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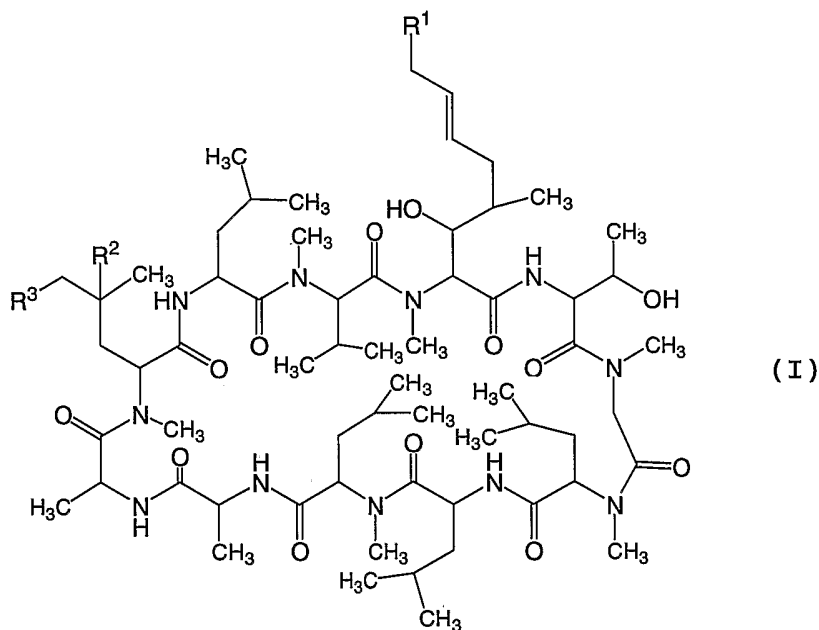
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[Continued on next page]

(54) Title: NEW CYCLIC PEPTIDE COMPOUND



(57) Abstract: A cyclic peptide compound of the following general formula (I): Wherein R^1 , R^2 and R^3 are defined in the description, or a salt thereof. The compound (I) is useful for the prophylactic and/or therapeutic treatment of hepatitis C.



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DESCRIPTION

NEW CYCLIC PEPTIDE COMPOUND

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TECHNICAL FIELD

The present invention relates to a new compound. More particularly, the present invention relates to a new cyclic peptide compound or a salt thereof having inhibitory activity against the RNA replication of hepatitis C virus (hereafter referred to as HCV) replicon. In particular, the present invention relates to a new peptide compound or a salt thereof, to a process for preparation thereof, to a pharmaceutical composition comprising the new cyclic peptide compound or a salt thereof, and to a method for the prophylactic and/or therapeutic treatment of hepatitis C in a human being or animal.

15

BACKGROUND ART

The estimated number of HCV carriers is about 170 million worldwide (about 3%) and about 1.5 million in Japan. Even in the combination therapy of using interferon (hereafter referred to as IFN) and ribavirin (Virazole), available as a first option for treatment, its effectiveness is 40% for all types of HCV. Furthermore, its effectiveness is only 15 to 20% for IFN-resistant virus (genotype 1b), particularly abundantly found in Japan. On the other hand, the combination therapy has side effects frequently. It is thus difficult to get rid of the virus completely by using currently available treatment methods. In the case when chronic hepatitis cannot be cured completely, the hepatitis will securely develop into cirrhosis hepatitis (30%) or hepatocellular carcinoma (25%). In Europe and the United States, hepatitis C has been a major indication for liver transplant. However, the redevelopment of HCV occurs frequently even in transplanted livers. For these reasons, the needs for

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new agents being improved in both effectiveness and safety, having higher antiviral effects and capable of inhibiting hepatitis C are very strong in society.

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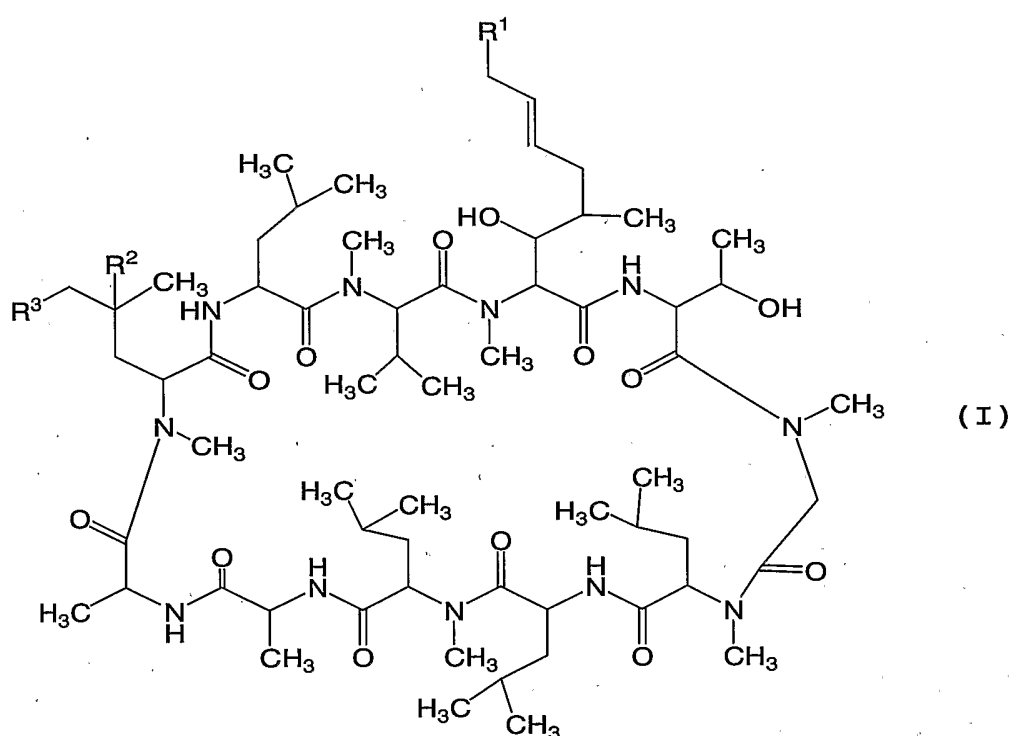
DISCLOSURE OF THE INVENTION

The object cyclic peptide compound in the present invention is a new compound, and can be represented by the following general formula (I):

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wherein

R^1 is hydrogen or hydroxy,

R^2 is hydrogen or hydroxy, and

R^3 is hydrogen or hydroxy,

Providing that when R^1 is hydrogen,

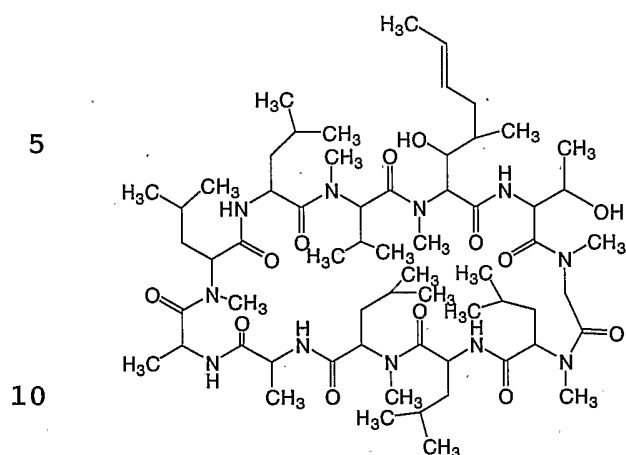
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then at least one of R^2 and R^3 is hydroxy,

or a salt thereof.

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The compound (I) or a salt thereof in the present invention can be prepared by the processes as illustrated in the following reaction schemes.

Process 1

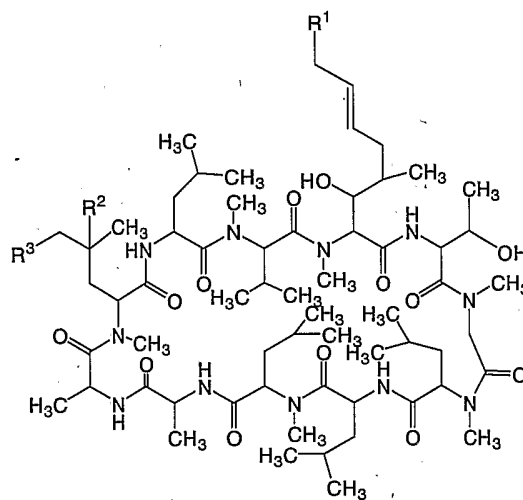
(II)

or a salt thereof

an enzyme obtained by culturing
an enzyme-producing strain of
microorganism belonging to
the genus *Lentzea*

15

20



(I)

or a salt thereof

25 wherein

R^1 , R^2 and R^3 are as defined above.

Suitable salts of the object compound (I) are conventional pharmaceutically acceptable and non-toxic salts, and may be a salt with a base or an acid addition salt, for example, a salt with an inorganic base (such as an alkali metal salt, e.g. sodium salt, potassium salt, etc., an alkaline earth metal salt, e.g. calcium salt, magnesium salt, etc., an ammonium salt), a salt with an organic base (such as an organic amine salt, e.g. triethylamine salt, diisopropylethylamine salt, pyridine salt, picoline salt,

ethanolamine salt, triethanolamine salt, dicyclohexylamine salt, N'N'-dibenzylethylenediamine salt, etc.), an inorganic acid addition salt (such as hydrochloride, hydrobromide, sulfate, phosphate, etc.), an organic carboxylic acid or sulfonic acid addition salt (such as formate, acetate, trifluoroacetate, maleate, tartrate, gluconate, fumarate, methanesulfonate, benzenesulfonate, toluenesulfonate, etc.), a salt with a basic or acidic amino acid (such as arginine, aspartic acid, glutamic acid, etc.) and the like.

The compound (II) or a salt thereof can be produced by fermentation of fungus (*Stachybotrys chartarum* No. 19392: deposit number FERM BP-3364) according to the method described in Japanese Laid-open Patent Application Hei 5-271267, for example.

The process for preparing the object compound (I) or a salt thereof of the present invention is explained in detail below.

20 Process 1

The object compound (I) or a salt thereof can be prepared by contacting a compound (II) or a salt thereof with an enzyme, which can be obtained by fermentation of an enzyme-producing strain of microorganism belonging to the genus *Lentzea*.

25

The fermentation process is explained in detail below.

(1) Microorganism:

The enzyme can be obtained by fermentation of an enzyme-producing strain of microorganism belonging to the genus *Lentzea* such as *Lentzea* sp. No. 7887 in a nutrient medium.

Strain No. 7887 was isolated from a soil sample collected at Ibaraki Prefecture, Japan. For the taxonomic study of strain No. 7887, the methods and media described by Hamada¹⁾ were

employed.

