AU9461862

(12) PATENT ABRIDGMENT (11) Document No. AU-B-61862/94
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 682341

(54) Title
SUBSTITUTED VALERIC ACID

International Patent Classification(s)
(51)[5] **C07C 059/215** **C07D 307/60** **C12P 007/42** **A23L 001/226**

(21) Application No. : **61862/94** (22) Application Date : **03.05.94**

(30) Priority Data

(31) Number	(32) Date	(33) Country
1394/93	**06.05.93**	**CH SWITZERLAND**

(43) Publication Date : **10.11.94**

(44) Publication Date of Accepted Application : **02.10.97**

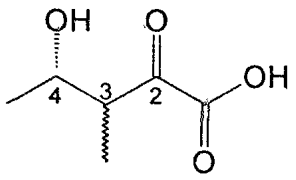
(71) Applicant(s)
GIVAUDAN-ROURE (INTERNATIONAL) SA

(72) Inventor(s)
KONRAD LERCH

(74) Attorney or Agent
SPRUSON & FERGUSON , GPO Box 3898, SYDNEY NSW 2001

(57) Claim

1. A 4S-hydroxy-3-methyl-2-ketopentanoic acid of the formula:

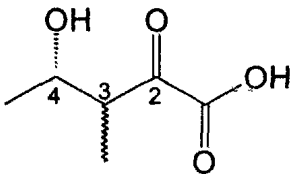


I

substantially free of the 4R-isomer.

2. A process for the manufacture of the compound according to claim 1, which process comprises reacting L-4-hydroxyisoleucine (2S,3R,4S) with a L-amino acid oxidase or a microorganism containing this enzyme, wherein these microorganisms are conveniently cultivated in a, conveniently, submersed, culture on a suitable nutrient medium prior to the actual biotransformation.

The invention is concerned with two newly, optically active forms of 2-keto-3-methyl-4-hydroxy-valeric acid of the formula

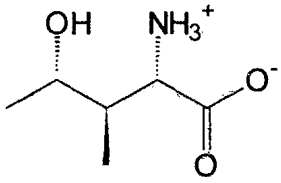


I

substantially free of the 4R-isomer, wherein the C atom 4 has the (S)-configuration. The C atom 3 can have the (R)-configuration or the (S)-configuration.

The invention is also concerned with a microbiological process for the manufacture of the compounds I, which process comprises reacting L-4-hydroxyisoleucine (2S,3R,4S) with a L-amino acid oxidase or a microorganism containing this enzyme, wherein these microorganisms are conveniently cultivated in a, conveniently, submersed, culture on a suitable nutrient medium prior to the actual biotransformation.

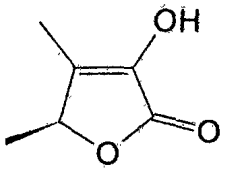
This process comprises reacting 4-hydroxyisoleucine (2S,3R,4S) (= L-4-hydroxy-isoleucine) of the formula



II

with microorganisms which have L-amino acid oxidase (EC 1.4.3.2.) activity or with a L-amino acid oxidase.

The compounds I are interesting intermediates for the production of the optically active (5S)-3-hydroxy-4,5-dimethyl-2(5H)-furanone of the formula



III

substantially free of the 5R-isomer.

The racemate of III is an important flavorant having an extremely low threshold value (H. Sulser, M. Habegger, W. Büchi, *Z. Lebensm. Unters. Forsch.* (1972), **148**, 215).

AUSTRALIAN PATENT OFFICE

The present invention enables III to be produced from a natural raw material using a process which is considered to be natural.

It has thus been possible to develop a process which permits this substance III to be produced in a biotechnological manner.

Seeds of fenugreek *Trigonellum foenum graecum L.* are especially suitable as the raw material for I and, respectively, III.

Since fenugreek seeds contain relatively large amounts (5 g/kg seeds) of free L-4-hydroxyisoleucine (L. Fowden, H. M. Pratt, A. Smith, *Phytochemistry* (1973), **12**, 1707), the free amino acid II present in the Trigonellum seeds can be converted in a simple manner firstly into I using the discovered biotransformation system.

