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(54) DESACETYL ANTIBIOTICS 890A$_1$ AND 890A$_3$

(71) We, MERCK & CO. INC., a corporation duly organized and existing under the laws of the State of New Jersey, United States of America, of Rahway, New Jersey, United States of America, 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 discovery of the remarkable antibiotic properties of penicillin stimulated great interest in this field which has resulted in the finding of many other valuable antibiotic substances such as other penicillins, cephalosporins, streptomycin, bacitracin, tetracyclines, chloramphenicol and erythromycins. In general, each of these antibiotics do not act against certain clinically important pathogenic bacteria. For example, some are principally active against only gram-positive types of bacteria. Furthermore, acquired resistance over the course of widespread use of existing antibiotics in the treatment of bacterial infection has caused a serious resistance problem to arise.

Accordingly, the deficiencies of the known antibiotics have stimulated further research to find other antibiotics which will be active against a wider range of pathogens as well as resistant strains of particular microorganisms.

This invention provides antibiotic substances herein called desacetyl 890A$_1$ and desacetyl 890A$_3$. The invention encompasses the antibiotics in dilute forms, as crude concentrates and in pure forms.

In accordance with the present invention, the antibiotic substances of the present invention are produced by hydrolysing the N-acetyl group of 890A$_1$ and 890A$_3$ using an amidohydrolase capable of hydrolysing the N-acetyl group. A convenient source of an amidohydrolase with this capability is amidohydrolase-producing strains of the microorganism *Protaminobacter ruber*. The particular enzyme produced by *Protaminobacter ruber* is N-acetylthienamycin amidohydrolase, a member of the sub-group of enzymes designated E.C. 3.5.1 according to the recommended enzyme nomenclature of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. The present invention also provides a process for producing thienamycin that comprises intimately contacting N-acetyl thienamycin with the enzyme N-acetylthienamycin amidohydrolase produced by an amidohydrolase-producing strain of the microorganism *Protaminobacter ruber*. It should be noted that thienamycin and its preparation by fermentation of *Streptomyces cattleya* are claimed in the specification of our copending application No. 48208/75 (1498087).

The microorganism capable of carrying out the deacetylation process was isolated from a soil sample and, based upon taxonomic studies, was identified as belonging to the species *Protaminobacter ruber* and has been designated MB-3528 in the culture collection of Merck & Co., Inc., Rahway, N.J. A culture thereof has been placed on unrestricted permanent deposit with the culture collection of the Northern Regional Research Laboratories, Northern Utilization Research and Development Division, Agricultural Research Service, U.S.Department of Agriculture, Peoria, Ill. and has been assigned accession No. NRRL B-8143.

The morphological and cultural

characteristics of *Protaminobacter ruber* NRRL B-8143 as well as carbon and nitrogen utilization and biochemical reactions are as follows:—

Morphology

Cells are rod-shaped with rounded ends, 0.9—1.2×2.3—4.6 microns, occurring singly or in pairs. Twenty-four and forty-eight hour cells stain Gram-negative with a granular appearance. The granules, especially the polar granules, stain black with Sudan Black B. Cells are motile at 28°C., but motility is questionable at 37°C.

Cultural Characteristics

Nutrient agar colonies are at first thin, punctiform, semi-transparent and colorless; then becoming low convex, opaque, smooth, edge entire, somewhat dry in consistency and pigmented rose to rose-red.

Nutrient broth cultures are uniformly turbid with no pellicle.

Pigment production is not dependent on light or temperatures tested (28°C. and 37°C.). Pigment is soluble in acetone but insoluble in water or chloroform.

Growth on nutrient agar and brain-heart infusion agar under aerobic conditions is somewhat slow but good at 28°C; growth is moderate to good but slower at 37°C; there is no growth at 50°C.

Utilization of Carbon and Nitrogen Sources

Using a basal salts medium with ammonium sulfate as nitrogen source, growth is good with arabinose, moderate with xylose, and poor with dextrose, fructose, mannose, rhamnose, lactose, maltose, sucrose, raffinose, cellulose, inositol and mannitol.

N-acetylethanolamine can be utilized as the sole carbon and nitrogen source.

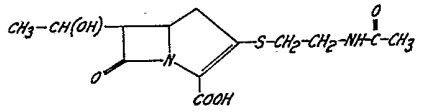
No acid or gas is produced from dextrose or lactose in OF Basal Medium (Difco Laboratories, Detroit, Michigan) under aerobic or anaerobic conditions.

Biochemical Reactions

The biochemical reactions are based on standard methods as described in *Manual of Microbiological Methods* edited by the Society of American Bacteriologists, McGraw-Hill Book Co., New York, 1957.

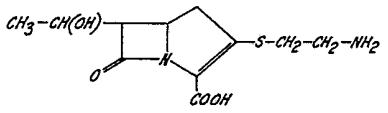
Catalase—positive
Oxidase—negative
Starch not hydrolysed
Casein not hydrolysed
Gelatin not liquefied
Litmus milk unchanged in consistency but becomes slightly alkaline after 7 days.
Indol—negative
H_2S—negative
Nitrates not reduced
Urease—positive
Lysine and ornithine decarboxylase—negative

N-Acetyl thienamycin, 890A_1 and 890A_3 are the terms applied to isomers of the antibiotic having the structure:



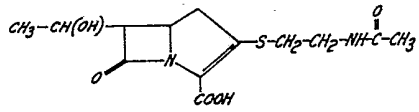
N-Acetyl thienamycin and its production are described and claimed in the specification of our copending application No. 48232/76 (Serial No. 1561107); 890A_1 and 890A_3 and their production are described and claimed in the specification of our copending application No. 48235/76 (Serial No. 1561109).

The novel antibiotics of the present invention, viz. desacetyl 890A_1 and desacetyl 890A_3, are isomers of the structural formula:



and are prepared by enzymatic hydrolysis of 890A_1 and 890A_3, respectively using an amidohydrolase present in species of genus *Protaminobacter*.