The observations were made after 14 days cultivation at 30 °C. The morphological observations were made on the cultures grown on 1/5 yeast extract-starch agar containing 0.4 g of yeast
5 extract(Daigo Eiyo, Osaka, Japan), 2 g of soluble starch, and 16 g of agar in 1000 ml of tap water (adjusted to pH 7.2 with 1N NaOH before sterilized), by using an optical microscope and a scanning electron microscope. Carbon utilization was determined on Pridoham and Gottlieb's medium²⁾. The color names used in this
10 study were taken from Methuen Handbook of Colour³⁾. The chemotaxonomic characterization was performed by procedure of Suzuki et al⁴⁾. The phylogenetic analysis of the 16S rDNA sequence was performed by the method of Nakagawa et al.⁵⁾. The 16S rDNA sequences of type strains were obtained from DDBJ data base⁶⁾.
15 The phylogenetic tree was constructed by the neighbor-joining methods⁷⁾ in CLUSTAL X package (version 1.8)⁸⁾.

(2) Morphological characteristics:

The substrate mycelium was developed well and branched
20 irregularly and penetrated the agar, forming compact colonies on the agar surface. The aerial mycelium was moderately developed on 1/5 yeast extract-starch agar, glycerol-asparagine agar, tyrosine agar, Czapek agar, yeast-starch agar and glucose-asparagine agar, and fragmented into rod-shaped elements.
25 Sclerotic granules, sporangia and motile spores or fragments were not observed.

(3) Cultural and physiological characteristics:

The results of cultural and physiological characteristics
30 are shown in Table 1 and 2 respectively. Reverse side color of growth were reddish brown, orange red, brownish red, brownish orange, light orange, orange, and pale orange. Melanoid pigments were not produced in trypton-yeast extract broth and pepton-yeast extract-iron agar. Soluble pigments were produced in yeast

extract-malt extract agar, oatmeal agar, and inorganic salts-starch agar, glycerol-asparagine agar, Tyrosine agar, Bennett's agar, Czapek agar, yeast-starch agar,

5 glucose-asparagine agar, sucrose-nitrate agar. Mycelial mass color and soluble pigments were not pH sensitive. L-Arabinose, D-xylose, D-glucose, D-fructose, sucrose, inositol, D-mannitol, cellobiose, dextrin, D-galactose, glycerol, D-mannose, Maltose, D-melibiose, soluble starch, and trehalose were utilized as
10 carbon sources but L-rhamnose, raffinose, adonitol, dextran, dulcitol, inulin, lactose, D-melezitose, sorbitol, L-sorbose, and xylitol were not utilized.

Strain No. 7887 was able to grow in the temperature range of from 10.5 to 32.5 °C, with the growth optimum at 31.0 °C.

15

(4) Chemotaxonomical characteristics:

The cell wall contained meso-diaminopimelic acid and no characteristic sugars (wall chemotype III).

20 (5) Analysis of 16S rDNA sequences:

The partial sequence of strain No.7887 is shown in Table 3. Similarity values of the 16S rDNA between strain No.7887 and members of the genus *Lentzea* are 97.2-99.1%, and they make single cluster on the phylogenetic tree.

25

(6) Classification:

Based on the morphological and chemical characteristics and phylogenetic analysis described above, strain No. 7887 is considered to belong to the genus *Lentzea*^{9, 10, 11}). Therefore, this
30 strain was designated as *Lentzea* sp. No. 7887.

A culture of *Lentzea* sp. No. 7887 thus named has been deposited at the International Patent Organism Depositary (IPOD) National Institute of Advanced Industrial Science and Technology,

AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi,
IBARAKI, 305-8566, JAPAN, on August 3, 2004 under the number of
FERM BP-10079.

Table 1. Cultural characteristics of strain No. 7887

	<u>Medium</u>	<u>Cultural characteristics</u>
5	Yeast extract-malt extract agar (ISP-2)	G: Good A: Thin, white R: Reddish brown (8E8) S: Brown
10	Oatmeal agar (ISP-3)	G: Moderate A: Thin, white R: Orange red (8A8) S: Orange
15	Inorganic salts-starch agar (ISP-4)	G: Good A: Scant, white R: Brownish red (9C7) S: Light brown
20	Glycerol-asparagine agar (ISP-5)	G: Good A: Moderate, white R: Brownish orange (7C7) S: Brown
	Peptone-yeast extract-iron agar (ISP-6)	G: Moderate A: Scant, white R: Light orange (5A5) S: Not detected
25	Tyrosine agar (ISP-7)	G: Good A: Moderate, white R: Brownish orange (7C7) S: Brown
30	Bennett's agar	G: Good A: Thin, white R: Orange red (8B8) S: Brown
	Czapek agar	G: Moderate A: Moderate, white

		R: Orange (6A6)
		S: Light orange
	Yeast-starch agar	G: Moderate
5		A: Moderate, white
		R: Orange red (8B8)
		S: Light brown
	Glucose-asparagine agar	G: Moderate
10		A: Moderate, yellowish white (4A2)
		R: Pale orange (5A3)
		S: Light brown
	Sucrose-nitrate agar	G: Moderate
15		A: Thin, yellowish white (4A2)
		R: Orange (5A6)
		S: Light brown
	Nutrient agar	G: moderate
20		A: Thin, white
		R: Light orange (5A5)
		S: Not detected

Abbreviation: G: growth, A: aerial mycelium, R: reverse side color,
S: soluble pigment

Table 2 Physiological characteristics of strain No. 7887

	Conditions	Characteristics
5	Temperature range for growth (°C)	10.5-35.2
	Optimum temperature for growth(°C)	31.0
	Production of Melanoid pigments	—
	Production of soluble pigments	+
	Hydrolysis of gelatin	+
10	Carbon utilization	
	L-Arabinose	+
	D-Xylose	+
	D-Glucose	+
	D-Fructose	+
15	Sucrose	+
	L-Rhamnose	—
	Raffinose	—
	Inositol	+
	D-Mannitol	+
20	Adonitol	—
	Cellobiose	+
	Dextran	—
	Dextrin	+
	Dulcitol	—
25	D-Galactose	+
	Glycerol	+
	Inulin	—
	Lactose	—
	D-Mannose	+
30	Maltose	+
	D-Melezitose	—
	D-Melibiose	+
	Sorbitol	—
	Soluble starch	+

	L- Sorbose	—
	Trehalose	+
	Xylitol	—
5	Sodium acetate (0.1% v/v)	±
	tri-Sodium Citrate (0.1% v/v)	±
	Malonic acid disodium salt (0.1% v/v)	±
	Propionic acid sodium salt (0.1% v/v)	±
	Pyruvic acid sodium salt (0.1% v/v)	±

10

+: positive, ±: weakly positive, -: negative

Table 3 The partial 16S rDNA sequence of strain No. 7887

CGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCCTTCGGGGTACACG
 5 AGCGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCTGTACTCTGGGATAAGCCTTGGA
 AACGAGGTCTAATACCGGATACGACCATTTGATCGCATGATCGGTGGTGGAAAGTTCCGGCG
 GTATGGGATGGACCCGCGGCCTATCAGCTTGTGGTGGGGTAATGGCCTACCAAGGCGACG
 ACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
 TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCG
 10 GTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAAGTGACG
 GTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG
 GAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTTCGCGTCGGCCGTG
 AAAACTTGGGGCTTAACTCCAAGCTTTCGGTCGATACGGGCAGACTTGAGTTCCGGCAGGGG
 AGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGA
 15 AGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATT
 AGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTGCTAGGTGTGGGGGGCTTCCACGCCC
 TCTGTGCCGCAGCTAACGCATTAAGCACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAC
 TCAAAGGAATTGACGGGGGGCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACG
 CGAAGAACCTTACCTGGGCTTGACATGGACTAGAAAGCTCTAGAGATAGAGCCTCCCTTGT
 20 GGCTGGTTTCACAGGTGGTGCATGGCTGTCTGTCAGCTCGTGTCTGAGATGTTGGGTTAAGT
 CCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTAATGGTGGGGACTCATGGGA
 GACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGT
 CCAGGGCTTCACACATGCTACAATGGCCGGTACAAAGGGCTGCTAAGCCGTGAGGTGGAGC
 GAATCCCATAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGG
 25 AGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACA
 CCGCCCGTCACGTACGAAAGTCGGTAACACCCGAAGCCCGTGGCTCAACCCGCAAGGGGG
 AGAGCGGTCTGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGA
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(7) The preparation of the object compound (I) or a salt thereof

by contacting a compound (II) or a salt thereof with an enzyme:

5 The object compound (I) or a salt thereof can be obtained by contacting the compound (II) or a salt thereof in an aqueous solvent with a crude or purified enzyme solution.

Suitable example of the enzyme may include the one produced by certain microorganisms of the *Lentzea* or the like, such as *Lentzea* sp. No.7887 (FERM BP-10079).

10 This reaction is usually carried out in an aqueous solution with one or more solvent(s) such as methanol or any other solvent which does not adversely influence the reaction.

The reaction temperature is not critical and the reaction can be carried out at room temperature or under warming.

15 More specifically, the object compound can be prepared by the processes described in Examples in the present application or similar processes.

The compounds obtained by the above-mentioned Process 1 can be isolated and purified by a conventional method, such as pulverization, recrystallization, column chromatography, high performance liquid chromatography (HPLC), reprecipitation and demineralized resin column chromatography.

25 In order to show the usefulness of the object compound (I) or a salt thereof in the present invention, a pharmacological test example of a representative compound in the present application is shown as follows.

30 Test example

HCV replicon assay

The inhibitory activity against the replication of HCV replicon was evaluated by determining the amount of replicon RNA, purified from cell lysate with a conventional column method, 35 using real-time RT-PCR based on Taq-Man chemistry. The assay was

carried out with the modified method reported by Lohmann et al., Science 285: 100 (1999) and Takeuchi et al., Gastroenterology 116: 636-642 (1999). The details thereof are described in the following.

1) Addition of agent to cells

7.5 x 10³ HCV replicon cells in 0.1 ml of D-MEM medium containing 5% fetal bovine serum and 300 µg/ml of G418 (Kishine et al., B.B.R.C. 293: 993-999 (2002)) were seeded in each well of a 96-well microtiter plate (Corning Inc.). After the plate was incubated at 37°C for 16 hours in 5% CO₂, the medium was replaced with the above-mentioned medium in which the test compound was dissolved.

2) Extraction of RNA from cells

After cultivation for two more days, total RNA was extracted from the cells according to the protocol of the RNA extraction column RNeasy96 (Qiagen Inc.).

3) Determination of the amount of replicon RNA according to the real-time RT-PCR method

The real-time RT-PCR was carried out by adding the appropriate primer set for amplifying the part of the HCV gene sequence and the complementary probe (all of these were produced by Takara Shuzo Co., Ltd.).

The RNA extracted at 2) was diluted with Nuclease-free water containing RNase inhibitor to 25 ng/µl, and dispensed to a 384-well PCR plate, 2 µl in each well. As the reaction solution for RT-PCR, TaqMan Ez RT-PCR Core Reagent (Applied Biosystems Inc.) was mixed according to the protocol and added, 8 µl in each well.

RT-PCR was carried out by using the ABI PRISM 7900HT sequence detection system (Applied Biosystems Inc.), and the gene copy number of the HCV replicon RNA in each cell was determined.

Various concentrations of HCV RNA standard solutions, 10-fold serially diluted were used for the calibration curve creation. The reaction for negative control was carried out without RNA.

