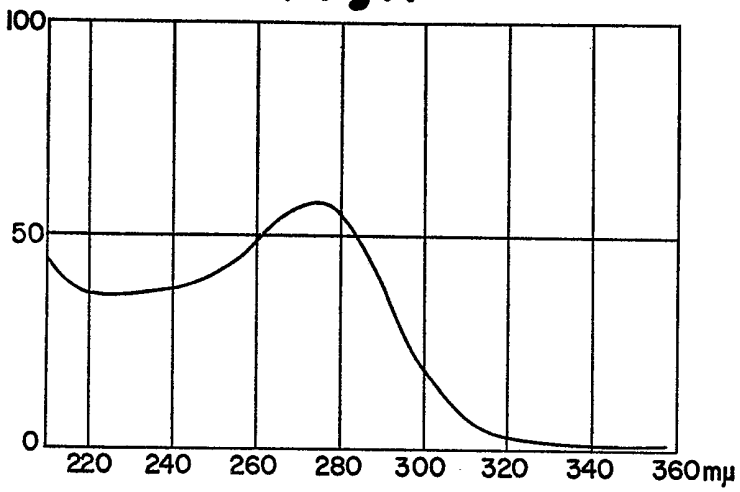


Fig.6

Fig.7



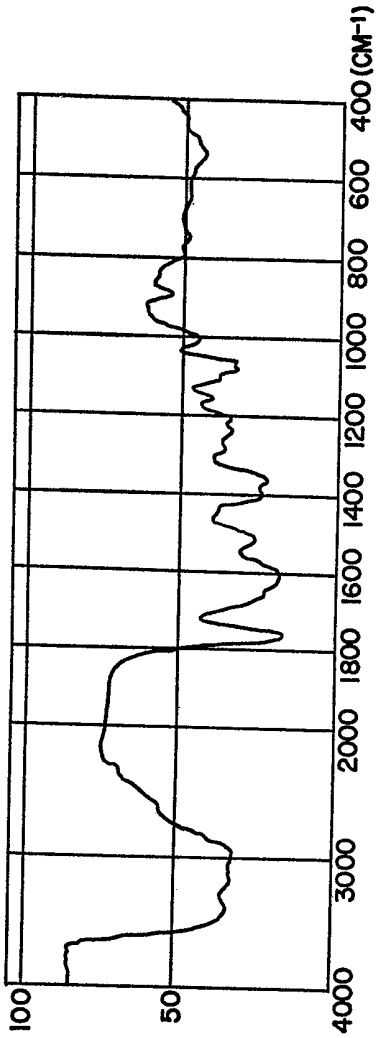
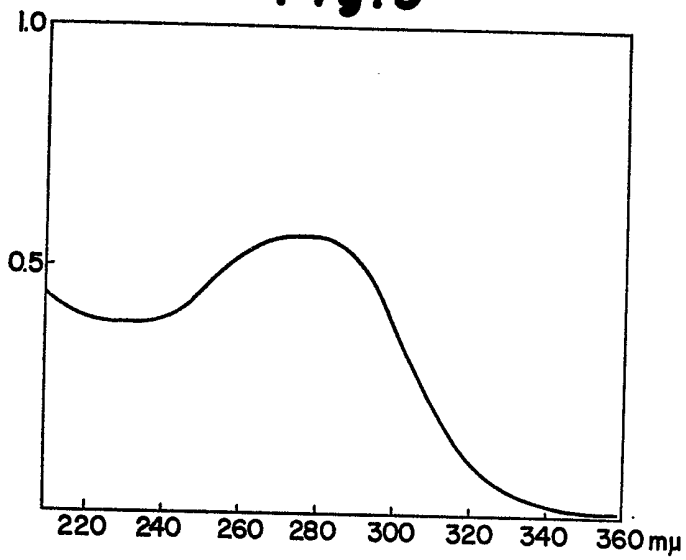
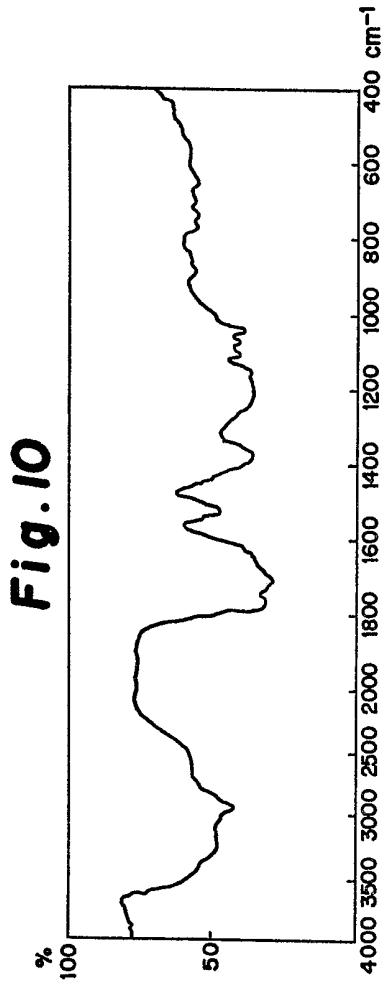