The novel process of the present invention involves to the cleavage of the N-acetyl group of a compound of the formula:



by intimately contacting the said compound with an amidohydrolase capable of hydrolysing the N-acetyl group. More specifically the process of the present invention provides for the N-deacetylation of N-acetyl thienamycin, 890A_1 and 890A_3 by intimately contacting those compounds with N-acetylthienamycin amidohydrolase.

An unexpected homology between N-acetylethanolamine and N-acetylthienamycin is set forth, whereby extracts of microorganisms with the hitherto undescribed enzyme, N-acetylethanolamine amidohydrolase, are in many cases able to hydrolyse N-acetylthienamycin. Furthermore, amidohydrolases capable of hydrolysing N-acetylthienamycin are also found to be active in the conversion of antibiotics 890A_1 and 890A_3 to their novel desacetyl forms. Another novel aspect of the present invention relates to the process for obtaining such microorganisms, wherein the process comprises selecting the strains capable of using N-acetylethanolamine for

growth and then testing these microorganisms for the presence of N-acetylthienamycin amidohydrolase.

The antibiotic thienamycin, obtained by the novel N-deacetylation of N-acetyl thienamycin is a useful antibiotic. Its description, method of production and utility are set forth in the specification of our copending application No. 48208/75 (1498087).

One aspect of the present invention is the deacetylation of N-acetyl thienamycin. There are two sources of N-acetyl thienamycin. N-acetyl thienamycin is prepared by the fermentation of broth with the microorganism *Streptomyces cattleya* NRRL 8057. This microorganism also produces thienamycin, which may be chemically, N-acetylated.

Based upon extensive taxonomic studies, *Streptomyces cattleya*, isolated from a soil sample, was identified as an actinomycete and has been designated MA-4297 in the culture collection of Merck & Co., Inc., Rahway, N.J. A culture thereof has been placed on unrestricted permanent deposit with the culture collection of the Northern Regional Research Laboratories, Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill. and has been assigned accession No. NRRL 8057.

The classification keys for the genus *Streptomyces* and the culture descriptions of *Streptomyces* species found in Bergey's *Manual of Determinative Bacteriology* (7th Edition, 1957) and in *The Actinomycetes*, Vol. II (1961) by S. A. Waksan and in "Cooperative Descriptions Of Type Cultures of *Streptomyces*" by E. B. Shirling and D. Gottleib, *International Journal of Systematic Bacteriology*, *18*, 69—189 (1968), *18* 279—392 (1968), *19*, 391—512 (1969) and *22*, 265—394 (1972) were searched for a *Streptomyces* species having morphological and cultural characteristics similar to those of MA-4297. In these aforementioned classical references, no *Streptomyces* species is described to have the orchid pigmentation of the aerial mycelium, the morphological characteristics and the absence of diffusible pigment which together comprise distinctive characteristics of MA-4297. These considerations made the assignment of a new *Streptomyces* species justified and necessary.

The morphological and cultural characteristics of *Streptomyces cattleya* are set forth in the specification of our copending application No. 48232/76 (Serial No. 1561107).

Another aspect of the present invention is the novel deacetylation of $890A_1$ and $890A_3$. $890A_1$ and $890A_3$ are prepared by fermentation of broth with the microorganism *Streptomyces flavogriseus* NRRL 8139. The material $890A_1$ may also be prepared by fermentation of *Streptomyces flavogriseus* NRRL 8140.

Based upon extensive taxonomic studies the strains of microorganisms were identified as belonging to the species *Streptomyces flavogriseus* and have been designated MA-4434a and MA-4600a in the culture collection of Merck & Co., Inc., Rahway, N.J. A culture of each thereof has been placed on irrevocable permanent deposit with the culture collection of the Northern Regional Laboratories, Northern, Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill., and have been assigned accession No. NRRL 8139 and 8140, respectively.

Streptomyces flavogriseus NRRL 8139 produces both antibiotics $890A_1$ and $890A_3$ which are isolated in substantially pure form from the fermentation broth. *Streptomyces flavogriseus* NRRL 8140 produces antibiotic $890A_1$ without any detectable amount of $890A_3$.

The morphological and cultural characteristics of *Streptomyces flavogriseus* NRRL 8139 and *Streptomyces flavorgriseus* NRRL 8140 are set forth in the specification of our copending application No. 48235/76 (Serial No. 1561109).

$890A_1$ and $890A_3$ are produced during the aerobic fermentation, under controlled conditions, of suitable aqueous nutrient media inoculated with strains of the organism, *Streptomyces flavogriseus*. Aqueous media, such as those used for the production of other antibiotics, are suitable for producing $890A_1$ and $890A_3$. Such media contain sources of carbon, nitrogen and inorganic salts assimilable by the microorganism.

Desacetyl $890A_1$ and desacetyl $890A_3$, the compounds of this invention, are valuable antibiotics active against various gram-positive and gram-negative bacteria and, accordingly, find utility in human and veterinary medicine. The compounds of this invention can be used as antibacterial drugs for treating infections caused by gram-positive or gram-negative bacteria, for example against susceptible strains of *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Pseudomonas aeruginosa*. The antibacterial materials of the invention may further be utilized as additives to animal feedingstuffs, for preserving foodstuffs and as disinfectants. For example, they may be employed in aqueous compositions in concentrations ranging from 0.1 to 100 parts, preferably 1 to 10 parts, of antibiotic per million parts of

solution in order to destroy and inhibit the growth of harmful bacteria on medical and dental equipment and as bactericides in industrial applications, for example in water-based paints and in the white water of paper mills to inhibit the growth of deleterious bacteria.

The antibiotics of this invention may be used in any one of a variety of pharmaceutical preparations as the sole active ingredient or in combination either with one or more other antibiotics or with one or more pharmacologically active substances. As an example of the former, an aminocyclitol antibiotic such as gentamicin may be coadministered in order to minimize any chance that resistant organisms will emerge. As an example of the latter, diphenoxylate and atropine may be combined in dosage forms intended for the therapy of gastroenteritis. The antibiotics may be employed in capsule form or as tablets, powders or liquid solutions or as suspensions or elixirs. They may be administered orally, topically, intravenously or intramuscularly.