5

4) Measurement of intrinsic control RNA

As a further control for RNA recovery in each assay well, the copy number of cellular Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was quantified by real-time RT-PCR with specific primer set and probe (all of these were produced by Takara Shuzo Co., Ltd.) as described above (part3) .

10

Estimation of cellular toxicity

The alamarBlue metabolic assay (Biosource International Inc.) was used to estimate the cytotoxicity in HCV replicon cells. After HCV replicon cells were cultured in the same conditions as those of the evaluation system of the replicon inhibitory activity, 5µl of alamarBlue was added in each well, and the cells were further incubated at 37°C for 2 hours in 5% CO₂. The fluorescence of each well was measured by spectrofluorometer (excitation 544nm, emission 595nm).

20

Test Method

The copy numbers of the replicon RNA in replicon cell treated at each concentrations of the compound, corrected by the intrinsic control GAPDH values at each point, were employed for the calculation of EC50 value of the compound, which gave the compound concentration indicating 50% RNA level to the control (no drug group, containing only DMSO). Similarly, the fluorescence values of the cells treated at each concentration of the compound were employed for the calculation of CC50 value, which gave the compound concentration indicating 50% level to the control (no drug group, containing only DMSO).

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Test resultTest compound

The compound(2) of the Example 3.

5

Table 4

Compound	HCV replicon inhibitory activity: EC50 (μ M)
Compound(2)	<3

From the result of the above-mentioned test example, it is realized that the object compound (I) or a salt thereof of the present invention possesses an anti-hepatitis C virus activity.

The anti-HCV agent in the present invention, containing the compound (I) or a salt thereof as an active ingredient, can be used in the form of a pharmaceutical preparation, for example, in a solid, semisolid or liquid form, in admixture with an organic or inorganic carrier or excipient suitable for oral; sublingual; buccal; nasal; respiratory; parenteral (intracutaneous, intraorgan, subcutaneous, intradermal, intramuscular, intra-articular, central-venous, hepatic-venous, peripheral-venous, lymphatic, cardiovascular, arterial, ocular including injection around eye or intravenous drip around eye); intravenous drip into eyeball, augen structure or augen layer; aural including auditory canal, papillary chamber, external and internal auditory canals, drum membrane, tympanum, internal-auditory including spiralis cochleae ganglion, labyrinth, etc.; intestinal; rectal; vaginal; ureteral; and vesical administration. With respect to intrauterine and perinatal adaptation diseases, parenteral administration is preferable since administration is carried out in maternal blood

vessels, or in vacancies, such as maternal organs including uterus, uterine cervix and vagina; fetal embryo, fetus, neonate, and combination tissue; and amnion, umbilical cord, umbilical artery and vein; placenta, and the like. Use of these passages is changed depending on the condition of each patient.

The compound (I) or a salt thereof can be administered independently as a therapeutic agent or may be desired to be used as part of prescribed drugs. The "anti-HCV agent" in accordance with the present invention can be used in the form of a pharmaceutical preparation, for example, in a solid, semisolid or liquid form, in admixture with at least one or some suitable organic or inorganic carriers or excipients, or other pharmacological therapeutic agents. The active ingredient can be compounded with, for example, usual pharmacologically acceptable and non-toxic carriers in a solid form, such as granules, tablets, pellets, troches, capsules or suppositories; creams; ointments; aerosols; powders for insufflation; in a liquid form, such as solutions, emulsions or suspensions for injection; oral ingestion; eye drops; and any other forms suitable for use. And, if necessary, there may be included in the above preparations auxiliary substances, such as stabilizing, thickening, wetting, hardening and coloring agents; perfumes or buffers; or any other additives used commonly.

The compound (I) or a pharmaceutically acceptable salt thereof is/are included in the pharmaceutical composition in an amount sufficient to produce the desired anti-hepatitis C effect upon the process or condition of diseases.

The combination use of IFN and/or ribavirin with the compound (I) or a salt thereof is effective against hepatitis C.

For applying the composition to humans, it is preferable to apply it by intravenous, intramuscular, pulmonary, oral administration, eye drop administration or insufflation. While the dosage of therapeutically effective amount of the compound (I) varies from and also depends upon the age and condition of each individual patient to be treated, in the case of intravenous administration, a daily dose of 0.001-400 mg of the compound (I) per kg weight of human being in the case of intramuscular administration, a daily dose of 0.1-20 mg of the compound (I) per kg weight of human being, in case of oral administration, a daily dose of 0.5-50 mg of the compound (I) per kg weight of human being is generally given for treating or preventing hepatitis C. However, these doses may be required to exceed the limit thereof to obtain therapeutic results.

The amount of the compound (I) or its pharmaceutically acceptable salt contained in the composition for a single unit dosage of the present invention is 0.1 to 400 mg, more preferably 1 to 200 mg, still more preferably 5 to 100 mg, specifically 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 and 100 mg.

An article of manufacture, comprising packaging material and the compound (I) identified in the above contained within said packaging material, wherein said the compound (I) is therapeutically effective for preventing or treating hepatitis C, and wherein said packaging material comprises a label or a written material which indicates that said compound (I) can or should be used for preventing or treating hepatitis C.

A commercial package comprising the pharmaceutical composition containing the compound (I) identified in the above and a written matter associated therewith, wherein the written matter states that the compound (I) can or should be used for

preventing or treating hepatitis C.

5 It is to be noted that the compound (I) or a salt thereof may include one or more stereoisomer(s), such as optical isomer(s) and geometrical isomer(s), due to asymmetric carbon atom(s) and double bond(s), and that all such isomers and the mixture thereof are included within the scope of the present invention.

10 The compound (I) or a salt thereof may include solvated compound (e.g. hydrate, ethanolate, etc.).

The compound (I) or a salt thereof may include both the crystal form and non-crystal form.

The compound (I) or a salt thereof may include the prodrug form.

15

In addition the object compound (I) is useful as an intermediate for preparing a compound having an anti-hepatitis C virus activity.

20 The patent specifications and publications cited herein are incorporated in this specification by reference.

The following Examples are given for the purpose of illustrating the present invention. However, the present invention is not limited to these Examples.

25

Example 1

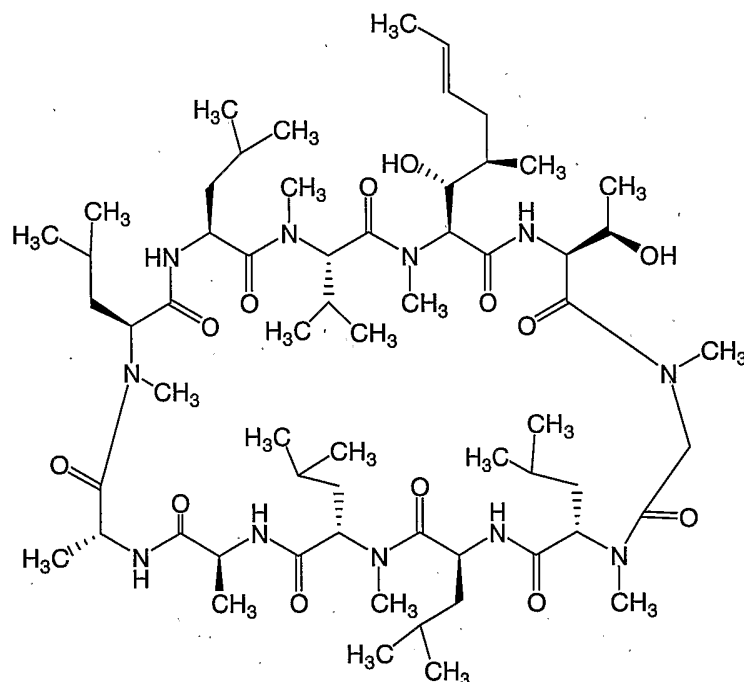
(1) Fermentation of *Lentzea* sp. No.7887

30 A stock culture of *Lentzea* sp. No.7887 is prepared and maintained on agar slant. A loopful of the slant culture was inoculated into a seed medium consisting of corn flour 1.0%, modified starch 6.0%, pharmamedia 1.2%, dried yeast 0.8%, KH_2PO_4 0.3% and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3% and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02% (pH 6.5 adjusted with 6N NaOH). The inoculated vegetative medium (60 ml) was
35 shaken on a rotary shaker (220 rpm, 5.1 cm throw) in a 225 ml

Erlenmeyer flask at 30 °C for about 72 hours. And 3.2 ml of the seed culture was transferred to 160 ml of the sterile seed medium consisting of sucrose 0.5%, glucose 0.5%, oatmeal 0.5%, yeast extract 0.2%, peptone 0.5%, peanut powder 0.5%, "HUMAS" (Aiaisi kabushikikaisha, Osaka, Japan) as humic acid 0.01%, polyoxyethylene sorbitan monooleate 0.1% and CaCO_3 0.2% (pH 7.0 adjusted with 6N NaOH) in the 500-ml Erlenmeyer flasks. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 30 °C for about 72 hours, and 7.2 L (45 flasks) of the second culture was inoculated to sterile production medium (160 L and 200 L) consisting of corn flour 1.0%, modified starch 6.0%, pharmamedia 1.2%, dried yeast 0.8%, KH_2PO_4 0.3% and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%, Adekanol LG-109 0.05% and Silicone KM-70 0.05% (pH 6.5 adjusted with 6N NaOH). The inoculated production medium was allowed to ferment in 200 and 300 L jar fermentors at a temperature of 30 °C for about 72 hours. The fermentation medium was stirred with conventional agitators at 200 rpm and aerated at 160 L (200 L jar fermenter) or 200 L (300 L jar fermenter) per minute. The cultured broth was used as enzyme to produce the compound (I) from the compound (II).

(2) Reaction condition

To a solution of the compound (IIb) disclosed below (180 g) in methanol (7.2 L) was added 360 L of the cultured broth obtained by above-mentioned fermentation. The mixture was carried out at 30 °C with stirring for 13 hours to give a reaction mixture (A). Increase of the compound(1), compound (2), compound (3), compound(4), compound(5), compound(6) and compound(7) were monitored by analytical HPLC indicated below.

Compound (IIb)

Analytical HPLC condition for compound(1), compound(2), compound(3) and compound(4)

Column	Mightysil RP-8 GP 150-4.6 (150mm L. X 4.6mm I.D., Kanto Chemical Co., Inc.)	
Eluent	60% aqueous acetonitrile containing 0.1%TFA	
Flow rate	1 ml/min.	
Detection	UV at 210nm	
Retention time	Compound(1)	7.6 min.
	Compound(2)	4.9 min.
	Compound(3)	6.4 min.
	Compound(4)	5.6 min.

Analytical HPLC condition for compound(5), compound(6) and compound(7)

Column	Mightysil RP-18 GP 250-4.6 (250mm L. X 4.6mm I.D., Kanto Chemical Co., Inc.)	
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Eluent 50% aqueous acetonitrile containing 0.1%TFA

Flow rate 1 ml/min.