Fig.8

Fig.9




%
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4000 3500 3000 2500 2000 1800 1600 1400 1200 1000 800 600 400 cm⁻¹

Fig. 10

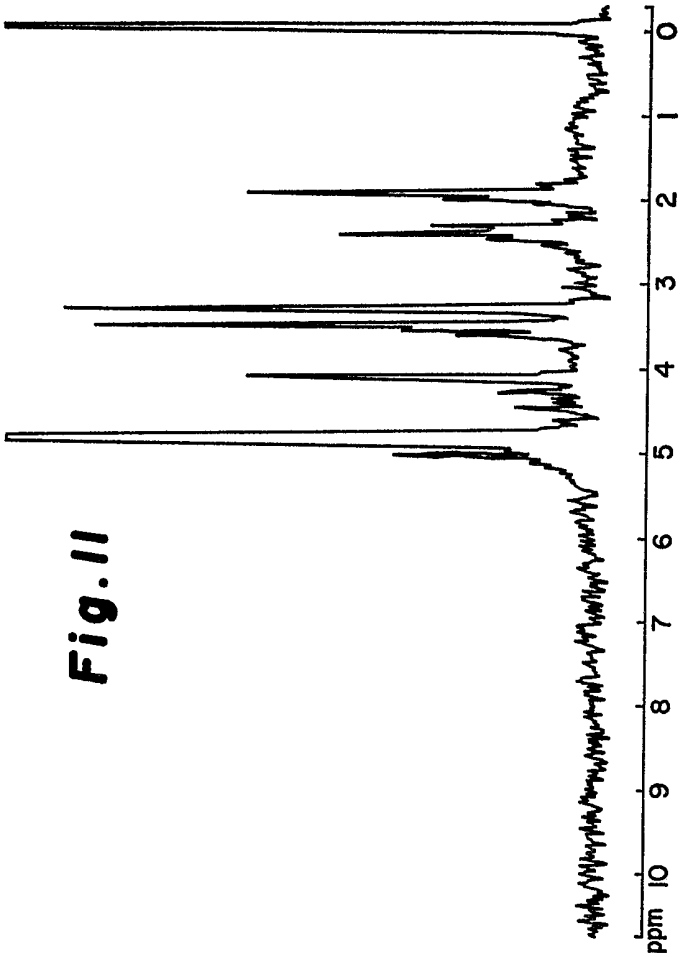
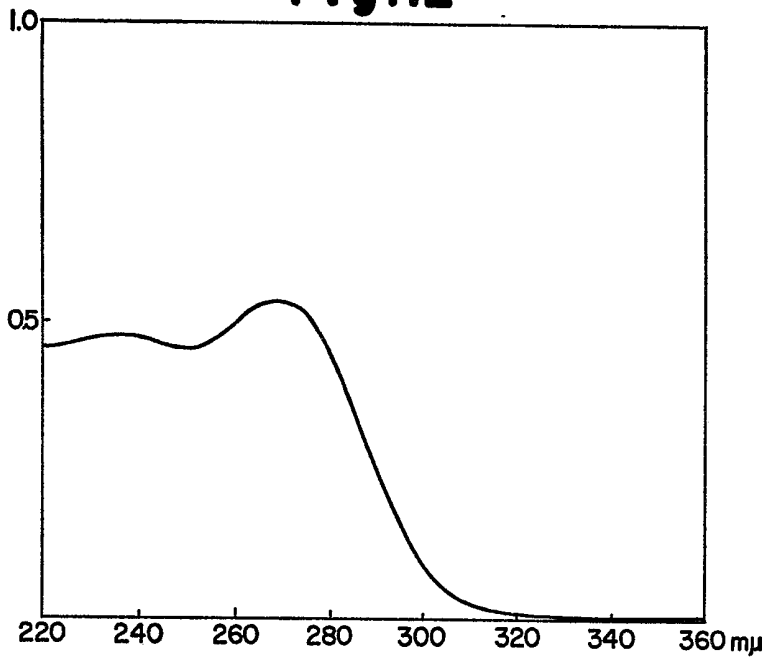