Tablets and capsules for oral administration may be in unit dose presentation form, and may contain conventional excipients such as binding agents, for example, syrup, acacia, gelatin, sorbitol, tragacanth, or polyvinyl-pyrrolidone; fillers, for example, lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; lubricants, for example, magnesium stearate, talc, polyethylene glycol, silica; disintegrants, for example, potato starch or acceptable wetting agents such as sodium lauryl sulphate. The tablets may be coated according to methods well known in the art. Oral liquid preparations may be in the form of aqueous or oily suspension, solution, emulsions, syrups or elixirs or may be presented as a dry product, for reconstitution with water or other suitable vehicles before use. Such liquid preparations may contain conventional additives such as suspending agents, for example, sorbitol syrup, methyl cellulose, glucose/sugar syrup, gelatin, hydroxyethyl cellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats; emulsifying agents, for example lecithin, sorbitan monooleate or acacia; nonaqueous vehicles which may include edible oils, for example, almond oil, fractionated coconut oil, oily esters, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl *p*-hydroxybenzoates or sorbic acid. Suppositories will contain conventional suppository bases, e.g. cocoa butter or other glyceride.

Compositions for injection may be presented in unit dose form in ampoules, or in multidose containers with an added preservative. The compositions may take such forms as suspensions, solutions, emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for reconstitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

The compositions may also be prepared in suitable forms for absorption through the mucous membranes of the nose and throat or bronchial tissues and may conveniently take the form of powder or liquid sprays or inhalants, lozenges or throat paints. For medication of the eyes or ears, the preparations may be presented as individual capsules, in liquid or semi-solid form, or may be used as drops. Topical applications may be formulated in hydrophobic or hydrophilic bases as ointments, creams, lotions, paints or powders.

Also, in addition to a carrier, the compositions may include other ingredients such as stabilizers, binders, antioxidants, preservatives, lubricators, suspending agents, viscosity agents or flavouring agents.

In veterinary medicine, such as in the treatment of chickens, cows, sheep or pigs, the compositions may, for example, be formulated as intramammary preparations in either long acting or quick-release bases.

The dosage to be administered depends to a large extent upon the condition of the subject being treated, the weight of the host and the type of infection, the route and frequency of administration, the parenteral route being preferred for generalized infections and the oral route for intestinal infections.

In the treatment of bacterial infections in man, the compounds of this invention are administered orally or parenterally, in accordance with conventional procedures for antibiotic administration, in an amount of from 2 to 600 mg./kg./day and preferably 5 to 100 mg./kg./day in preferably divided dosage, e.g. three or four times a day. They may be administered in dosage units containing, for example, 25, 250, 330, 400 or 1000 mg. of active ingredient with suitable physiologically acceptable carriers or excipients. The dosage units are in the form of liquid preparations such as solutions or suspensions or as solids in tablets or capsules. It will, of course, be understood that the optimum dose in any given instance will depend upon the type and severity of infection to be treated, and that smaller doses will be employed for pediatric use, all of such adjustments being within the skill of the practitioner in the field.

It is to be understood that the antibiotic thienamycin is also administered in the

manner set forth above for the antibiotics desacetyl 890A_1 and desacetyl 890A_3.

Assay Procedures for N-acetyl Thienamycin

I. Bioassay

Assays of antibacterial activity are run according to the following disc-diffusion method using either *Vibrio percolans* ATCC 8461 or *Staphylococcus aureus* ATCC 6538P as tester organism.

Plates containing *Vibrio percolans* ATCC 8461 are prepared as follows:

A lyophilized culture of *Vibrio percolans* ATCC 8461 is suspended in 15 ml. of a sterilized medium containing 8 g./l. of Difco Nutrient Broth ("Difco" is a trade mark) and 2 g./l. of yeast extract in distilled water (hereinafter designated (NBYE). The culture is incubated overnight on a rotary shaker at 28°C. This culture is used to inoculate the surface of slants containing 1.5% agar in NBYE, and the inoculated slants are incubated overnight at 28°C. and then stored in a refrigerator.

The refrigerated slants prepared from a single lyophilized culture are used for up to four weeks from their preparation, as follows: A loop of inoculum from the slant is dispersed in 50 ml. of NBYE contained in a 250 ml. Erlenmeyer flask. The culture is incubated overnight on a rotary shaker at 28°C., and is then diluted to a density giving 50% transmittance at 660 nm. 33.2 ml. of this diluted culture is added to 1 liter of NBYE containing 15 g. of agar and maintained at 46°C. The inoculated agar-containing medium is poured into 100x15 mm. plastic petri dishes, 5 ml. per dish, chilled, and maintained at 2—4°C. for up to 5 days before using.

Plates containing *Staphylococcus aureus* ATCC 6538P are prepared as follows:

An overnight growth of the assay organism, *Staphylococcus aureus* ATCC 6538P, in nutrient broth plus 0.2% yeast extract is diluted with nutrient broth plus 0.2% yeast extract to a suspension having 55% transmittance at a wavelength of 660 nm. This suspension is added to Difco nutrient agar supplemented with 2.0 g./l. Difco yeast extract at 47°C. to 48°C., to make a composition containing 33.2 ml. of the suspension per liter of agar. Five ml. of this suspension is poured into petri dishes of 85 mm. diameter, and these plates are chilled and held at 4°C. until used (5 day maximum).

Samples of antibiotic to be assayed are diluted to an appropriate concentration in phosphate buffer at pH 7. Filter paper discs, 1/4 or 1/2 inch diameter, are dipped into the test solution and placed on the surface of the assay plate. The plates are incubated at 37°C. overnight, and the zone of inhibition is measured as mm. diameter. The zone of inhibition measured in mm. determines relative potencies.

II. Hydroxylamine-extinguishable absorbance

The proportion of absorbance measured at 301 nm which can be attributed to the antibiotic content in impure samples is determined by the selective extinction of this absorbance (with concomitant inactivation of antibiotic activity) upon reaction with dilute hydroxylamine.