Detection UV at 210nm

5	Retention time	Compound(5)	9.4 min.
		Compound(6)	7.6 min.
		Compound(7)	8.3 min.

Example 2

- 10 The reaction mixture (A) obtained in Example 1(2) (360 L) was extracted with an equal volume of acetone at room temperature. The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (14L) of DIAION HP-20 (Mitsubishi Chemical Co.,
- 15 Ltd.) packed with 25% aqueous acetone. The column was washed with 25% aqueous acetone (42 L) and then eluted with methanol (40 L). Active fraction (7-37 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% aqueous
- 20 methanol. The column was washed with 50% aqueous acetonitrile (28 L) and eluted with 60% aqueous acetonitrile (24 L). Active fraction (12-24 L) was diluted with equal volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile.
- 25 The column was eluted with 60% aqueous acetonitrile (7.5 L). Active fraction (5.1-7.1 L) was diluted with equal volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted with 60% aqueous
- 30 acetonitrile (14 L). Active fraction (5.8-8.1 L) was diluted with equal volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted with 70% aqueous acetonitrile containing 0.1% TFA (6.4 L). Active fraction
- 35 (2.8-3.6 L) was diluted with equal volume of water and passed

through a column (2 L) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 35% aqueous acetonitrile containing 0.05% TFA. The column was washed with water (4 L) and eluted with ethyl acetate (1.8 L). Active fraction (0-0.8 L) was concentrated to dryness under reduced pressure. The dried materials were dissolved in a small amount of chloroform, and were applied onto silica gel (Silica Gel 60 N, spherical, neutral, 40-100 μ m, KANTO CHEMICAL Co., INC., 120 g). The column was washed with chloroform-methanol (98:2) and eluted with chloroform-methanol (97:3) and chloroform-methanol (95:5). Active fractions were collected and concentrated under reduced pressure to dryness. The dried materials were dissolved in a small amount of ethyl acetate and added to a large amount of n-hexane. And then, the compound (1) was precipitated, and filtered with glass filter. This precipitate was dried up under reduced pressure to give 3.7 g of the compound (1) as white powder.

The compound (1) has the following physico-chemical properties.

Appearance:

White powder

Nature:

Neutral substance

Melting point:

157-160 °C (dec.)

Specific rotation:

$[\alpha]_D^{23} -214^\circ$ (c 1.0, CH_2Cl_2)

Molecular formula:

$\text{C}_{62}\text{H}_{111}\text{N}_{11}\text{O}_{14}$

Molecular weight:

ESI-MS(+) m/Z 1235(M+H)

5 Solubility:

Soluble: CHCl₃, MeOH, Ethyl acetate, Acetone, DMSO,
Pyridine

Slightly soluble: H₂O

Insoluble: n-Hexane

10

Color reaction:

Positive: iodine vapor reaction

Negative:

15 Thin layer Chromatography (TLC):

Silica gel 60 F254(Merck) CHCl₃:MeOH = 10:1

Rf 0.34

Infrared absorption spectrum: KBr

20 3420, 3330, 2960, 1630, 1520, 1410, 1100 cm⁻¹

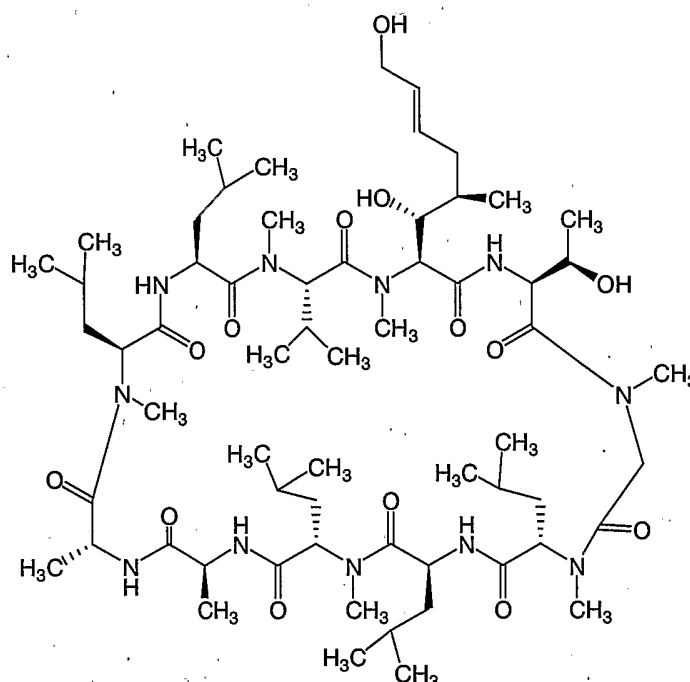
The compound (1) exists in several stable conformations in a common organic solution. For one example, the ¹³C-NMR chemical shifts due to the major conformer of the compound (1) in
25 pyridine-d₅ were listed as follows.

¹³C-NMR (pyridine-d₅, 125 MHz)

30 δ: 175.1 (s), 174.4 (s), 174.4 (s), 173.4 (s), 173.0 (s),
172.9 (s), 171.0 (s), 171.0 (s), 170.4 (s), 169.5 (s), 169.0 (s),
133.9 (d), 128.7 (d), 74.9 (d), 69.6 (d), 63.1 (t), 61.4 (d), 59.3
(d), 57.3 (d), 55.7 (d), 55.6 (d), 54.6 (d), 49.0 (t), 48.6 (d),
48.1 (d), 47.4 (d), 46.6 (d), 41.2 (t), 41.1 (t), 39.1 (q), 38.5
(t), 38.2 (t), 37.3 (d), 36.8 (t), 36.7 (t), 34.1 (q), 31.0 (q),
30.8 (q), 29.9 (q), 28.8 (q), 27.6 (d), 25.9 (d), 25.6 (d), 25.3
35 (d), 24.9 (d), 24.8 (d), 24.1 (q), 23.6 (q), 23.3 (q), 23.2 (q),

23.2 (q), 23.2 (q), 22.8 (q), 21.5 (q), 21.2 (q), 20.9 (q), 19.7 (q), 19.3 (q), 18.3 (q), 17.4 (q), 16.2 (q), 15.4 (q)

From the analysis of the above physical and chemical properties, and the result of the further investigation of identification of chemical structure, the chemical structure of the compound (1) has been identified and assigned as follows.



Example 3

The reaction mixture (A) obtained in Example 1(2) (360 L) was extracted with an equal volume of acetone at room temperature. The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (14L) of DIAION HP-20 (Mitsubishi Chemical Co., Ltd.) packed with 25% aqueous acetone. The column was washed with 25% aqueous acetone (42 L) and then eluted with methanol (40 L). Active fraction (7-37 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 25% aqueous methanol. The column was washed with 50% aqueous acetonitrile (28 L) and

eluted with 60% aqueous acetonitrile (24 L). Active fraction (0-12 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted with 60% aqueous acetonitrile (31 L). Active fraction (15.8-20.7 L) was diluted with equal volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was washed with water (5 L) and eluted with ethyl acetate (3.5 L). Active fraction (1-3 L) was concentrated to dryness under reduced pressure. The dried materials were dissolved in ethyl acetate (80 mL) and added dropwise to n-hexane (2.4 L). And then, the compound (2) was precipitated, and filtered with glass filter. This precipitate was dried up under reduced pressure to give 32.8 g of the compound (2) as white powder.

The compound (2) has the following physico-chemical properties.

Appearance:

White powder

Nature:

Neutral substance

Melting point:

170-173 °C (dec.)

Specific rotation:

$[\alpha]_D^{23} -222^\circ$ (c 1.0, CH₂Cl₂)

Molecular formula:

C₆₂H₁₁₁N₁₁O₁₄

Molecular weight:

ESI-MS(+) m/Z 1235(M+H)

5 Solubility:

Soluble: CHCl₃, MeOH, Ethyl acetate, Acetone, DMSO,
Pyridine

Slightly soluble: H₂O

Insoluble: n-Hexane

10

Color reaction:

Positive: iodine vapor reaction

Negative:

15 Thin layer Chromatography (TLC):

Silica gel 60 F254(Merck) CHCl₃:MeOH = 9:1

Rf 0.55

Infrared absorption spectrum: KBr

20

3420, 3330, 2960, 1640, 1530, 1410, 1100 cm⁻¹

The compound (2) exists in several stable conformations in a common organic solution. For one example, the ¹³C-NMR chemical shifts due to the major conformer of the compound (2) in

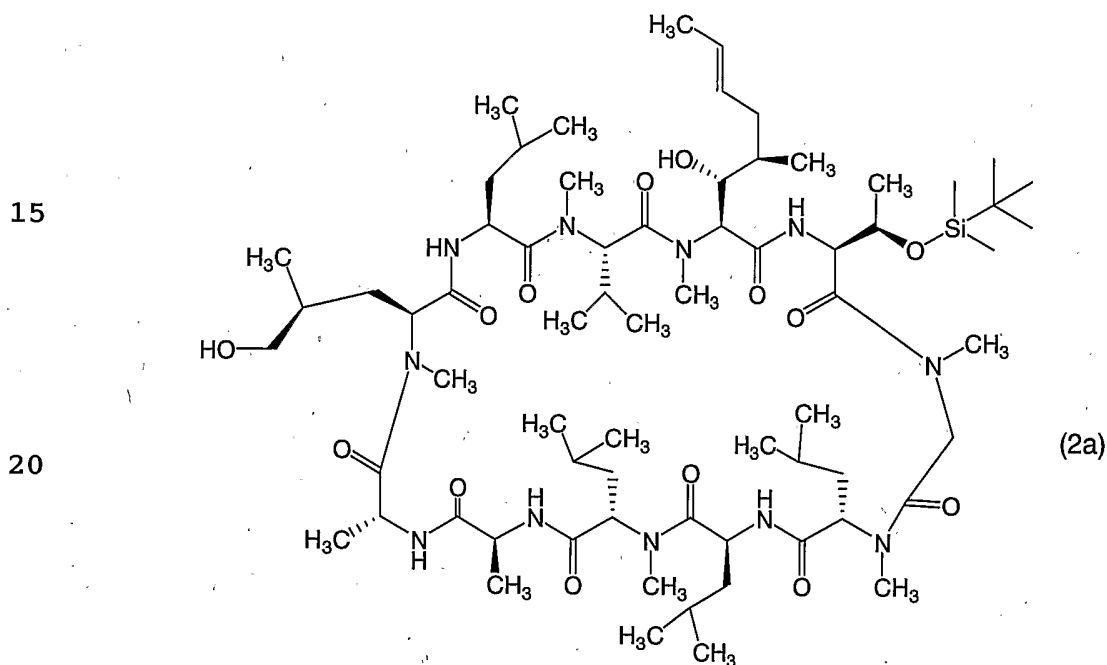
25 pyridine-d₅ were listed as follows.