Fig. 11

Fig.12



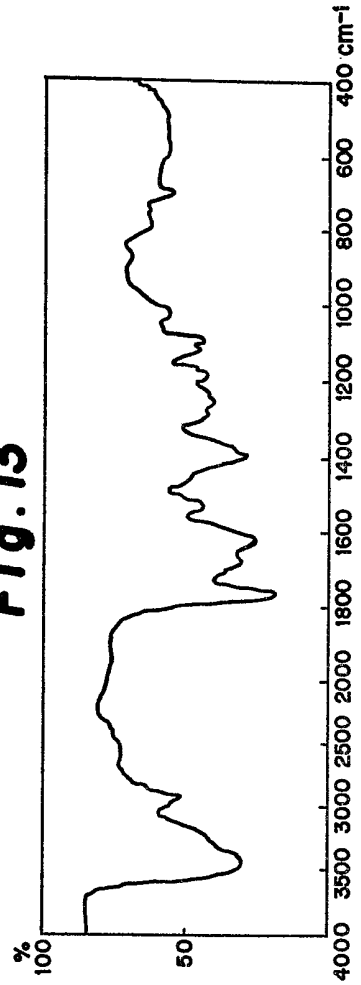

%
100
50
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400 cm-1

Fig. 13

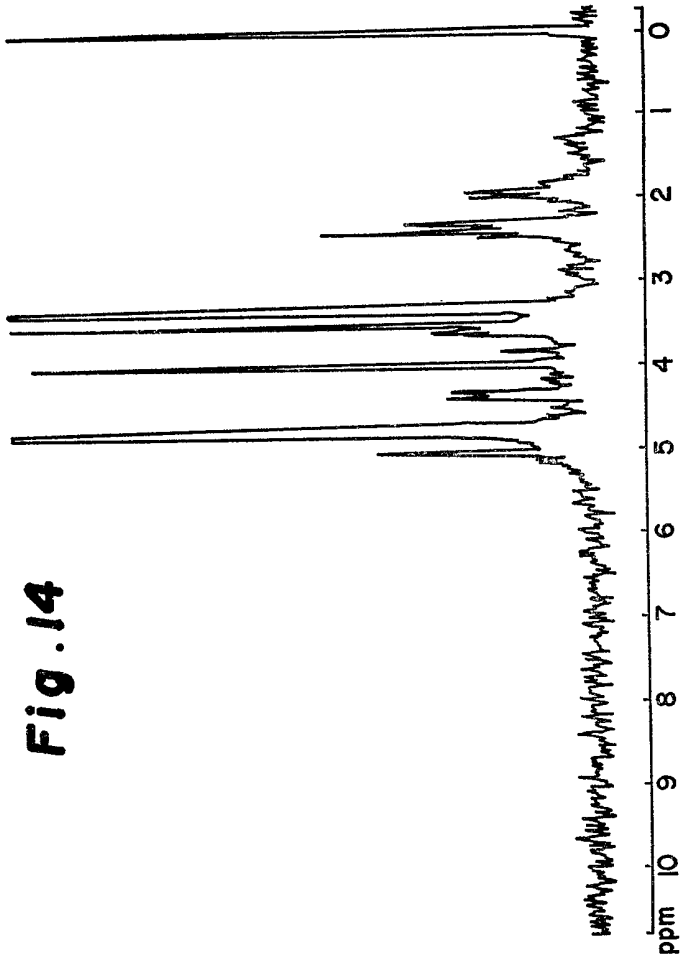
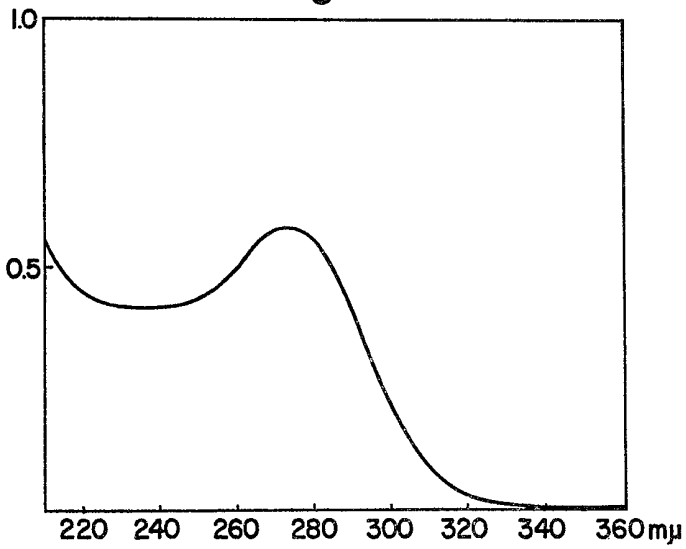
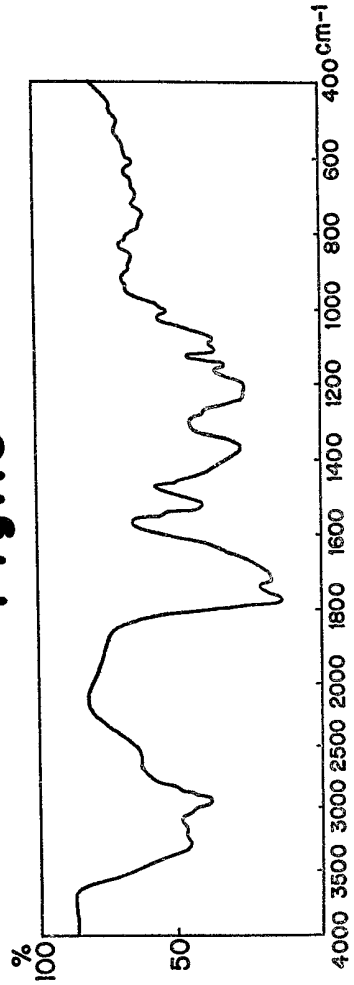