Samples containing antibiotic to be tested are prepared in 0.01*M* potassium phosphate buffer at pH 7 to have an initial A_{301} between 0.1 and 1.0. Freshly prepared, neutralized hydroxylamine (NH_2OH . HCl plus NaOH to a final pH of 7) is added to a final concentration of 0.01*M*, and the reaction is allowed to progress at room temperature for at least 30 minutes. The resulting A_{301} when subtracted from the initial reading (after correction for dilution by added reagent) yields the hydroxylamine-extinguishable absorbance. Solutions of pure N-acetyl thienamycin show a hydroxylamine-extinguishable absorbance of 96.0%.

Assay Procedures for Thienamycin

I. Bioassay

Assays of antibacterial activity are run according to the following disc-diffusion procedure unless otherwise indicated. The assay plates are prepared in the following manner. An overnight growth of the assay organism, *Staphylococcus aureus* ATCC 6538P, in nutrient broth plus 0.2% yeast extract is diluted with nutrient broth plus 0.2% yeast extract to a suspension having 55% transmittance at a wavelength of 660 mμ. This suspension is added to Difco nutrient agar supplemented with 2.0 g./l. Difco yeast extract, at 47°C. to 48°C., to make a composition containing 33.2 ml. of the suspension per liter of agar. Five ml. of this suspension is poured into petri dishes of 85 mm. diameter, and these plates are chilled and held at 4°C. until used (5 day maximum).

Samples of the antibiotic to be assayed are diluted to an appropriate concentration in phosphate buffer at pH 7. Filter-paper discs, 0.5-inch in diameter, are dipped into the test solution and placed on the surface of the assay plate; two discs for each sample are normally placed on one plate opposite to one another. The plates are incubated overnight at 37°C. and the zone of inhibition is measured as mm. diameter. The zone of inhibition measured in mm. determines relative potencies or, when compared with a purified reference standard such as cephalothin, the potency of antibiotic in units/ml. The unit of activity is based on

cephalothin standard solutions of 8, 4, 2 and 1 μg./ml. One unit is defined as the amount which calculates to produce the same inhibition as 1 μg. of cephalothin/ml., that zone of inhibition being between 16 and 21 mm. diameter.

II. Hydroxylamine-Extinguishable Absorbance

The proportion of absorbance measured at 297 nm which can be attributed to the antibiotic content in impure samples is determined by the selective extinction of this absorbance (with concomitant inactivation of antibiotic activity) upon reaction with dilute hydroxylamine.

Samples are prepared in 0.01*M* potassium phosphate buffer at pH 7.0 to have an initial A_{297} between 0.05 and 2.0. Freshly prepared neutral hydroxylamine ($NH_2OH.HCl$ plus NaOH to a final pH of 7) is added to a final concentration of 0.01 *M*, and reaction is allowed to progress at room temperature for at least 30 min. The resulting A_{297} when subtracted from the initial reading (after correction for dilution by added reagent) yields the hydroxylamine-extinguishable absorbance. Solutions of pure thienamycin show a hydroxylamine-extinguishable absorbance of 94.5%.

Assay procedures for $890A_1$ and $890A_3$ are set forth in the specification of our copending application No. 48235/76. (Serial No. 1561109).

In the Examples, which follow, the words 'Dowex','Selas', 'Millipore', 'Branson' and 'Sonifier' are trade marks and mesh and capsule sizes are U.S. standards. The Examples illustrate methods of preparing the compounds of the present invention.

EXAMPLE 1

Method of Isolation of N-acetylthienamycin amidohydrolase - producing Organisms

A 1% (w/v) suspension of fertile lawn soil is prepared by suspending 1 gm. of lawn soil in 100 ml. sterile phosphate-buffer-saline solution has the following composition:

Phosphate Buffer-Saline Solution

NaCl	8.8 g.
1*M* Phosphate Buffer, pH 7.5*	10 ml.
Distilled H_2O	1000 ml.

*1M Phosphate Buffer, pH 7.5

16 ml. of 1*M* KH_2PO_4 is mixed with 84 ml. 1M K_2HPO_4. The pH of the phosphate buffer is adjusted to 7.5 by adding small quantities of either 1M KH_2PO_4 or 1M K_2HPO_4.

Aliquot portions of this 1% stock soil suspension are used to prepare 10x, 100x and 1,000x dilutions.

One-ml. portions of the stock suspension or 1 ml. portions of the 10x, 100x and 1,000x dilutions are added to 2-ml. portions of sterile, 1.0% agar solutions at 48°C. The mixtures are quickly poured over the surface of sterile petri dishes of 85 mm. diameter containing 20 ml. of Medium A. Medium A has the following composition:

Medium A

K_2HPO_4		3.0 g.
K_2HPO_4	7.0 g.	7.0 g
$MgSO_4$		0.1 g.
Distilled H_2O		1000 ml.
N-Acetylethanolamine solution*		8.5 ml.

*N-acetylethanolamine Solution

N-acetylethanolamine is diluted 10x in H_2O and membrane sterilized. This solution is added after autoclaving.

For solid media: Add 20 g. agar.

The petri dishes are incubated for 18 days at 28°C. Well-isolated colonies are picked and streaked on Medium B. Medium B has the following composition:

Medium B

Tomato paste	40 g.
Ground oatmeal	15 g.
Distilled H_2O	1000 ml.

pH: adjust to 6 using NaOH

For solid media: add 20 g. agar

Individual clones are selected and grown for 2 days at 28°C. on slants of Medium B.

A portion of the growth of the slants is used to inoculate a 250-ml. Erlenmeyer flask containing 50 ml. of Medium A; a 250-ml. Erlenmeyer flask containing 50 ml. supplemented Medium B (supplemented after autoclaving with 0.4 ml. of a membrane-sterilized solution of N-acetylethanolamine diluted 10x with water); and a 250-ml. Erlenmeyer flask containing 50 ml. Medium C. Medium C has the following composition:

Medium C

Dextrose	20 g.
Pharmamedia	8 g.
Corn Steep Liquor (wet basis)	5 g.
Distilled H_2O	1000 ml.

pH: adjust to 7 with NaOH or HCl

N-acetylethanolamine solution*	8.5 ml.

*N-acetylethanolamine Solution

N-acetylethanolamine is diluted 10x in H_2O and membrane sterilized. This solution is added after autoclaving.

