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Structure of an α-Amylase Inhibitor Produced by Marine Actinomycete and its Lowering Effects *in vivo* of Glucose and Lipid in Blood*1

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A fraction of α-amylase inhibitor was extracted from the mycelia of a marine *Actinomycetes, Streptomyces* sp. No.2020 found in seawater. The fraction was purified using activated charcoal and Dowex 50W column chromatography. The basic structure of the fraction of α-amylase inhibitor (AI-2020) was identified as oxireno [e] pyrrolo [2, 1-b] benzoxazole-2, 3, 5, 6-tetraol, decahydro-7- (1-hydroxyethyl) 1a- (hydroxymethyl) -, structure formula 11, on the basis of the elementary analysis, mass spectrum, infrared (IR) and nuclear magnetic resonance (NMR) spectra of the hydrolyzate of AI-2020. The AI-2020 suppressed the elevation of blood glucose *in vivo* after oral administration of starch. In streptozotocin diabetic rats receiving AI-2020, triglyceride, free fatty acid, total cholesterol and β-lipoprotein were reduced, and AI-2020 ameliorated diabetes syndrome including symptoms such as hyperglycemia. These findings indicate that AI-2020 may be useful for control of some carbohydrate-dependent diseases such as diabetes, obesity and hyperlipemia.

1 Introduction

Many amylase inhibitors (AI) were discovered frequently in plants and in land microorganisms, and they are considered to have preventative and therapeutic effects for geriatric diseases such as diabetic mellitus, obesity and hyperlipidemia. At present, α-glycosidase inhibitor, which has a low molecular weight, Acalbose[1-3] developed by Bayer Pharmaceutical Co. and AO-128[4] developed by Takeda Pharmaceutical Co., are being investigated as remedies for diabetes mellitus. The AI, has a high molecular weight, which has been identified hitherto, could be developed as functional foods because the effect of AI might be consequently almost the same as that of food fiber[5] which is currently employed in supplemental remedies for the above diseases. While AIs reported until now were all derived from soil

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organisms, Imada et al[6,7] isolated an AI-producing microbe at the Aburatsubo Bay and determined the whole amino acid sequences of Marinostatin from *Alteromonas* sp..

We isolated and cultivated an AI-producing *Actinomycetes* in samples of seawater collected from the Indian Ocean and the Pacific Ocean. AI was isolated and purified, and the structure of the AI hydrolysate was partially elucidated. Furthermore, the effects of the AI were studied on carbohydrate (glucose) and lipid metabolism in streptozotocin-dosed rats.

2 Materials and methods

2.1 Collection and Isolation of Microorganisms

The samples of seawaters were collected by training ship,"Koyo-Maru", from surface seawater near Shimonoseki, Brisbane, Auckland, Nukualofa, Honolulu and from October 28, 1987 to January 22, 1988. More, the samples of seawater were collected by the other training ship,"Tenyo-Maru", from the depth of 100 to 500 m in the Indian Ocean from May 18, 1988 to May 23, 1988. All samples were kept frozen until the start of the investigation. The detailed sampling sites are shown in Fig.1. After applying the sample seawater aseptically to