Fig. 14

Fig.15




%
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4000 3500 3000 2500 2000 1800 1600 1400 1200 1000 800 600 400 cm-1

Fig.16

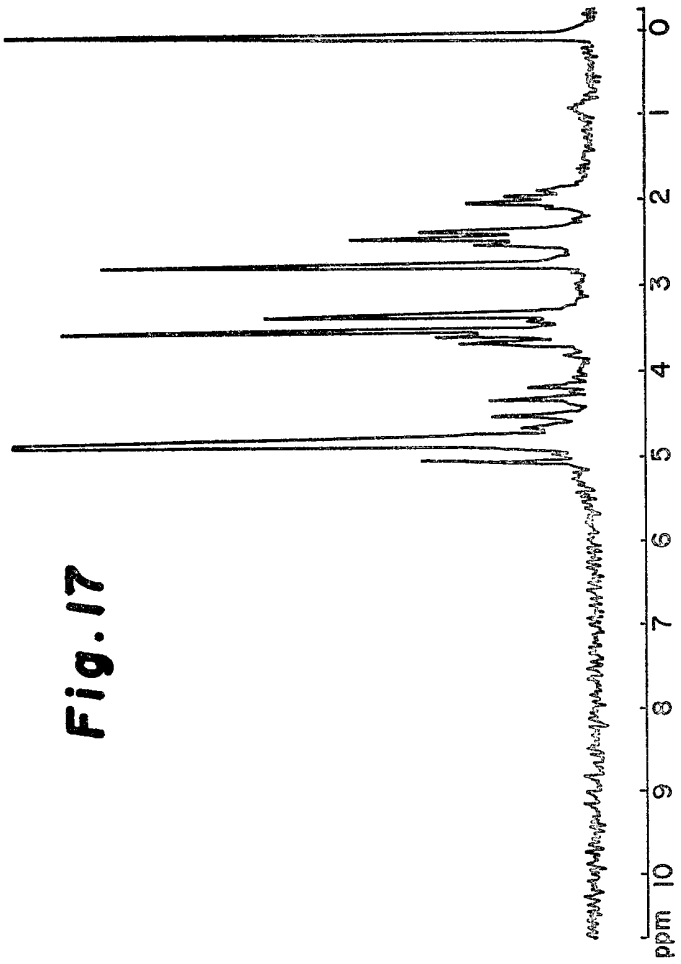
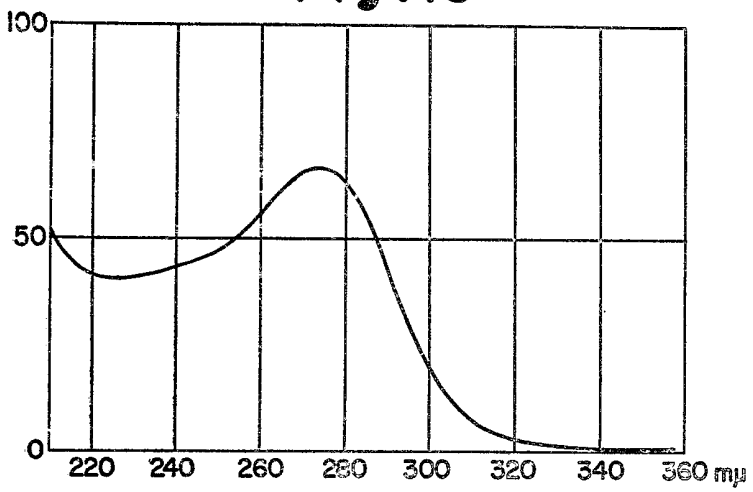