The flasks are shaken at 28°C. on a 220 rpm (2" throw) shaker for 4 days. A 30-ml. portion from each flask is centrifuged for 15 minutes at 8,000 rpm. The supernatant portion is removed, leaving only enough to form a thick suspension of cells and media solids. Half of the suspension is subjected to ultrasonic disruption using a Branson Instrument Model LS-75 Sonifier with a 1/2-inch probe. The input power is set at position No. 4 and four successive 15 second cycles of irradiation are used, while chilling the suspension in ice water during and between disruption. To test for the presence of N-acetyl-thienamycin amidohydrolase activity in either the whole cell preparation or the sonicate, aliquot portions of both whole cell suspensions and sonic disrupted suspensions are assayed by incubating 5 μl. portions of the suspensions with solutions containing 20 μl. of a 1.586 mg./ml. solution of N-acetyl thienamycin in 0.01*M* potassium phosphate buffer, pH 7 and 10 μl. 0.2*M* potassium phosphate buffer, pH 7.4. Controls containing antibiotic and buffer alone and also cell suspensions without antibiotic are also run. After incubation of these mixtures overnight at 28°C., 2 μl. aliquot portions are removed and applied on cellulose-coated thin-layer-chromatography (TLC) plates, and the TLC plates developed in EtOH:H_2O, 70:30. After air drying, the TLC plates are placed on a *Staphylococcus aureus* ATCC 6538P assay plates for 5 minutes. The TLC plaates are removed and the assay plates incubated overnight at 37°C.

The assay plates are prepared as follows: An overnight growth of the assay organism, *Staphyllococcus aureus* ATCC 6538P, in nutrient broth plus 0.2% yeast extract is diluted with nutrient broth, plus 0.2% yeast extract to a suspension having 60% transmittance at a wavelength of 660 nm. This suspension is added to Difco nutrient agar supplemented with 2.0 g./l. Difco yeast extract at 47°C. to 48°C., to make a composition containing 33.2 ml. of the suspension per liter of agar. Forty ml. of this suspension is poured into 22.5 cm.x22.5 cm. petri plates, and these plates are chilled and held at 4°C. until used (5-day maximum).

Activity of N-acetylthienamycin amidohydrolase in the incubation mixtures is indicated by the presence of a bioactive area at R_f 0.44—0.47 due to thienamycin. The unreacted bioactive N-acetyl thienamycin appears at R_f 0.7—0.89. The process of this Example provides one with the ability to isolate N-acetylethanolamine-amidohydrolase-producing microorganisms with N-acetylthienamycin amidohydrolase activity.

EXAMPLE 2

Deacetylation of N-acetyl Thienamycin

A 1% (w/v) suspension of fertile lawn soil is prepared by suspending 1 gm. of lawn soil in 100 ml. sterile - phosphate - buffer - saline solution where the phosphate - buffer - saline solution has the following composition:

Phosphate-Buffer-Saline Solution	
NaCl	8.8 g.
1*M* Phosphate Buffer, pH 7.5*	10 ml.
Distilled H_2O	1000 ml.

*1M Phosphate Buffer, pH 7.5

16 ml. 1M KH_2PO_4 is mixed with 84 ml. 1*M* K_2HPO_4. The pH of the phosphate buffer is adjusted to 7.5 by adding small quantities of either 1*M* KH_2PO_4 or 1*M* K_2HPO_4.

Aliquot portions of this 1% stock soil suspension are used to prepare 10x, 100x and 1,000x dilutions.

One-ml. portions of the stock suspension or 1-ml. portions of the 10x, 100x and 1,000x dilutions are added to 2-ml. portions of steriles 1.0% agar solutions at 48°C. The mixtures are quickly poured over the surface of sterile petri dishes of 85 mm. diameter containing 20 ml. of Medium A. Medium A has the followong composition:

Medium A	
KH_2PO_4	3.0 g.
K_2HPO_4	7.0 g.
$MgSO_4$	0.1 g.
Distilled H_2O	1000 ml.
N-Acetylethanolamine solution*	8.5 ml.

*N-acetylethanolamine Solution

N-acetylethanolamine is diluted 10x in H_2O and membrane sterilized. This solution is added after autoclaving.

For solid media: Add 20 g. agar

The petri dishes are incubated for 18 days at 28°C. A well-isolated colony is picked and streaked on a petri dish containing Medium B. Medium B has the following composition:

Medium B	
Tomato paste	40 g.
Ground oatmeal	15 g.
Distilled H_2O	1000 ml.

pH: adjust to 6 using NaOH

For solid media: Add 20 g. agar

An individual clone is selected and grown for 2 days at 28°C. on a slant of Medium B.

A portion of the growth on this slant is streaked on the surface of six slants prepared from Medium B. These slants are incubated for 2 days at 28°C. This culture was identified as *Protaminobacter ruber* and has been designated MB-3528 in the culture collection of Merck & Co., Inc., Rahway, New Jersey.

A portion of the growth on the slant of *Protaminobacter ruber* MB-3528 is used to inoculate a 250 ml. Erlenmeyer flask containing 50 ml. of Medium C. Medium C has the following composition:

Medium C	
Dextrose	20 g
Phramamedia	8 g
Corn Steep Liquor (wet basis)	5 g
Distilled H_2O	1000 ml

pH: adjust to 7 with NaOH or HCl

N-acetylethanolamine solution*	8.5 ml.

*N-acetylethanolamine Solution

N-acetylethanolamine is diluted 10x in H_2O and membrane sterilized. This solution is added after autoclaving.