¹³C-NMR (pyridine-d₅, 125 MHz)

δ : 175.3 (s), 174.5 (s), 174.4 (s), 173.4 (s), 173.0 (s),
172.9 (s), 171.0 (s), 170.9 (s), 170.5 (s), 169.5 (s), 169.0 (s),
30 129.8 (d), 127.1 (d), 75.2 (d), 69.6 (d), 67.9 (t), 61.5 (d), 59.3
(d), 57.3 (d), 55.7 (d), 55.3 (d), 54.6 (d), 49.0 (t), 48.6 (d),
48.1 (d), 47.4 (d), 46.6 (d), 41.2 (t), 41.0 (t), 39.1 (q), 38.5
(t), 38.2 (t), 37.6 (d), 37.3 (t), 34.1 (q), 33.5 (d), 31.5 (t),
31.0 (q), 30.8 (q), 29.9 (q), 28.8 (q), 27.7 (d), 25.9 (d), 25.6
35 (d), 24.9 (d), 24.8 (d), 24.1 (q), 23.3 (q), 23.2 (q), 23.2 (q),

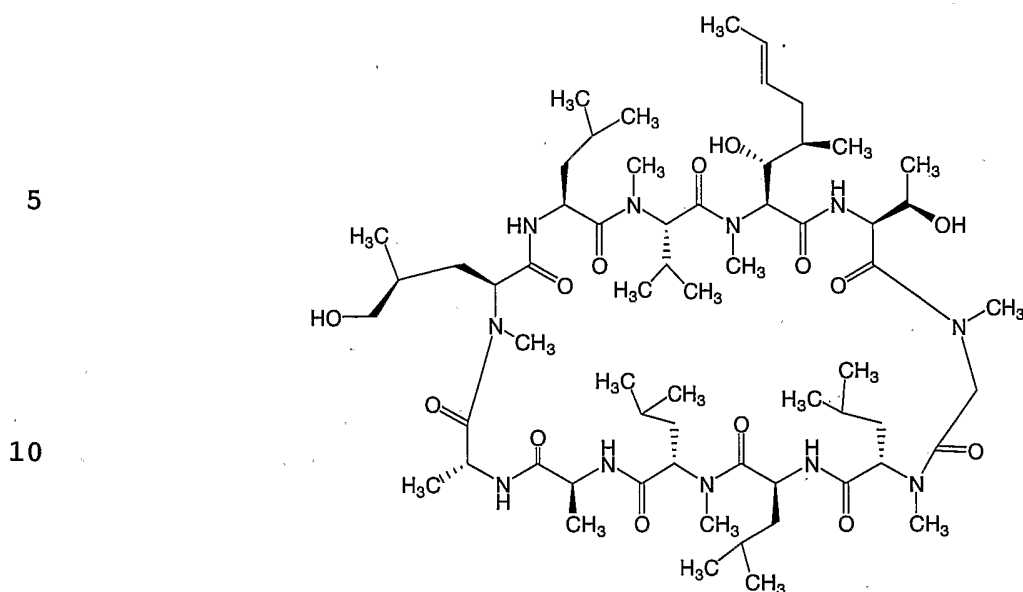
23.2 (q), 22.8 (q), 21.2 (q), 20.9 (q), 19.6 (q), 19.3 (q), 18.3 (q), 18.1 (q), 17.4 (q), 16.2 (q), 16.1 (q), 15.4 (q)

5 The hydroxy group of the 2-threonine residue of the p-nitorophenylcarbonate derived from the compound(2) was converted into the t-butyldimethylsilyl ether. And it was hydrolyzed with 1N NaOH to give a compound(2a). The compound(2a) was crystallized from ethyl acetate and the absolute
10 configuration was determined by X-ray analysis.



25

From the analysis of the above physical and chemical properties, the chemical structure of the compound (2) including
30 stereochemistry has been identified and assigned as follows.



Example 4

15 The reaction mixture (A) obtained in Example 1(2) (360 L) was extracted with an equal volume of acetone at room temperature. The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (14L) of DIAION HP-20 (Mitsubishi Chemical Co.,

20 Ltd.) packed with 25% aqueous acetone. The column was washed with 25% aqueous acetone (42 L) and then eluted with methanol (40 L). Active fraction (7-37 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% aqueous methanol. The

25 column was washed with 50% aqueous acetonitrile (28 L) and eluted with 60% aqueous acetonitrile (24 L). Active fraction (0-12 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted

30 with 60% aqueous acetonitrile (31 L). Active fraction (20.7-24.7 L) was diluted with equal volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted with 60% aqueous acetonitrile (9 L). Active fraction

35 (6.7-8.7 L) was diluted with equal volume of water and passed

through a column (1 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted with 60% aqueous acetonitrile (4 L). Active
5 fraction (3.0-3.7 L) was concentrated in vacuo to give 322 mg of the compound (3) as pale yellow powder.

The compound (3) has the following physico-chemical properties.

10

Appearance:

Pale yellow powder

Nature:

15

Neutral substance

Melting point:

162-165 °C (dec.)

20

Specific rotation:

$[\alpha]_D^{23} -200^\circ$ (c 1.0, CH₂Cl₂)

Molecular formula:

C₆₂H₁₁₁N₁₁O₁₄

25

Molecular weight:

ESI-MS(+) m/Z 1235 (M+H)

Solubility:

30

Soluble: CHCl₃, MeOH, Ethyl acetate, Acetone, DMSO, Pyridine

Slightly soluble: H₂O

Insoluble: n-Hexane

35

Color reaction:

Positive: iodine vapor reaction

Negative:

5 Thin layer Chromatography (TLC):

Silica gel 60 F254(Merck) CDCl_3 :MeOH = 9:1

Rf 0.50

Infrared absorption spectrum: KBr

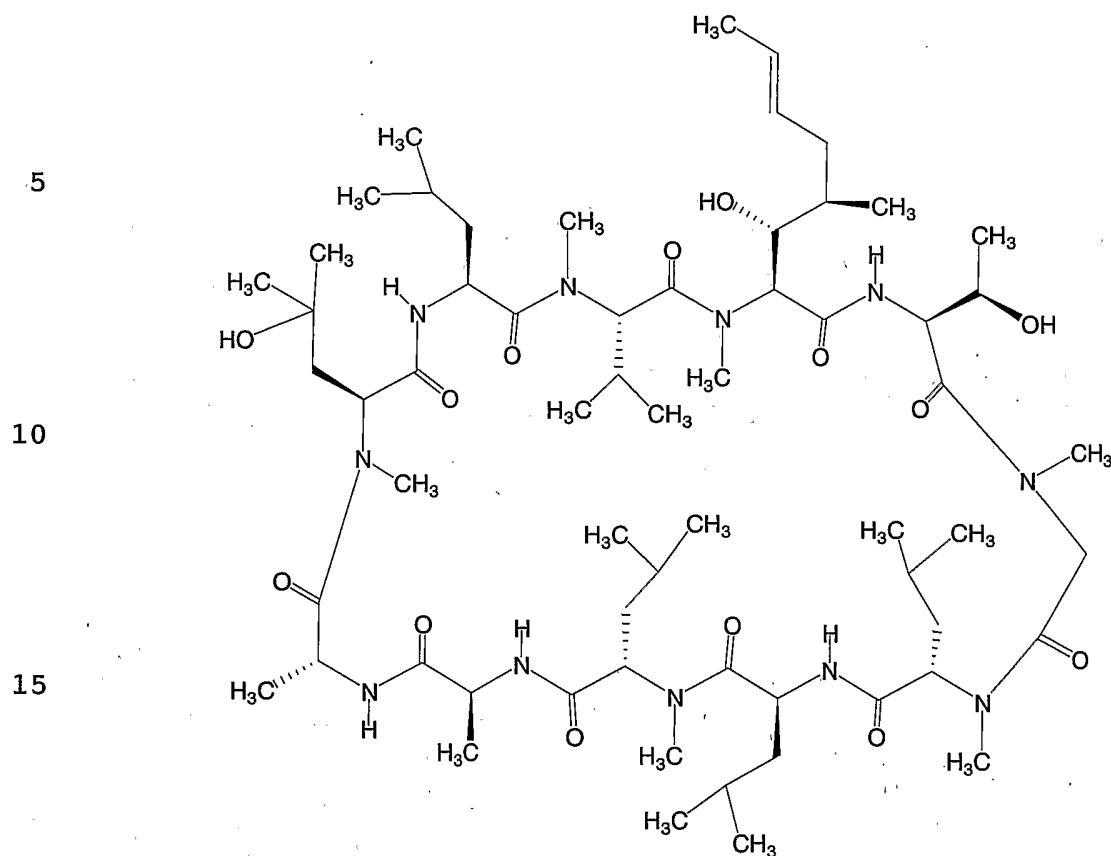
10 3420, 3330, 2960, 1640, 1530, 1410, 1280, 1100 cm^{-1}

The compound (3) exists in several stable conformations in a common organic solution. For one example, the ^{13}C -NMR chemical shifts due to the major conformer of the compound (3) in
15 pyridine- d_5 were listed as follows.

^{13}C -NMR (pyridine- d_5 , 125 MHz)

δ : 174.7 (s), 174.5 (s), 174.3 (s), 173.4 (s), 173.0 (s),
172.9 (s), 171.1 (s), 171.0 (s), 170.9 (s), 169.5 (s), 169.0 (s),
20 129.8 (d), 127.1 (d), 75.2 (d), 69.6 (d), 69.1 (s), 61.5 (d), 59.3
(d), 57.3 (d), 55.7 (d), 55.0 (d), 54.6 (d), 49.0 (t), 48.9 (d),
48.1 (d), 47.4 (d), 46.6 (d), 41.3 (t), 41.3 (t), 41.0 (t), 39.1
(q), 38.6 (t), 38.2 (t), 37.6 (d), 37.3 (t), 34.1 (q), 31.8 (q),
31.0 (q), 30.4 (q), 29.9 (q), 29.8 (q), 28.8 (q), 27.6 (d), 25.9
25 (d), 25.6 (d), 24.9 (d), 24.8 (d), 24.1 (q), 23.3 (q), 23.3 (q),
23.2 (q), 23.2 (q), 22.8 (q), 21.2 (q), 20.9 (q), 19.6 (q), 19.3
(q), 18.3 (q), 18.1 (q), 17.4 (q), 16.1 (q), 15.0 (q)

From the analysis of the above physical and chemical
30 properties, and the result of the further investigation of
identification of chemical structure, the chemical structure of
the compound (3) has been identified and assigned as follows.



Example 5

The reaction mixture (A) obtained in Example 1(2) (360 L) was extracted with an equal volume of acetone at room temperature. The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (14L) of DIAION HP-20 (Mitsubishi Chemical Co., Ltd.) packed with 25% aqueous acetone. The column was washed with 25% aqueous acetone (42 L) and then eluted with methanol (40 L). Active fraction (7-37 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% aqueous methanol. The column was washed with 50% aqueous acetonitrile (28 L) and eluted with 60% aqueous acetonitrile (24 L). Active fraction (0-12 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted

with 60% aqueous acetonitrile (31 L). Active fraction (20.7-24.7 L) was diluted with equal volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted with 60% aqueous acetonitrile (9 L). Active fraction (5.1-6.1 L) was diluted with equal volume of water and passed through a column (1 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 30% aqueous acetonitrile. The column was eluted with 60% aqueous acetonitrile (4.7 L). Active fraction (2.7-2.9 L) was concentrated in vacuo to give 740 mg of the compound (4) as pale yellow powder.