This is possible, as indicated earlier, by reacting II with *microorganisms which have L-amino acid oxidase* (EC 1.4.3.2.) activity or with a L-amino acid oxidase itself.

The reaction proceeds as follows:

$$II + O_2 + H_2O \rightarrow I + NH_3 + H_2O_2$$

The diastereomeric forms of 2-keto-3-methyl-4-hydroxy-valeric acid (I) which thereby result can be isolated e.g. in a simple manner as metal salts such as the alkali metal or alkaline earth metal salts, for example the sodium or potassium salts. The free acids are very labile and cyclize spontaneously to the optically active lactone III.

The widest variety of microorganisms, such as bacteria and fungi, and algae, which can transform L-4-hydroxyisoleucine into I are suitable for the biotransformation of II.

Preferred organisms are those having high L-amino acid oxidase activity. To these belong algae of the genus *Amphiroa, Gymnogongrus* etc; fungi of the genus *Neurospora* etc; bacteria of the genus *Proteus, Providencia, Morganella, Corynebacterium* etc. Bacteria of the genus *Proteus, Providencia* and *Morganella,* e.g.

Proteus vulgaris (DSM 2140, 3265)
Proteus mirabilis (ATCC 15290)
Providencia rettgeri (ATCC 9250)
Morganella morganii (DSM 30117),

or fungi of the genus *Neurospora* such as e.g.
Neurospora crassa (FGSC 988 and 2963),

are especially well suited.

Prior to the actual biotransformation, the microorganisms are conveniently cultivated in a submersed culture on a suitable nutrient medium. Both conventional complex and chemically defined media can be used for this purpose, provided that a (novel) biosynthesis of the L-amino acid oxidase required for the biotransformation occurs. These details are easily verified by the man skilled in the are involved. In addition to suitable carbon and nitrogen sources, the nutrient medium conveniently contains inorganic salts, trace elements and free amino acids.

Sugar, preferably glucose or saccharose, is conveniently used as the carbon source. The nitrogen source can be inorganic compounds and organic compounds, for example ammonium salts, preferably ammonium sulphate and ammonium nitrate, nitrates, yeast extract or peptone etc.

Usually, the nutrient medium also contains salts, e.g. sulphates, phosphates or chlorides, especially of the elements magnesium, potassium and calcium, as well as the trace elements iron, zinc, manganese, boron, cobalt, copper and molybdenum. In

the case of *Neurospora crassa*, the addition of the vitamin biotin is indispensable.

The free amino acids used conveniently originate from protein hydrolysates, preferably from casein and soya protein hydrolysates, or in the case of individual amino acids from corresponding fermentations. Leucine, methionine and phenylalanine are preferred.

The ratio of the aforementioned nutrients conveniently depends on the mode of fermentation and is conveniently determined on a case by case basis in a manner which will be known to a person skilled in the art. For example, glucose or saccharose concentrations in the range of about 5 to about 50 g/l are suitable for carrying out the process in accordance with the invention, with concentrations in the range of about 10 to about 20 g/l being preferred. The ammonium sulphate or ammonium nitrate which is the preferred nitrogen source is incorporated in the range of about 0.03 to about 5 g/l, especially of about 0.06 to about 2 g/l.

The mineral salts which are used are conveniently incorporated in the range of about 0.05 to about 10 g/l, especially of about 0.1 to about 5 g/l. The trace elements are usually used in a concentration range of in each case about 0.005 to about 20 mg/l, especially of about 0.01 to about 5 mg/l, e.g. incorporated as an aqueous solution.

The protein hydrolysates which are used as the source of amino acids are conveniently incorporated in the range of about 5 to about 50 g/l, especially of about 10 to about 20 g/l. With respect to the addition of free amino acids such as leucine, methionine or phenylalanine, the chosen amount is conveniently in the range of about 0.1 to about 5 g/l, especially of about 0.2 to about 2 g/l.

The vitamin biotin which is required for the cultivation of *Neurospora crassa* is conveniently incorporated in the range of about 1 to about 20 µg/l, especially of about 2 to about 5 µg/l.