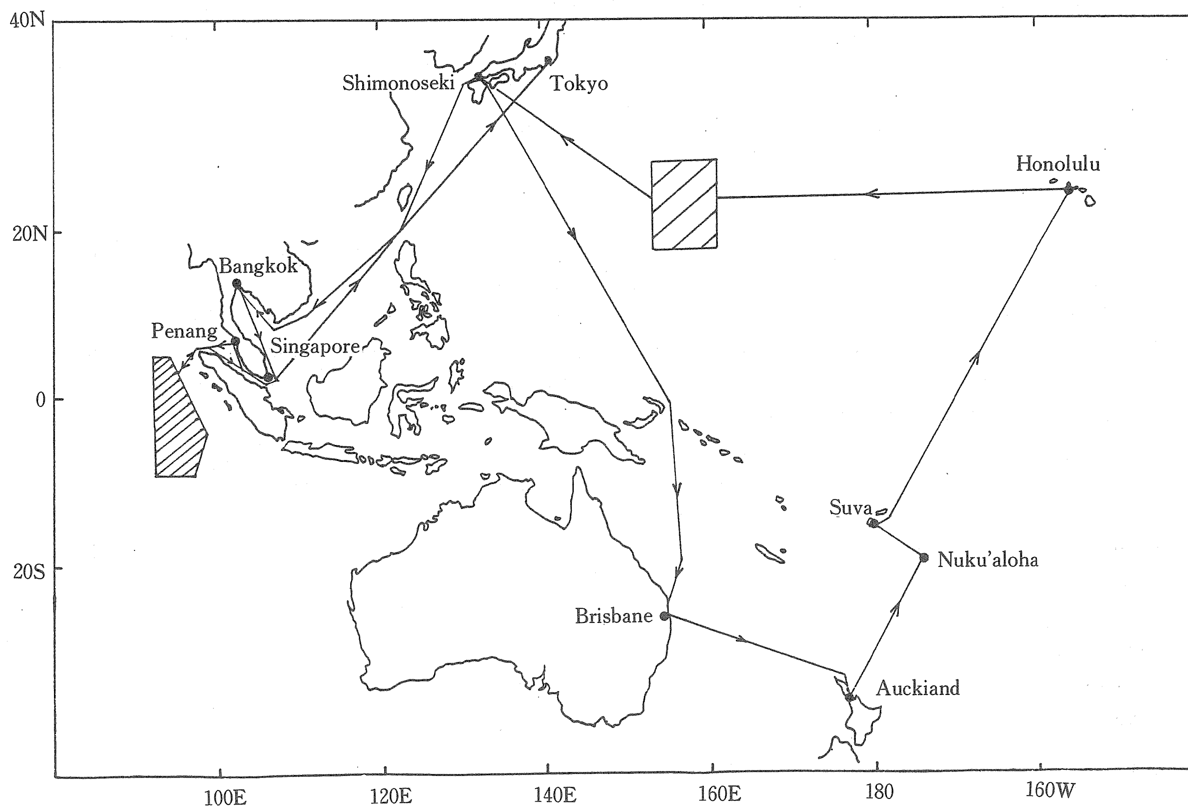

Fig. 1. Sampling station.

medium B for *Actinomycetes* developed by Imada et al. (Glucose 10.0 g, L-Asparagine 0.5 g, KH_2PO_4 0.5 g, Bactoagar 15.0 g, seawater 1.0 l, pH7.0) in a plate (ϕ 90mm), we conducted the slant-culture was at 24 ± 5℃ for 14 days and finally isolated the colonies of AI-producing *Actinomycetes* were isolated.

2.2 Primary Screening for AI and Measurement of Amylase Inhibitory Activity

The slant-cultured mycelia were incubated in a 500ml Sakaguchi flask containing 100ml of AI-producing liquid medium (Soluble starch 40.0 g, Polypeptone 5 g, Beef extract 5 g, pH7.2) at 28±2℃ for 6 days on a culture shaker. After completion of incubation, the medium solution was filtered with a filter paper (Toyo filter No.2).

The amylase inhibitory activity of the filtrate (AI solution) was measured as follows; 0.9 ml of α-amylase (liquid type originating from bacteria, Sigma Co.) solution that was adjusted 20 μg/ml in 0.2M acetate buffer solution (pH5.5) and 1.0ml of 2.5% soluble starch solution were added to 0.1ml of AI solution and incubated at 37±2℃ for 10 min. Then the reaction was stopped by adding 5ml of 1N acetic acid. After 10ml of 5×10^{-4}N iodine solution was added to 0.3ml of the reaction solution, the absorbance at 680nm was measured by a Blue Value method.[8] The inhibition percentage was showed as in the following formula; The inhibition (%) = $(E_c - E_s) / (E_c - E_B) \times 100$, wherein Es means the absorbance of the AI-solution, Ec indicates the absorbance when a 0.2M acetate buffer solution was applied instead of the AI-solution and E_B is the absorbance when the acetic acid solution to stop the reaction is added in advance of the reaction.