The compound (4) has the following physico-chemical properties.

Appearance:

Pale yellow powder

Nature:

Neutral substance

Melting point:

162-165 °C (dec.)

Specific rotation:

$[\alpha]_D^{23} -215^\circ$ (c 1.0, CH₂Cl₂)

Molecular formula:

C₆₂H₁₁₁N₁₁O₁₄

Molecular weight:

ESI-MS(+) m/z 1235 (M+H)

Solubility:

Soluble: CHCl_3 , MeOH, Ethyl acetate, Acetone, DMSO,
Pyridine

Slightly soluble: H_2O

5 Insoluble: n-Hexane

Color reaction:

Positive: iodine vapor reaction

Negative:

10

Thin layer Chromatography (TLC):

Silica gel 60 F254(Merck) CDCl_3 :MeOH = 9:1

Rf 0.5

15 Infrared absorption spectrum: KBr

3420, 3330, 2960, 1880, 1640, 1520, 1410, 1280, 1100 cm^{-1}

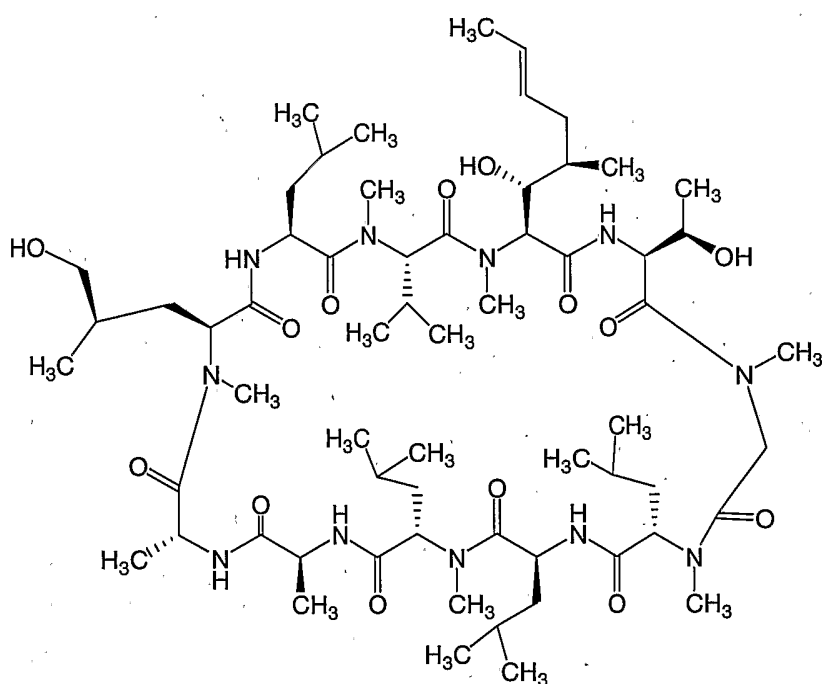
The compound (4) exists in several stable conformations in
a common organic solution. For one example, the ^{13}C -NMR chemical
20 shifts due to the major conformer of the compound (4) in
pyridine- d_5 were listed as follows.

^{13}C -NMR (pyridine- d_5 , 125 MHz)

δ : 175.1 (s), 174.4 (s), 174.4 (s), 173.4 (s), 173.0 (s),
25 172.9 (s), 171.0 (s), 170.9 (s), 170.4 (s), 169.5 (s), 169.0 (s),
129.8 (d), 127.1 (d), 75.2 (d), 69.6 (d), 66.1 (t), 61.5 (d), 59.3
(d), 57.3 (d), 55.7 (d), 55.7 (d), 54.6 (d), 49.0 (t), 48.6 (d),
48.1 (d), 47.4 (d), 46.6 (d), 41.2 (t), 41.2 (t), 39.1 (q), 38.5
(t), 38.2 (t), 37.6 (d), 37.3 (t), 34.1 (q), 34.0 (d), 31.6 (t),
30 31.1 (q), 31.0 (q), 29.9 (q), 28.8 (q), 27.7 (d), 25.9 (d), 25.6
(d), 24.9 (d), 24.8 (d), 24.1 (q), 23.2 (q), 23.2 (q), 23.2 (q),
23.2 (q), 22.8 (q), 21.3 (q), 20.9 (q), 19.7 (q), 19.3 (q), 18.4
(q), 18.1 (q), 18.1 (q), 17.4 (q), 16.1 (q), 15.3 (q)

From the analysis of the above physical and chemical properties, and the result of the further investigation of identification of chemical structure, the chemical structure of the compound (4) has been identified and assigned as follows.

And the stereochemistry of N-methyl-hydroxyleucine in the compound(4) was determined by ^{13}C -NMR data comparison with that of the compound(2).



25 Example 6

The reaction mixture (A) obtained in Example 1(2) (360 L) was extracted with an equal volume of acetone at room temperature. The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (14L) of DIAION HP-20 (Mitsubishi Chemical Co., Ltd.) packed with 25% aqueous acetone. The column was washed with 25% aqueous acetone (42 L) and then eluted with methanol (40 L). Active fraction (7-37 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% aqueous

methanol. The column was eluted with 50% aqueous acetonitrile (28 L). Active fraction (15-28 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% aqueous acetonitrile. The column was eluted with 50% aqueous acetonitrile. Active fraction (18.8-20.8 L) was concentrated under reduced pressure to remove acetone and extracted with 1 L of ethyl acetate. The solvent extract (upper layer) was concentrated under reduced pressure to an oily residue. The dried materials were dissolved in a small amount of chloroform, and were applied onto silica gel (Silica Gel 60N, spherical, neutral, 40-100 μ m, KANTO CHEMICAL Co., INC., 50 g). The column was washed with chloroform-methanol (97:3) and eluted with chloroform-methanol (96:4) and chloroform-methanol (95:5). Active fractions were collected and concentrated under reduced pressure to dryness. The dried materials were dissolved in a small amount of methanol and subjected to preparative HPLC, (Mightysil RP-18 GP 250-20 (5 mm), KANTO CHEMICAL Co., INC.). The column was developed with 50% aqueous acetonitrile containing 0.1%TFA at a flow rate of 10 ml/minute. The active fraction was diluted with a equal volume of water and passed through a column (19 ml) of Daisogel SP-120-ODS-B (15/30mm, DAISO Co., Ltd., Japan) packed with 25% aqueous methanol containing 0.05% TFA. The column was washed with water (50 ml) and eluted with ethyl acetate (50 ml). The elute was concentrated to dryness under reduced pressure. The dried materials were dissolved in a small amount of ethyl acetate and added to a large amount of n-hexane. And then, the compound (5) was precipitated, and filtered with glass filter. This precipitate was dried up under reduced pressure to give 205 mg of the compound (5) as white powder.

The compound (5) has the following physico-chemical properties.

Appearance:

white powder

Nature:

5 Neutral substance

Melting point:

163-167 °C (dec.)

10 Specific rotation:

 $[\alpha]^{23}_D -199^\circ$ (c 1.0, CH₂Cl₂)

Molecular formula:

C₆₂H₁₁₁N₁₁O₁₅

15

Molecular weight:

ESI-MS(+) m/z 1251 (M+H)

Solubility:

20 Soluble: CHCl₃, MeOH, Ethyl acetate, Acetone, DMSO,
PyridineSlightly soluble: H₂O

Insoluble: n-Hexane

25 Color reaction:

Positive: iodine vapor reaction

Negative:

Thin layer Chromatography (TLC):

30 Silica gel 60 F254(Merck) CHCl₃:MeOH = 10:1

Rf 0.42

Infrared absorption spectrum: KBr

3420, 3330, 2960, 1630, 1530, 1410, 1100 cm⁻¹

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The compound (5) exists in several stable conformations in a common organic solution. For one example, the ^{13}C -NMR chemical shifts due to the major conformer of the compound (5) in pyridine- d_5 were listed as follows.

5

^{13}C -NMR (pyridine- d_5 , 125 MHz)

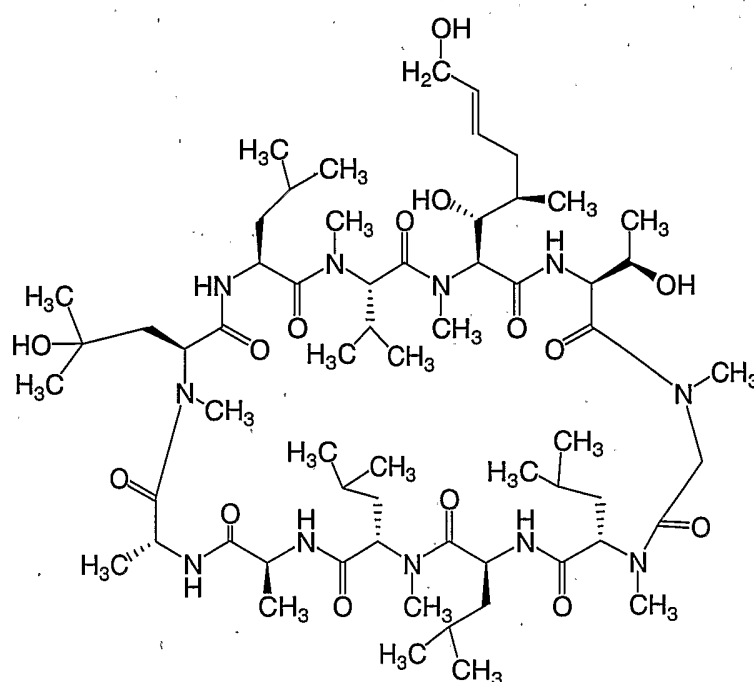
δ : 174.7 (s), 174.5 (s), 174.3 (s), 173.4 (s), 173.0 (s),
172.9 (s), 171.1 (s), 171.0 (s), 170.9 (s), 169.5 (s), 169.0 (s),
133.9 (d), 128.7 (d), 74.9 (d), 69.6 (d), 69.0 (s), 63.1 (t), 61.4
10 (d), 59.3 (d), 57.3 (d), 55.7 (d), 55.0 (d), 54.6 (d), 49.0 (t),
48.9 (d), 48.1 (d), 47.4 (d), 46.6 (d), 41.3 (t), 41.3 (t), 41.1
(t), 39.1 (q), 38.6 (t), 38.2 (t), 37.3 (d), 36.8 (t), 34.1 (q),
31.7 (q), 31.0 (q), 30.4 (q), 29.9 (q), 29.8 (q), 28.8 (q), 27.6
(d), 25.9 (d), 25.6 (d), 24.9 (d), 24.8 (d), 24.1 (q), 23.3 (q),
15 23.3 (q), 23.2 (q), 23.2 (q), 22.8 (q), 21.2 (q), 20.9 (q), 19.7
(q), 19.3 (q), 18.3 (q), 17.5 (q), 16.2 (q), 15.0 (q)

From the analysis of the above physical and chemical
properties, and the result of the further investigation of
20 identification of chemical structure, the chemical structure of
the compound (5) has been identified and assigned as follows.