The fermentation temperature depends essentially on the microorganism which is used and conveniently ranges between about 10 and about 50°C, especially between about 20 and about 37°C. The pH value of the nutrient medium normally lies in the range of about 4 to about 10, preferably of about 5 to about 8. The preferred fermentation period, i.e. to the completion of growth, lies between about 10 and about 100 hours, especially between about 18 and about 72 hours.

After the fermentation the cells are conveniently separated mechanically from the nutrient medium, washed and used directly for the transformation. However, immobilized cells or crude extracts prepared from cells as well as, e.g. isolated, L-amino acid oxidase preparations in free or immobilized form can also be used for the conversion of L-4-hydroxyisoleucine (II). Since the basis of the conversion is an oxidative process, the reaction mixture is preferably shaken intensively or in addition aerated.

The isolation of the enzyme from the cells, where desired, can be carried out in a manner which will be known to a person skilled in the art, e.g. by mechanical disintegration, and purification, e.g. ion exchange chromatography and/or adsorption chromatography, etc.

The biotransformation is preferably carried out in a pH range of about 4 to about 8, especially of about 5 to about 7. The conversion in accordance with the invention is usually carried out at a temperature of about 10 to about 40°C, preferably at 20 to 37°C. The educt L-4-hydroxyisoleucine (II) is incorporated into the reaction solution in an amount of about 0.5 to about 5% (w/v), preferably of about 1 to about 2%.

The II:enzyme ratio is conveniently chosen so that the reaction does not require too much time, e.g. does not exceed about 6 to about 24 hours, which can be elucidated by means of a few experiments. After termination of the reaction, the course of which can be followed by conventionel amino acid analysis -

for disappearance of the compound II -, the reaction mixture is lactonized in a manner known for γ-hydroxy-acids, i.e. is conveniently acidified with acid, e.g. with dilute phosphoric acid, to a pH value of about 1 to about 3, preferably of about 1.5 to about 2.5, and subsequently lactonized by heating. The heat treatment is conveniently carried out in a temperature range between about 70 to about 95°C, preferably at about 80°C, for about 20 to about 90 minutes, preferably about 30 to about 60 minutes.

The solution can be extracted with an organic solvent such as an ester, especially ethyl acetate, or an ether such as methyl tert.butyl ether according to known methods, for example in a countercurrent extraction apparatus. Subsequently, the solvent is conveniently removed by distillation and the product is taken up in a carrier which is customary in the flavour industry such as e.g. an oil, primarily peanut oil, or e.g. triethyl citrate.

The cell-free reaction mixture containing I obtained after the biotechnological conversion of (II) can, however, also be mixed directly with a carrier such as maltodextrin or another starch derivative and (spray) dried. This product can be used as a flavor precursor of (III) for the flavouring of selected food products. These are food products which are to be subjected to a thermal treatment. A heat treatment (boiling, baking, frying, grilling, roasting, microwave treatment etc) is conveniently carried out in a temperature range between about 80 and about 250°C for about 2 to about 30 minutes. The amount of the precursor (I) which is used can be varied, for example, between about 1 ppb to about 5 ppm (in the final product).

The following Examples illustrate the present invention.

Example 1

Isolation and characterization of 2-keto-3-methyl-4-hydroxy-valeric acid (= 4-hydroxy-3-methyl-2-keto-pentanoic acid) (I)

a) Isolation of L-4-hydroxyisoleucine (II) from seeds of fenugreek *Trigonella foenum graecum L.*

The extraction and purification of L-4-hydroxyisoleucine was carried out according to L. Fowden, H. M. Pratt and A. Smith *(Phytochemistry* (1973), **12**, 1707). From 4 kg of seeds of *Trigonella foenum graecum L.* there were isolated 12 g of purified L-4-hydroxyisoleucine (yield 62%). Purity: >97% (amino acid analysis). Optical rotation: $[\alpha]_D^{20} = +32.3^\circ$ (c 1 (i.e. 1 g/100 ml), H_2O); literature value: 31.0°.

b) Biotransformation of L-4-hydroxyisoleucine (II) with *Morganella morganii*

Morganella morganii (DSM No. 30117) was cultivated in nutrient medium A (Table 1) for 20 hours in a fermenter at pH 7 and a temperature of 30°C. Subsequently, the cells were centrifuged, washed with 0.02M sodium phosphate buffer (pH 7.0), resuspended in 0.05M sodium phosphate buffer pH 7.0 and the mixture was adjusted to an optical density of 20 at 650 nm (or O.D. 0.2 after 100-fold dilution). This cell suspension was added to a shaking flask and treated with 7.7 mmol of L-4-hydroxy-isoleucine per litre. After incubation for 6 hours at 30°C on a shaking machine the amount of L-4-hydroxyisoleucine provided had reacted completely. The cells were separated from the reaction mixture by centrifugation and the supernatant obtained was lyophilized.