2.3 Preparation of α-Amylase Inhibitor

Two hundred seventy six grams of the activated charcoal powder (Wako Chemical Co.) was added to 9.2 l of the culture supernatant, which was obtained by centrifugation of 10 l of the cultivated broth at 10,000rpm for 10 min at 10℃, and then the mixture was stirred for 1 h. The activated charcoal powder which was obtained by suction filtration, was sufficiently washed with deionized water. Two point five liters of 30% isopropyl alcohol which had been adjusted to pH2.5 with 1N-HCl, was added to the activated charcoal powder, and the mixture was stirred for 30 min, and then 2.4 l of the filtrate was obtained by suction filtration. Five hundred milliliters of the filtrate which was obtained by concentration in vacuo at 38℃ was applied to Dowex 50W column (H^+ form, 20 × 400mm), and the column was sufficiently washed with deionized water. After elution with 1 l of 2N-NH_4OH, the eluate was concentrated in vacuo at 38℃ and dried under vacuum to obtain 10.7g of crude AI.

2.4 Preparation of Amino Oligosaccharide by Hydrolysis of AI

One hundred milliliters of 1N-HCl were added to 10.7 g of the crude AI powder and the powder was hydrolysed in a sealed tube in an oil bath (120℃) for 3 h under reflux. After cooling and removing as much HCl as possible concentration in vacuo, the hydrolyzate was absorbed onto an activated charcoal powder column (23 × 323mm) and the column was sufficiently washed and eluted by gradient method from 1 l of deionized water to 1 l of 3% butyl alcohol. The amino oligosaccharide in the eluate was detected with a gas chromatography (GC). Reagent; TMS-PZ (trimethylsilylating reagent, Tokyo Kasei Co.), column; 3% SE-30 Chromosorb W-HP (60/80, 3.0 × 1,000mm, Gasukuro Kogyo Co.), column temp.; 130-230℃, programmed temperature rising at 10℃ /min., inject temp.; 260℃, carrier gas; He, flow rate; 40 ml/min.. The eluate containing amino oligosaccharide was adsorbed again on to Dowex

50W column (H^+ form, 24 × 124mm) and washed sufficiently with deionized water. After eluting with 300ml of 2N-NH_4OH, the eluate was concentrated in vacuo at 38℃. To purify further, the amino oligosaccharide was applied to high performance liquid chromatography (HPLC). Reversible column; dextropak (8 × 100mm, Waters Ltd.), mobile phase; distilled water, flow rate; 1 ml/min., detection; differential refractometer (Refractive Index; RI).

2.5 Instrumental Analyses of Amino Oligosaccharide

Various spectra of amino oligosaccharide were measured on the following apparatus; Shimadzu UV-300 spectrophotometer for UV spectrum, Shimadzu FTIR-4000 spectrophotometer for IR spectrum, Hitachi double beam mass spectrometer for FD-MS, JEOL FX-200 and GX-270 for ^1H-^1H and ^{13}C-^1H COSY NMR spectra, and chemical shifts were given in ppm (in δ) relative to TMS (0 ppm) as an employed internal standard. Elementary analysis was conducted using a Yanagimoto MT-3 to measure carbon (C), nitrogen (N) and hydrogen (H).

2.6 Effects of AI on Blood Glucose Levels in Mice Dosed with Starch Orally

The animals employed were SPF, 4 week old male CD-1 (ICR) mice, weighing 20 g, purchased from Nippon SLC Co.. After acclimatizing for one week in a room with constant temp. and humidity (23 ± 3℃, 60 ~ 65%), they, weighing 30 ± 1 g, were used.

After fasting for 24 h, 0.25ml of soluble starch (1g per kg of mice, Kanto Chemicals Co.) dissolved in a physiological saline (0.9% NaCl) solution was orally given to AI-dosed groups (10, 20 and 40 mg/kg). Afer dosing, blood samples of 50μl each were taken sequentially from orbital plexus venosus, and blood glucose levels were measured with a Wako kit (Wako Chemical Co.).