25

30

35



Example 7

The reaction mixture (A) obtained in Example 1(2) (360 L) was extracted with an equal volume of acetone at room temperature.

5 The mixture was filtered with an aid of diatomaceous earth. A part of filtrate was diluted with an equal volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% acetone. The column was eluted with 50% aqueous acetonitrile (10 L). Active fraction
10 (2.6-4.6 L) was diluted with equal volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% aqueous acetonitrile. The column was eluted with 45% aqueous acetonitrile containing 0.1% TFA (13 L). Active fraction (8.6-9.6 L) was diluted with equal
15 volume of water and passed through a column (2 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 22.5% aqueous acetonitrile containing 0.05% TFA. The column was washed with water (5 L) and eluted with ethyl acetate (2 L). Active fraction (0-0.5 L) was concentrated to dryness under reduced
20 pressure. The dried materials were dissolved in a small amount of ethyl acetate and added to a large amount of n-hexane. And then, the compound (6) was precipitated. This precipitate was dried up under reduced pressure to give 1.3 g of the compound (6) as pale yellow powder.

25

The compound (6) has the following physico-chemical properties.

Appearance:

30 Pale yellow powder

Nature:

Neutral substance

35 Melting point:

163-167 °C (dec.)

Specific rotation:

5 $[\alpha]^{23}_D -207^\circ$ (c 1.0, CH₂Cl₂)

Molecular formula:

C₆₂H₁₁₁N₁₁O₁₅

10 Molecular weight:

ESI-MS(+) m/z 1251 (M+H)

Solubility:

15 Soluble: CHCl₃, MeOH, Ethyl acetate, Acetone, DMSO,
Pyridine
Slightly soluble: H₂O
Insoluble: n-Hexane

Color reaction:

20 Positive: iodine vapor reaction
Negative:

Thin layer Chromatography (TLC):

25 Silica gel 60 F254(Merck) CHCl₃:MeOH = 9:1
R_f 0.29

Infrared absorption spectrum: KBr

3420, 3330, 2960, 1630, 1530, 1410, 1100 cm⁻¹

30 The compound (6) exists in several stable conformations in a common organic solution. For one example, the ¹³C-NMR chemical shifts due to the major conformer of the compound (6) in pyridine-d₅ were listed as follows.

35 ¹³C-NMR (pyridine-d₅, 125 MHz)

δ: 175.2 (s), 174.5 (s), 174.4 (s), 173.4 (s), 173.0 (s),

172.9 (s), 171.0 (s), 170.9 (s), 170.5 (s), 169.5 (s), 169.0 (s),
133.9 (d), 128.7 (d), 75.0 (d), 69.6 (d), 67.9 (t), 63.1 (t), 61.4
(d), 59.3 (d), 57.3 (d), 55.7 (d), 55.3 (d), 54.6 (d), 49.0 (t),
48.6 (d), 48.1 (d), 47.4 (d), 46.6 (d), 41.2 (t), 41.1 (t), 39.1
5 (q), 38.5 (t), 38.2 (t), 37.3 (d), 36.8 (t), 34.1 (q), 33.5 (d),
31.5 (t), 31.0 (q), 30.8 (q), 29.9 (q), 28.8 (q), 27.6 (d), 25.9
(d), 25.6 (d), 24.9 (d), 24.8 (d), 24.1 (q), 23.3 (q), 23.2 (q),
23.2 (q), 23.2 (q), 22.8 (q), 21.2 (q), 20.9 (q), 19.7 (q), 19.3
(q), 18.3 (q), 17.4 (q), 16.2 (q), 16.2 (q), 15.4 (q)

10

From the analysis of the above physical and chemical
properties, and the result of the further investigation of
identification of chemical structure, the chemical structure of
the compound (6) has been identified and assigned as follows.

15

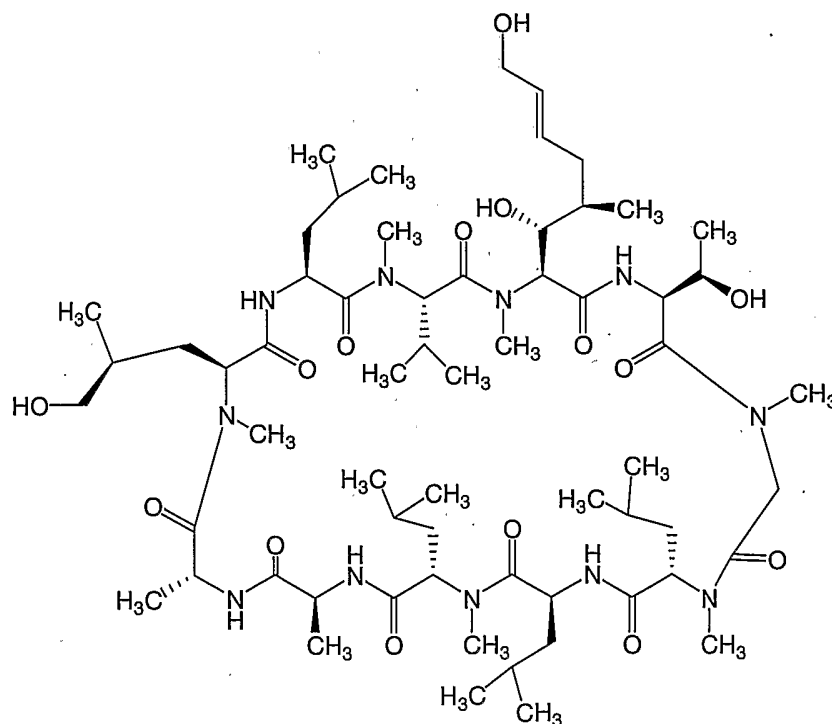
And the stereochemistry of N-methyl-hydroxyleucine in the
compound(6) was defined by ^{13}C -NMR data comparison with that of
the compound(2).

20

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30

35



Example 8

The reaction mixture (A) obtained in Example 1(2) (360 L) was extracted with an equal volume of acetone at room temperature.

5 The mixture was filtered with an aid of diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a column (14L) of DIAION HP-20 (Mitsubishi Chemical Co., Ltd.) packed with 25% aqueous acetone. The column was washed with 25% aqueous acetone (42 L) and then eluted with methanol (40

10 L). Active fraction (7-37 L) was diluted with equal volume of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% aqueous methanol. The column was eluted with 50% aqueous acetonitrile (28 L). Active fraction (15-28 L) was diluted with equal volume

15 of water and passed through a column (8 L) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd., Japan) packed with 25% aqueous acetonitrile. The column was eluted with 50% aqueous acetonitrile. Active fraction (17.8-18.8 L) was concentrated under reduced pressure to remove acetone and extracted with 500

20 ml of ethyl acetate. The solvent extract (upper layer) was concentrated under reduced pressure to an oily residue. The dried materials were dissolved in a small amount of chloroform, and were applied onto silica gel (Silica Gel 60 N, spherical, neutral, 40-100 μ m, KANTO CHEMICAL Co., INC., 50 g). The column was washed

25 with chloroform-methanol (97:3) and eluted with chloroform-methanol (96:4) and chloroform-methanol (95:5). Active fractions were collected and concentrated under reduced pressure to dryness. The dried materials were dissolved in a small amount of methanol and subjected to preparative HPLC,

30 (Mightysil RP-18 GP 250-20 (5 mm), KANTO CHEMICAL Co., INC.). The column was developed with 50% aqueous acetonitrile containing 0.1%TFA at a flow rate of 10 ml/minute. The active fraction was diluted with a equal volume of water and passed through a column (19 ml) of Daisogel SP-120-ODS-B (15/30 mm, DAISO Co., Ltd.,

35 Japan) packed with 25% aqueous methanol containing 0.05% TFA. The

column was washed with water (50 ml) and eluted with ethyl acetate (50 ml). The eluate was concentrated to dryness under reduced pressure. The dried materials were dissolved in a small amount of ethyl acetate and added to a large amount of n-hexane. And then, the compound (7) was precipitated, and filtered with glass filter. This precipitate was dried up under reduced pressure to give 227 mg of the compound (7) as white powder.

10 The compound (7) has the following physico-chemical properties.

Appearance:

White powder

15

Nature:

Neutral substance

Melting point:

20 163-167 °C (dec.)

Specific rotation:

$[\alpha]_D^{23} -199^\circ$ (c 1.0, CH₂Cl₂)

25 Molecular formula:

C₆₂H₁₁₁N₁₁O₁₅

Molecular weight:

ESI-MS(+) m/Z 1251 (M+H)

30

Solubility:

Soluble: CHCl₃, MeOH, Ethyl acetate, Acetone, DMSO,
Pyridine

Slightly soluble: H₂O

35

Insoluble: n-Hexane

Color reaction:

Positive: iodine vapor reaction

Negative:

5

Thin layer Chromatography (TLC):

Silica gel 60 F254(Merck) CHCl₃:MeOH = 10:1

Rf 0.41

10 Infrared absorption spectrum: KBr

3420, 3330, 2960, 1630, 1520, 1410, 1100 cm⁻¹

The compound (7) exists in several stable conformations in a common organic solution. For one example, the ¹³C-NMR chemical shifts due to the major conformer of the compound (7) in pyridine-d₅ were listed as follows.

¹³C-NMR (pyridine-d₅, 125 MHz)

δ : 175.1 (s), 174.4 (s), 174.4 (s), 173.4 (s), 172.9 (s), 172.9 (s), 171.0 (s), 170.9 (s), 170.4 (s), 169.5 (s), 169.0 (s), 133.9 (d), 128.7 (d), 75.0 (d), 69.6 (d), 66.1 (t), 63.1 (t), 61.4 (d), 59.3 (d), 57.3 (d), 55.7 (d), 55.7 (d), 54.6 (d), 49.0 (t), 48.6 (d), 48.1 (d), 47.4 (d), 46.6 (d), 41.3 (t), 41.2 (t), 39.1 (q), 38.5 (t), 38.2 (t), 37.3 (d), 36.8 (t), 34.1 (q), 34.0 (d), 31.6 (t), 31.1 (q), 31.0 (q), 29.9 (q), 28.8 (q), 27.7 (d), 25.9 (d), 25.6 (d), 24.9 (d), 24.8 (d), 24.1 (q), 23.2 (q), 23.2 (q), 23.2 (q), 23.2 (q), 22.8 (q), 21.2 (q), 20.9 (q), 19.7 (q), 19.3 (q), 18.4 (q), 18.1 (q), 17.4 (q), 16.2 (q), 15.3 (q)

From the analysis of the above physical and chemical properties, and the result of the further investigation of identification of chemical structure, the chemical structure of the compound (7) has been identified and assigned as follows.