The flask is shaken at 28°C. on a 220 rpm (2" throw) shaker for 4 days. A 25-ml. portion from the flask is centrifuged for 15 minutes at 8,000 rpm. The supernatant is removed and the cells on the surface of the media solids scraped off into 0.5 ml. 0.05*M* potassium phosphate buffer, pH 7.4. The resulting suspension is subjected to ultrasonic disruption using a Branson Instrument Model LS-75 Sonifier with a 1/2-inch probe at setting 4 for four 15-second intervals, while chilling the suspension in ice water during and between disruption. 10 μl. of the sonicate is mixed with a 25 μl. solution containing 840 μg./ml. of N-acetylthienamycin in 0.01*M* potassium phosphate buffer, pH 7 and incubated overnight at 28°C. Controls containing antibiotic and buffer alone, also sonicated cells and buffer without antibiotic, are run. After incubation overnight at 28°C., 2 μ5ml. quantities are applied on cellulose-coated TLCplates,which are developed in EtOH:H_2O, 70:30. After air drying, the TLC plate is placed on a *Staphylococcus aureus* ATCC 6538P assay plate for 5 minutes.

The assay plates are prepared as follows: An overnight growth of the assay organism, *Staphylococcus aureus* ATCC 6538P, in nutrient broth plus 0.2% yeast extract is diluted with nutrient broth plus 0.2% yeast extract to a suspension having 60% transmittance at a wavelength of 660 nm. This suspension is added to Difco nutrient agar supplemented with 2.0 g./l. Difco yeast extract at 47°C. to 48°C., to make a composition containing 33.2 ml. of the suspension per liter of agar. 40 ml. of this suspension is poured into 22.5 cm.x22.5 cm. petri plates, and these plates are chilled and held at 4°C. until used (5-day maximum).

The TLC plate is removed and the assay plate incubated overnight at 37°C. In addition to the unreacted bioactive N-acetyl thienamycin spot at R_f 0.7—0.89, a bioactive spot is observed at R_f 0.44—0.47 due to thienamycin. Control incubation mixtures of antibiotic plus buffer, cell sonicate plus buffer, and antibiotic plus buffer to which cell sonicate is added just prior to TLC application produce no bioactive material at R_f 0.44—0.47.

EXAMPLE 3

Deacetylation of 890A_1

A portion of the growth on the slant of *Protaminobacter ruber* MB-3528 is used to inoculate a 250-ml. Erlenmeyer flask containing 50 ml. of Medium C. Medium C has the following composition:

Medium C	
Dextrose	20 g.
Pharmamedia	8 g.
Corn Steep Liquor (wet basis)	5 g.
Distilled H_2O	1000 ml.

pH: adjust to 7 with NaOH or HCl

N-acetylethanolamine solution*	8.5 ml.

*N-acetylethanolamine Solution

c12

N-acetylethanolamine is diluted 10x in H_2O and membrane sterilized. This solution is added after autoclaving.

The flask is shaken at 28°C. on a 220 rpm (2" throw) shaker for 4 days. A 25-ml. portion from the flask is centrifuged for 15 minutes at 8,000 rpm. The supernatant is removed and the cells on the surface of the media solids scraped off into 0.5 ml. 0.05*M* potassium phosphate buffer, pH 7.4. The resulting suspension is subjected to ultrasonic disruption using a Branson Instrument Model LS-75 Sonifier with a 1/2-inch probe at setting 4 for four 15-second intervals, while chilling the suspension in ice water during and between disruption. A 10 μl. portion of the sonicate is mixed with 25 μl. of an 890A_1 solution containing 4.85 hydroxylamine-extinguishable optical density units at 300 nm/ml. Controls containing antibiotic and buffer alone, also sonicated cells and buffer without antibiotic, are run. After incubation overnight at 28°C., 5 μl. quantities are

applied on a cellulose-coated TLC plate, which is developed in EtOH:H_2O, 70:30. After air drying, the TLC plate is placed on a *Staphylococcus aureus* ATCC 6538P assay plate for 5 minutes.

The assay plates are prepared as follows: An overnight growth of the assay organism, *Staphylococcus aureus* ATCC 6538P, in nutrient broth plus 0.2% yeast extract is diluted with nutrient broth plus 0.2% yeast extract to a suspension having 60% transmittance at a wavelength of 660 nm. This suspension is added to Difco nutrient agar supplemented with 2.0 g./l. Difco yeast extract at 47°C. to 48°C. to make a composition containing 33.2 ml. of the suspension per liter of agar. 40 ml. of this suspension is poured into 22.5 cm.×22.5 cm. petri plates, and these plates are chilled and held at 4°C. until used (5-day maximum).

The TLC plate is removed and the assay plate incubated overnight at 37°C. In addition to the unchanged bioactive $890A_1$ spot at R_f 0.7—0.89, a new bioactive spot is observed at R_f 0.44—0.47 due to desacetyl $890A_1$. Control incubation mixtures of antibiotic plus buffer, and cell sonicate plus buffer, produce no bioactive material at R_f 0.44—0.47.

EXAMPLE 4

Deacetylation of $890A_3$

Antibiotic $890A_3$ is deacetylated by the process described in Example 3 for the deacetylation of $890A_1$ to provide desacetyl 890A3.

EXAMPLE 5

Preparation of desacetyl $890A_1$

Six 250 ml. Erlenmeyer seed flasks containing 50 ml. each Medium C are inoculated with a portion of a slant of *Protaminobacter ruber* MB-3528. Medium C has the following composition:

Medium C

Dextrose	20 g.
Pharmamedia	8 g.
Corn Steep Liquor (wet basis)	5 g.
Distilled H_2O	1000 ml.

pH: adjust to 7 with NaOH or HCl

N-acetylethanolamine solution*	8.5 ml.

*N-acetylethanolamine Solution

N-acetylethanolamine is diluted 10× in H_2O and membrane sterilized. This solution is added after autoclaving.

The flasks are shaken at 28°C. on a 220 rpm (2" throw) shaker for 1 day.

41 2-liter production flasks each containing 400 ml. of Medium C are inoculated with 7 ml. per flask of the growth from these seed flasks. These production flasks are shaken at 28°C. on a 220-rpm shaker (2" throw) for 6 days. The contents of the flasks are pooled and centrifuged at 8,000 rpm for 15 minutes.The cells are scraped off the media solids pellet into a final volume of 1,600 ml. 0.05*M* potassium phosphate buffer, pH 7.4. This suspension is again centrifuged at 8,000 rpm for 15 minutes. The cells are scraped off the media solids pellet into a final volume of 160 ml. 0.05*M* potassium phosphate buffer, pH 7.4. This suspension is chilled to 5°C. and aliquot portions of 15 ml. each are exposed to successive 15 second cycles of ultrasonic irradiation, employing the equipment described in Example 1, until no further diminution of turbidity is observed when a 500× dilution is made of the suspension into phosphate buffer-saline where the phosphate - buffer - saline solution has the compositions set forth in Example 2.