Table 1: Nutrient medium A

Glucose	10.0 g/l
Casein hydrolysate	10.0 g/l
$Na_2HPO_4 . 2H_2O$	11.9 g/l
KH_2PO_4	4.5 g/l
$(NH_4)_2SO_4$	1.0 g/l
$MgSO_4 . 7H_2O$	0.1 g/l
$CaCl_2 . 2H_2O$	4 mg/l
$FeSO_4 . 7H_2O$	0.5 mg/l

c) Isolation of the sodium salt of 2-keto-3-methyl-4-hydroxy-valeric acid (I).

The freeze-dried residue was extracted with methanol and the sodium salt of (I) was precipitated in the usual manner by the addition of methyl tert.butyl ether. The hitherto unknown substance exhibited the following physical properties:

Melting point:	144°C (dec.)
Optical rotation:	$[\alpha]_D^{20}$: + 18.49° (c 0.84 in methanol)
IR:	1720 cm^{-1}, 1630 cm^{1}, 1390 cm^{-1}, 1130 cm^{-1}
^{1}H-NMR:	δ = 4.18 ppm (2 x q), 3.18 (2 x q), 1.20 (2 x d), 1.05 (2 x d)
MS:	(electron spray ionization) 145.1 (M^-), 101.1, 57.2 313.1 ($2M^-Na^+$), 481.3 ($3M^-2Na^+$), 649.4 ($4M^-3Na^+$), 817.6 ($5M^-4Na^+$), (anion clusters).

d) Conversion of 2-keto-3-methyl-4-hydroxy-valeric acid (I) into (5S)-3-hydroxy-4,5-dimethyl-2(5H)-furanone (III)

840 mg of the sodium salt of (I) were dissolved in 50 ml of H_2O and brought to pH 2.0 with dilute phosphoric acid. The solution was held at 85°C for 30 minutes and, after cooling, extracted with 2 x 50 ml of ethyl acetate. 320 mg (yield 50%) of (III) separated after removal of the solvent. The substance (III), purified by preparative thin-layer chromatography (silica gel 60), exhibited an optical rotation of: $[\alpha]_D^{20}$: 21.66° (c 1.0 in chloroform).

Example 2

As described in Example 1, cells of *Morganella morganii* were cultivated in a fermenter, centrifuged, washed and subsequently adjusted to a density of 20 (650 nm). After adding

7.7 mmol of L-4-hydroxyisoleucine per litre the cell suspension was shaken at 30°C in an Erlenmeyer flask. The amount of L-4-hydroxyisoleucine provided had reacted completely after 18 hours. The cells were separated from the reaction mixture by centrifugation and the clear supernatant was adjusted to pH 2.5 with dilute phosphoric acid. After heating (30 minutes, 80°C) the solution was extracted twice with half volumes of ethyl acetate. A yellowish oil separated after removal of the solvent. Yield of (III): 65%.

Example 3

According to Example 1, cells of *Morganella morganii* were cultivated in a fermenter, centrifuged and subsequently washed with water. The cells were resuspended in 2% sodium alginate and introduced dropwise at room temperature into a stirred solution of $CaCl_2$ (2%). The immobilized cells which separated in small beads were washed thoroughly with water and subsequently used for the conversion of L-4-hydroxyisoleucine. For this purpose, a portion of sedimented, immobilized cells was treated with a solution of L-4-hydroxyisoleucine (11.25 mmol/l) and shaken at 30°C in an Erlenmeyer flask. The amount of 4-hydroxyisoleucine provided had reacted completely after 24 hours. The immobilized cells were separated from the reaction mixture over a suction filter and washed carefully with water. The clear filtrate was further treated as in Example 2. Yield of (III): 60%.