Fig. 17

Fig. 18



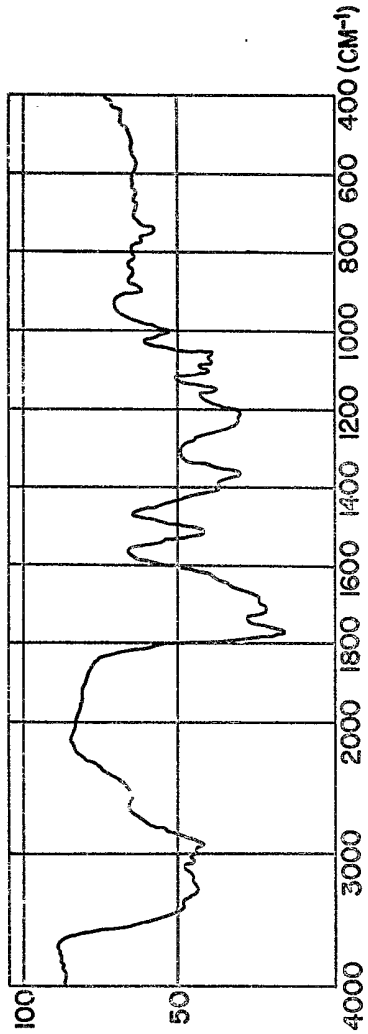


Fig.19

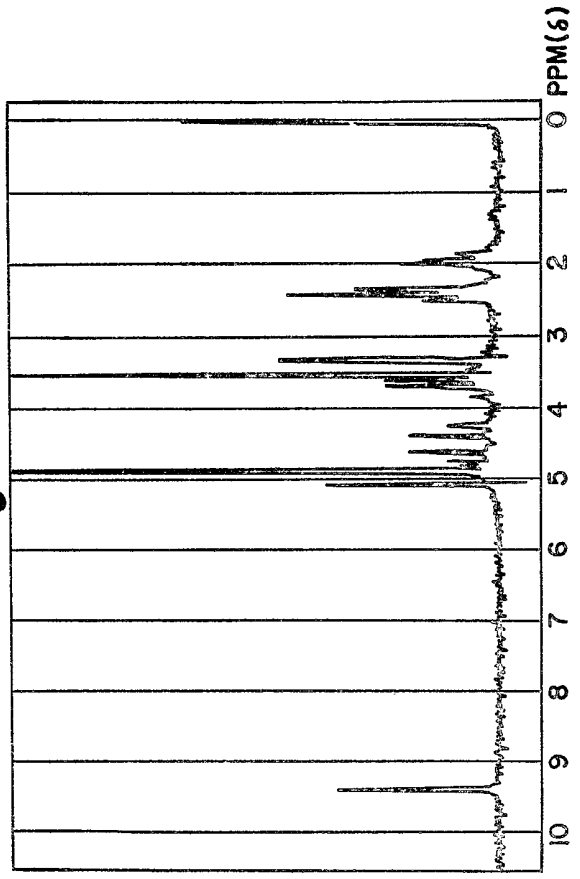


Fig.20