To 1 liter of 0.005*M* potassium phosphate buffer, pH 7.4, is added 250 mg. of the antibiotic $890A_1$. To this mixture is added 160 ml. of the sonic extract of *Protaminobacter ruber* containing N-acetylthienamycin amidohydrolase. The mixture is stirred slowly with a magnetic stirrer at 28°C. for 20 hours. The mixture is then centrifuged at 10,000 g. for 15 minutes and the supernatant removed, chilled to 5°C., and adjusted to pH 4.5±0.2 by the addition of acetic acid. Separation of desacetyl $890A_1$ from the unhydrolyzed antibiotic and from other constituents of the reaction mixture is effected in the following manner, a disc-diffusion bioassay against *Staphylococcus aureus* ATCC 6538P and measurements of hydroxylamine-extinguishable absorbance at 297 nm being used to monitor the performance of the purification procedures. (As described in Assay Procedures for Thienamycin).

The acidified, centrifuged reaction mixture is absorbed at the rate of 12 ml./min. on a 120 ml. bed of Dowex 50×4 sodium cycle, 20—50 mesh resin. The adsorbate is washed with 120 ml. of deionized water and then eluted with 2% aqueous pyridine at 6 ml./min. Following the emergence of 75 ml. of the latter eluant from the column, the succeeding 240 ml. is pooled and concentrated to 25 ml. and the concentrate adjusted to pH 7.

The 25-ml. concentrate is adsorbed at a rate of 1 ml./min. on a 25-ml. bed of Dowex-1×2 (50—100 mesh) chloride cycle resin. The resin is eluted with deionized water at the same rate. Following the emergence of 25 ml. of the eluant from the column, the succeeding 50 ml. is pooled, neutralized to pH 7 and concentrated to 10 ml. This

concentrate is adjusted to pH 6.3 with acetic acid and is applied to a bed of Dowex 50×8 (200—400 mesh) resin in the 2,6-lutidinium cycle, having a diameter of 1 cm. and height of 50 cm., which has previously been equilibrated with 0.1*M* 2,6-lutidine acetate buffer, pH 6.3. Elution is conducted with the buffer at the rate of 1 ml./min. Following the emergence of 25 ml. of the eluant from the column, the succeeding 35 ml. is pooled and freeze-dried.

The freeze-dried solids are dissolved in 0.5 ml. of 0.1*M* 2,6lutidine acetate buffer, pH 7.0. The solution is applied to a column of Bio-Gel P-2 (200—400 mesh), having a diameter of 1 cm. and height of 50 cm., which had previously been equilibrated with this buffer. The gel is then developed with the same buffer at a rate of 0.5 ml./min. Following the emergence of 25 ml. of eluant from the column, the succeeding 10 ml. is pooled and freeze-dried.

The freeze-dried solids are dissolved in 4 ml. distilled water and applied on a 1.7 cm. diameter column packed with 90 ml. prewashed XAD-2 and equilibrated at 5°C. with distilled water. The XAD-2 is washed prior to use successively with 1) 5 volumes of 1*N* NaOH followed by deionized H_2O until effluent is neutral; 2) 5 volumes 1*N* HCl followed by deionized H_2O until the effluent is neutral; 3) 5 volumes each of methanol, acetone, 0.001*M* EDTA tetrasodium, and finally, distilled H_2O. The sample is followed by two 2-ml. portions of distilled water. The column is developed with distilled water at the rate of 2 ml./min. Four-ml. fractions of eluate are collected. Fractions 25 to 58 are pooled and lyophilized to yield desacetyl $890A_1$.

The preparation of N-acetylthienamycin by fermentation is disclosed in Examples 3 and 4 of the specification of our copending application No. 48232/76 (Serial No. 1561107). Deacetylation of this material in accordance with the process of Example 2 affords the antibiotic thienamycin.

Examples 5 and 6 of specification No. 48232/76 Serial No. 1561107) disclose the preparation of N-acetylthienamycin by acetylation of thienamycin.

The preparation of Antibiotic $890A_3$ and that of Antibiotic $890A_1$ is disclosed in Examples 8 and 9 respectively of the specification of our copending application No. 48235/76 (Serial No. 1561109). Deacetylation of these materials in accordance with the processes of Examples 4 and 3 respectively affords the antibiotics desacetyl $890A_3$ and desacetyl $890A_1$ respectively.

Compositions containing desacetyl $890A_1$ and desacetyl $890A_3$, the antibiotics of this invention, and compositions containing thienamycin may be administered in several unit dosage forms, for example, in solid or liquid orally ingestible dosage form. The composition per unit dosage, whether liquid or solid may contain from 0.1% to 99% of active material, the preferred range being from 10 to 60%. The compositions will generally contain from 25 mg. to 1000 mg. by weight of the active ingredient based upon the total weight of the composition; however. in general, it is preferable to use a dosage in the range of from 250 mg, to 1000 mg. In parenteral administration the unit dosage is usually the pure compound in a slightly acidified sterile aqueous solution or in the form of a soluble powder intended for solution. Representative formulations can be prepared by the following procedures:

Capsules	Per Capsule
Desacetyl $890A_1$	400 mg.

Lactose, U.S.P., a sufficient quantity to fill No. 0 Capsules, approximately 475 mg. each

In this formulation the active compound and the diluent are mixed to produce a uniform blend, which is then filled into No. 0 hard gelatin capsules, by hand or on a suitable machine, as required. The mixing and filling is preferably done in an area having a relative humidity less than 40%.