Example 4

Providencia rettgeri (ATCC 9250) was cultivated for 20 hours in nutrient medium A (Table 1) in a fermenter at pH 7 and a temperature of 30°C. Subsequently, the cells were centrifuged, washed with 0.02M sodium phosphate buffer pH 7.0 and resuspended in 0.1M sodium phosphate buffer pH 7.2 and adjusted to an optical density of 20 at 650 nm. This cell suspension was added to a shaking flask and treated with 7.7 mmol of 4-hydroxyisoleucine per litre. After incubating at 30°C for 16 hours on a shaking machine the added amount of L-4-hydroxyisoleucine had

reacted completely. The cells were separated from the reaction mixture by centrifugation and the supernatant was treated further as in Example 2. Yield of (III): 60%.

Example 5

Neurospora crassa (FGSC 988) was cultivated for 48 hours in nutrient medium B (Table 2) in a 2 litre shaking flask at pH 6.0 and a temperature of 25°C. The mycelium was filtered through four layers of cotton wool gauze, washed well with water, freeze-dried and subsequently finely pulverized using a homogenizer. 5 g of mycelium powder were mixed with 50 ml of 0.1M sodium phosphate buffer pH 7.2 and stirred at 40°C for 30 minutes. The insoluble cell constituents were removed by centrifugation and the clear supernatant was treated with 7.7 mmol of L-4-hydroxyisoleucine per litre. After an incubation period of 18 hours at 30°C the amount of L-4-hydroxyisoleucine provided had reacted completely. The transformation of the resulting 4-hydroxy-ketoacid (I) into (III) was carried out according to Example 2. Yield: 60%.

Table 2: Nutrient medium B

Saccharose	20.0 g/l
Sodium citrate . 5 H_2O	3.0 g/l
KH_2PO_4	5.0 g/l
$(NH_4)NO_3$	0.08 g/l
$MgSO_4 . 7H_2O$	0.2 g/l
$CaCl_2 . 2H_2O$	0.1 g/l
L-Phenylalanine	0.73 g/l
$ZnSO_4 . 7 H_2O$	5.0 mg/l
$Fe(NH_4)_2(SO_4)_2 . 6H_2O$	1.0 mg/l
$CuSO_4 . 5 H_2O$	0.25 mg/l
$MnSO_4 . 1 H_2O$	0.05 mg/l
H_3BO_3	0.05 mg/l
$Na_2MoO_4 . 2 H_2O$	0.05 mg/l

Example 6

The crude extract described in Example 5 was used for the covalent immobilization of L-amino acid oxidase. 50 ml of extract were dialyzed against 1.0M potassium phosphate buffer pH 7.5 and mixed with 10 g of acrylic resin pearls Eupergit® (Röhm Pharma, Weiterstadt, Germany). This suspension was left to stand at room temperature for 24 hours and the resin was subsequently washed with 2 l of 0.1M potassium phosphate buffer pH 7.2. The immobilized enzyme preparation was reacted with L-4-hydroxyisoleucine (7.7 mmol) at 30°C for 24 hours as described in Example 3. Separation of the immobilized enzyme from the reaction mixture was carried out by suction filtration and the supernatant was treated further as described in Example 2. The yield of (III) was 55%.

Example 7

Maple syrup flavour

Two maple syrup flavours of the following composition were prepared:

	Parts by weight	
	A	B
Peru balsam	5	5
Vanilla extract	50	50
Butyric acid (natural)	5	5
Furonol (natural 15%)	10	10
Roast base (natural)	5	5
III (0.1% in triacetin) according to Example 2	-	150
Triacetin	925	775
	1000	1000

A comparison of the above flavours shows that the presence of 150 parts of (III) in B confers the typical sweet, caramel-like

spicy character of maple syrup to this flavour and contributes materially to the intensification of the body. This flavour can be used e.g. in a milk drink in a concentration of 0.1%.