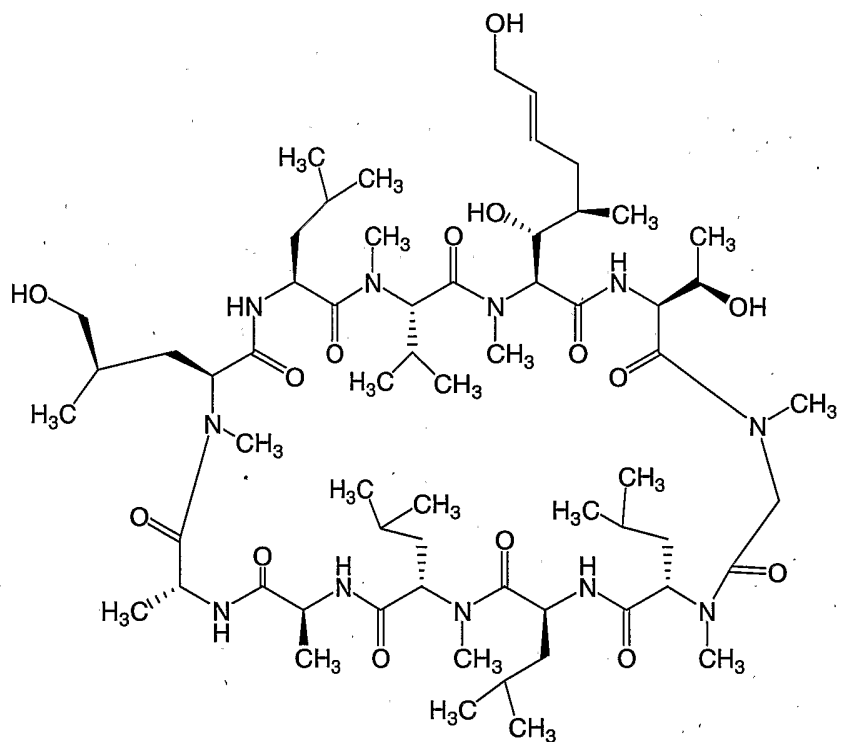
And the stereochemistry of N-methyl-hydroxyleucine in the compound(7) was determined by ¹³C-NMR data comparison with that

of the compound(4).

5

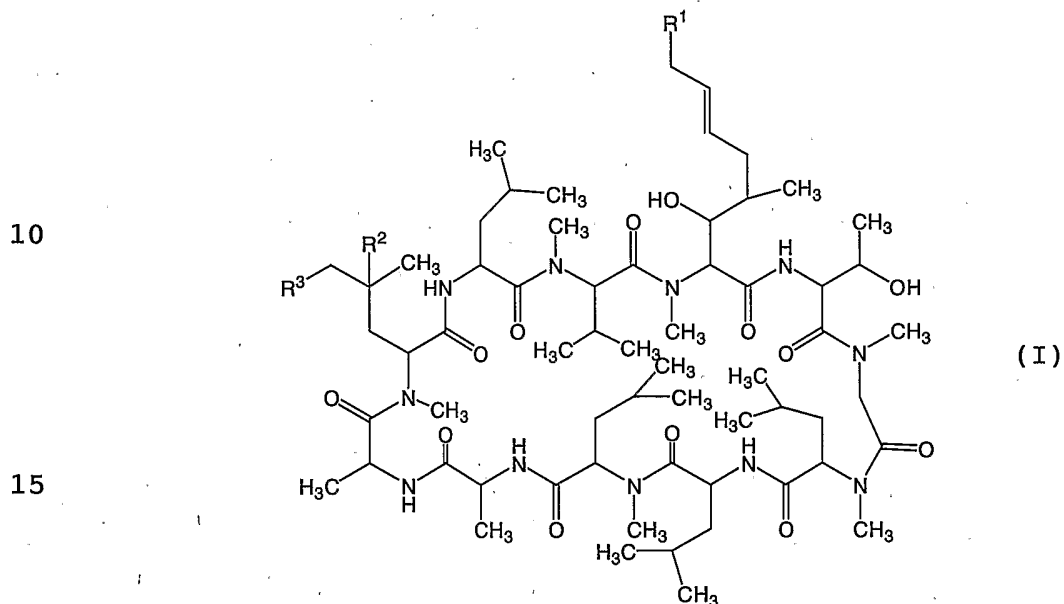
10

15



C L A I M S

1. A cyclic peptide compound of the following general
5 formula (I):



wherein

R^1 is hydrogen or hydroxy,

20 R^2 is hydrogen or hydroxy, and

R^3 is hydrogen or hydroxy,

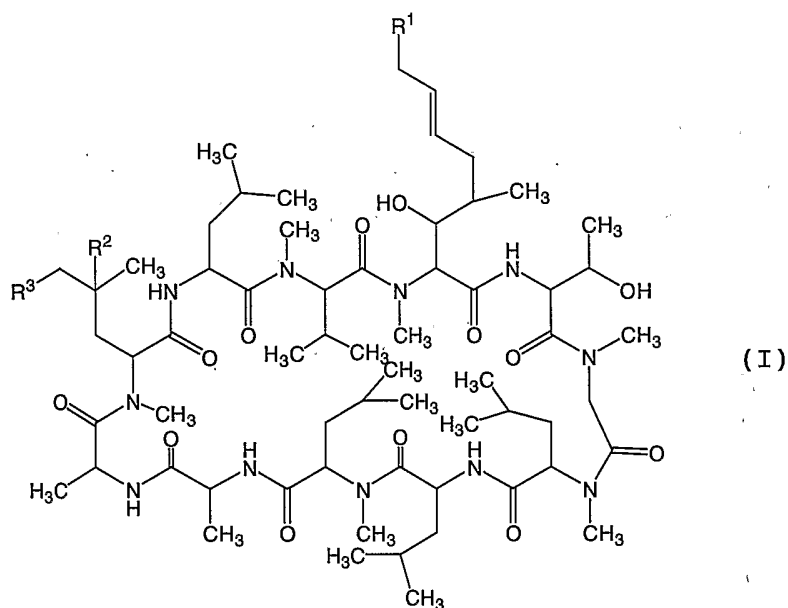
Providing that when R^1 is hydrogen,

then at least one of R^2 and R^3 is hydroxy,

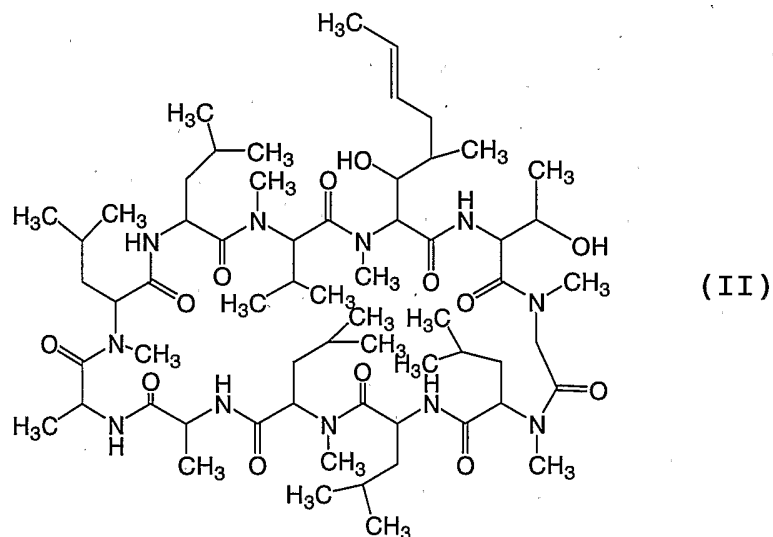
or a salt thereof.

25

2. A process of producing a cyclic peptide compound (I) of the
following formula (I):



15 [wherein R^1 , R^2 and R^3 are defined above], or a salt thereof, which comprises contacting a cyclic peptide compound (II) of the following formula (II):



30 or a salt thereof, with an enzyme obtained by culturing an enzyme-producing strain of microorganism belonging to the genus *Lentzea*.

3. The process of Claim 2, wherein the strain of microorganism is *Lentzea* sp. No. 7887.

4. *Lentzea* sp. No. 7887

5. A pharmaceutical composition which comprises, as an active
5 ingredient, a compound of claim 1 or a pharmaceutically
acceptable salt thereof in admixture with pharmaceutically
acceptable carriers or excipients.

6. Use of a compound of Claim 1 or a pharmaceutically
10 acceptable salt thereof for the manufacture of a medicament.

7. A compound of Claim 1 or a pharmaceutically acceptable salt
thereof for use as a medicament.

15 8. A method for the prophylactic and/or therapeutic treatment
of hepatitis C, which comprises administering a compound
of claim 1 or a pharmaceutically acceptable salt thereof
to a human being or an animal.

20 9. A commercial package comprising the pharmaceutical
composition of claim 4 and a written matter associated
therewith, wherein the written matter states that the
pharmaceutical composition can or should be used for
preventing or treating hepatitis C.

25 10. An article of manufacture, comprising packaging material
and the compound (I) identified in claim 1 contained within
said packaging material, wherein said the compound (I) is
therapeutically effective for preventing or treating
30 hepatitis C, and wherein said packaging material comprises
a label or a written material which indicates that said
compound (I) can or should be used for preventing or treating
hepatitis C.

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2005/021742

A. CLASSIFICATION OF SUBJECT MATTER C07K7/64 C12P21/02 C12R1/04 A61K38/13		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C07K C12P C12R A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, CHEM ABS Data, BIOSIS, WPI Data, PAJ		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/32447 A (FUJISAWA PHARMACEUTICAL CO., LTD; TOKYO METROPOLITAN ORGANIZATION FOR) 25 April 2002 (2002-04-25) The whole document; see particularly page 4, line 18	1-10
A	WATASHI K ET AL: "CYCLOSPORIN A SUPPRESSES REPLICATION OF HEPATITIS C VIRUS GENOME IN CULTURED HEPATOCYTES" HEPATOLOGY, WILLIAMS AND WILKINS, BALTIMORE, MD, US, vol. 38, no. 5, November 2003 (2003-11), pages 1282-1288, XP009047548 ISSN: 0270-9139 the whole document ----- -/-	1,5-10
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 7 March 2006		Date of mailing of the international search report 17/03/2006
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Groenendijk, M

INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2005/021742

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAKAGAWA MINA ET AL: "Specific inhibition of hepatitis C virus replication by cyclosporin A." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. 2 JAN 2004, vol. 313, no. 1, 2 January 2004 (2004-01-02), pages 42-47, XP004479114 ISSN: 0006-291X the whole document	1,5-10
A	SAKAMOTO K ET AL: "FR901459, A NOVEL IMMUNOSUPPRESSANT ISOLATED FROM STACHYBOTRYS CHARTARUM NO. 19392 TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES" JOURNAL OF ANTIBIOTICS, JAPAN ANTIBIOTICS RESEARCH ASSOCIATION, TOKYO, JP, vol. 46, no. 12, December 1993 (1993-12), pages 1788-1798, XP001109704 ISSN: 0021-8820 the whole document	1-10
A	YASSIN A F ET AL: "Lentzea gen. nov., a new genus of the order Actinomycetales" BIOSIS, 1995, XP002356553 abstract	2-4
P,A	WO 2005/032576 A (FUJISAWA PHARMACEUTICAL CO., LTD; TOKYO METROPOLITAN ORGANIZATION FOR) 14 April 2005 (2005-04-14) the whole document	1-10

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			JP 2004517818 T	17-06-2004
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WO 2005032576	A	14-04-2005	NONE	