Tablets	Per Tablet
Desacetyl $890A_1$	330 mg
Calcium phosphate	192 mg
Lactose, U.S.P.	190 mg
Cornstarch	80 mg
Magnesium stearate	8 mg
	800 mg

In this formulation, the active component is blended with the calcium phosphate, lactose and about half of the cornstarch. The mixture is granulated with a 15% by weight cornstarch paste and rough-screened and screened again through No. 16 screens. The balance of the cornstarch and the magnesium stearate is added and the mixture is compressed into tablets, approximately 1/2" in diameter, each weighing 800 mg.

Alternatively, the active component is blended with the calcium phosphate, lactose and one-half the cornstarch. The mixture is "slugged" on a heavy duty press to produce compacted tablet-like masses. These are broken down to a No. 16 mesh granule. The balance of the cornstarch and the magnesium stearate are added and the mixture is compressed into tablets approximately 1/2" in diameter, each weighing 800 mg.

Lyo Form (For Injection)	Per Vial
Desacetyl 890A$_1$	25 mg.
Water-for-Injection, U.S.P. to make	5 ml.

In this formulation the active component is dissolved in sufficient water-for-injection in the ratio shown. The solution is filtered through Selas candles or Millipore membrane filters to sterilize it. The solution is subdivided into sterile vials. The vials and contents are frozen, and the water is aseptically removed by lyophilization. The vials containing the sterile dry solid are aseptically sealed.

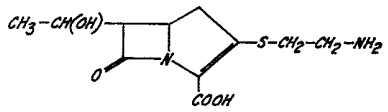
To restore for parenteral administration, 5 ml. of sterile water for injection is added to the contents of a vial.

Oral Liquid Forms	Per 1000 ml
Desacetyl 890A$_1$	1.0 gm
Sucrose	600.0 gm
Glucose	250.0 gm
Sodium Benzoate	1.0 gm
Concentrated Orange Oil	0.2 ml.
Purified water U.S.P. to make	1000.0 ml.

The sucrose and glucose are dissolved in about 400 ml. of water using heat to aid solution. This solution is cooled and the sodium benzoate is added, followed by the concentrated orange oil. The solution is brought to about 900 ml. volume with water and the antibiotic is added. The solution is clarified by filtration through a coarse filter.

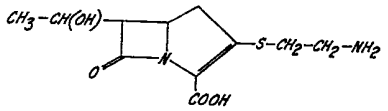
WHAT WE CLAIM IS:—

1. The compound desacetyl 890A$_1$, which has the formula:



or a pharmaceutically acceptable salt thereof.

2. The compound desacetyl 890A$_3$, which has the formula:



or a pharmaceutically acceptable salt thereof.

3. A process for producing the compound desacetyl 890A$_1$ that comprises intimately contacting the compound 890A$_1$ with an amidohydrolase capable of hydrolysing the N-acetyl group.

4. A process for producing the compound desacetyl 890A$_1$ that comprises intimately contacting the compound 890A$_3$ with an amidohydrolase capable of hydrolysing the N-acetyl group.

5. A process according to Claim 3 in which the amidohydrolase is an N-acetylthienamycin amidohydrolase.

6. A process according to Claim 4 in which the amidohydrolase is an N-acetylthienamycin amidohydrolase.

7. A process according to Claim 3 in which the amidohydrolase is an N-acetylethanolamine amidohydrolase.

8. A process according to Claim 4 in which the amidohydrolase is an N-acetylethanolamine amidohydrolase.

9. A process according to Claim 3 in which the amidohydrolase is one produced by an amidohydrolase-producing strain of the microorganism *Protaminobacter ruber*.

10. A process according to Claim 4 in which the amidohydrolase is one produced by an amidohydrolase-producing strain of the microorganism *Protaminobacter ruber*.

11. The process for producing thienamycin that comprises intimately contacting N-acetyl thienamycin with the enzyme N-acetylthienamycin amidohydrolase produced by an amidohydrolase-producing strain of the microorganism *Protaminobacter ruber*.

12. The process for producing desacetyl 890A$_1$ that comprises intimately contacting 890A$_1$ with the enzyme N-acetylthienamycin amidohydrolase produced by an amidohydrolase-producing strain of the microorganism *Protaminobacter ruber*.

13. The process for producing desacetyl 890A$_3$ that comprises intimately contacting 890A$_3$ with the enzyme N-acetylthienamycin amidohydrolase produced by an amidohydrolase-producing strain of the microorgansm *Protaminobacter ruber*.

14. A composition comprising an antibacterial effective amount of a compound as claimed in Claim 1 and a non-toxic pharmaceutically acceptable carrier.

15. A composition comprising an antibacterial effective amount of a compound as claimed in Claim 2 and a non-toxic pharmaceutically acceptable carrier.

16. An aqueous solution suitable for disinfecting medical or dental equipment containing 1 to 10 parts per million by weight of a compound as claimed in Claim 1 or 2.

17. A water-based paint or white water in a paper mill or containing as antibacterial agent a compound as claimed in Claim 1 or 2.

18. A composition as claimed in Claim 14 or 15 in the form of a tablet, capsule, powder, suspension or elixir.

19. A composition as claimed in Claim 14 or 15 in the form of a suppository.

20. A composition as claimed in Claim 14 or 15 enclosed in an ampoule.

21. A composition as claimed in Claim 14 or 15 in the form of a spray, inhalant, lozenge or throat paint.

22. A composition as claimed in Claim 14 or 15 in the form of an ointment, cream, lotion, powder or paint suitable for topical application.

23. A composition as claimed in Claim 14 or 15 in the form of an optically or aurally administrable material.

24. A composition as claimed in Claim 14 or 15 in the form of an intramammary preparation suitable for veterinary use.

25.A composition as claimed in any one of Claims 14, 15 and 18 to 24 also containing an aminocyclitol antibiotic.

26. A composition as claimed in any one of Claims 14, 15 and 18 to 24 also containing diphenoxylate and atropine.

27. A method of producing a compound as claimed in Claim 1 or 2 substantially as hereinbefore described.

28. The process that comprises testing microorganisms capable of utilizing N-acetylethanolamine for the presence of N-acetylthienamycin amidohydrolase and using N-acetylthienamycin amido hydrolase-containing microorganisms in a method as claimed in Claim 5, 6, 11, 12 or 13.

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