Example 8

Walnut flavour

	Parts by weight	
	A	B
Cocoa shell extract	50	50
Vanilla extract	10	10
Oleic acid	30	30
Walnut base (natural) III (0.1% in triacetin) according to Example 2	10	10
		120
Propylene glycol	900	780
	1000	1000

A comparison of the above flavours shows that the presence of 120 parts of (III) in B confers the typical walnut flavour character to this flavour and contributes materially to the intensification of the nutty and fatty notes. This flavour can be used e.g. in a milk drink in a concentration of 0.1%.

Example 9

HVP-free brown sauce

A HVP-free brown sauce of the following composition was prepared:

Ingredient	Parts by weight
White flour	370
Beef fat	145
Modified starch	145
Yeast extract	97
Cooking salt	80
Tomato powder	60
Maltodextrin	53.7
Monosodium glutamate	30
Caramel powder	10
Curcuma	4.5
Spice mixture (Spice'N'Easy®) (onion, garlic and rosemary extract)	4.8
	1000

When the sauce is heated in the presence of the flavour precursor I (2 ppm), the flavour is clearly intensified in the direction of meat, bouillon, HVP and salt.

Example 10

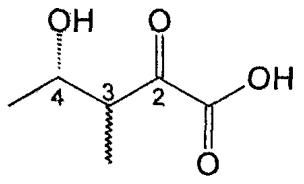
Crackers

Ingredient	Parts by weight
White flour	575
Vegetable fat	150
Skim milk powder	37
Powdered sugar	15
Sweetened whey powder	10.2
Yeast autolysate	10.0
Ammonium bicarbonate	10.0
Cooking salt	10.0
Sodium hydrogen pyrophosphate	1.0
White pepper	0.4
Paprika	0.4
Water	181
Flavour precursor (I)	1 ppm

When the cracker dough is baked (230°C, 10 min.) in the presence of the flavour precursor (I), the room flavour and the flavour of the product is intensified in a spicy, bouillon-like and herby direction.

The claims defining the invention are as follows:-

1. A 4S-hydroxy-3-methyl-2-ketopentanoic acid of the formula:

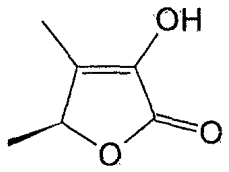


I

substantially free of the 4R-isomer.

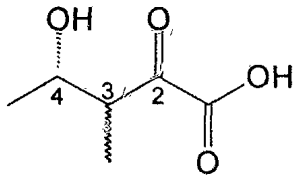
2. A process for the manufacture of the compound according to claim 1, which process comprises reacting L-4-hydroxyisoleucine (2S,3R,4S) with a L-amino acid oxidase or a microorganism containing this enzyme, wherein these microorganisms are conveniently cultivated in a, conveniently, submersed, culture on a suitable nutrient medium prior to the actual biotransformation.

3. A process for the production of optically active (5S)-3-hydroxy-4,5-dimethyl-2(5H)-furanone of the formula



III

substantially free of the 4R-isomer, which process comprises lactonizing a compound of the formula



I

in a manner known *per se*.

4. The use of the compound of claim 1 alone or in combination with a carrier as a flavour precursor of the compound III defined in claim 3.

Dated 10 July, 1997
Givaudan-Roure (International) SA

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

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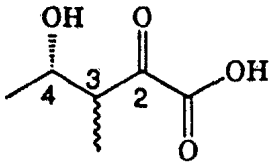
Substituted Valeric Acid

Abstract

The invention is concerned with two new, optically active forms of 2-keto-3-methyl-4-hydroxy-valeric acid of the formula

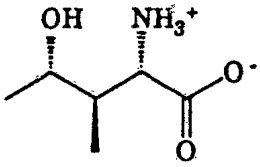


I

wherein the C atom 4 has the (S)-configuration. The C atom 3 can have the (R)-configuration or the (S)-configuration.

The invention is also concerned with a microbiological process for the manufacture of the compounds I.

This process comprises reacting 4-hydroxy-isoleucine (2S, 3R, 4S) (= L-4-hydroxyisoleucine) of the formula



II

with microorganisms which have L-amino acid oxidase (EC 1.4.3.2.) activity or with a L-amino acid oxidase.

The compounds I are interesting intermediates for the production of the optically active (5S)-3-hydroxy-4,5-dimethyl-2(5H)-furanone.