2.7 Effects of AI on Diabetic and Hyperlipidemic Rats Induced by Streptozotocin

Six week old, Wistar male rats, were acclimatized for a week with feeding food powder (CA-1, Nippon Crea Co.) and those weighing 170 ± 5 g were used.

Streptozotocin (Upjohn Co.) was dissolved in physiological saline solution at a concentration of 1ml/45mg/body weight (kg) and then it was injected via tail vein into the rats, which were then bred for one week. Blood samples were collected from a tail cut. The glucose levels were measured with Wako kits to select rats with levels between 270-330 mg/dl, which were regarded as STZ-diebetic rats and divided into groups.

CA-1 food powder and AI powder dissolved in water were mixed and 15 g was given to each rat daily. The AI-dose differed between as follows; 100, 200 and 300 mg/kg. After dosing for 3 weeks, the rats were subjected to thoracotomy under ether anesthesia and blood samples were collected by cardiac puncture. The sera obtained by centrifugation was measured for triglyceride (TG), free fatty acid (NEFA), total cholesterol (TCH), β-lipoprotein (β-LP) and blood glucose levels, with Wako kits.

3 Results

3.1 Screening of Amylase Inhibitor

From the surface seawater of the Pacific Ocean and the seawater in 100-500m depths in the Indian Ocean, 3,750 strains of microorganism colonies were isolated. The subcultured microorganisms were incubated with AI producing liquid media under shaker, and then amylase inhibitory activities of the culture filtrates were measured for the purpose of the AI

primary screening. After adsorbing and eluating AI by means of an activated charcoal treatment from broth cultures with strong amylase inhibitory activities, the eluate was further subjected to strong acid cation exchanger column chromatography to adsorb and eluate AI and finally crude AI powder was obtained. Furthermore, after acid hydrolysis (1N-HCl, 120℃, 3h) of the crude AI powder under reflux, amino oligosaccharide was isolated by means of an activated charcoal column chromatography. The AI secondary screening was conducted by detecting amino oligosaccharides with the GC method and by comparing them with well-known substances. As a result, 360 strains were isolated out of 3,750 strains in the AI primary screening. *Streptomyces* sp. N0.2020, which produced AI including amino oligosaccharide differed from well-known substances, was obtained for AI-producing strains on the AI secondary screening.

3.2 Isolation and Purification of Amino Oligosaccharide Derived from AI Hydrolysates

Ten point seven grams of the crude AI-2020 powder were obtained from 10 *l* of culture broth of *Streptomyces* sp. No.2020 by adsorption and elution of AI on an activated charcoal and then on a Dowex 50W (H^+ form) column. Furthermore, from the hydrolyzates of the crude AI-2020 powder, 680 mg of purified amino oligosaccharide was obtained by treatment with activated charcoal column, Dowex 50W (H^+ form) column chromatographies and HPLC finally.

3.3 Structure Elucidation of Amino Oligosaccharide

1) Inference of partical structures

The molecular formula was determined by means of mass spectral and elemental analysis. The FD-MS spectrum showed the molecular ion peak at m/z 319. Elementary analysis; found, C,48.71, H,6.59, N,4.32%. Calcd. for $C_{13}H_{21}NO_8$; C,48.88, H,6.78, N,4.38%.

Table 1. Network in ^{1}H-NMR spectrum

No. of proton	Conjugated bond	Network
1	1−9	
2	9−2−12	
3	3−6	1−9−2−12−10−13
4	4−8	
5	7−5−11	
6	3−6−11	---→
7	7−5	
8	8−4	---→3−6−11−5−7
9	1−9−2	
10	12−10−13	
11	5−11−6	
12	2−12−10	4−8
13	13−10	

The ^{1}H-^{1}H COSY NMR spectrum (shown in Fig.2, Table 1) and ^{13}C-^{1}H COSY NMR spectrum (shown in Fig.3, Table 2) elucidated the correlation of those carbons and protons. From the results of both COSY NMR, the following four partial structures were considered to exist (shown in Fig.4).

1, 2, 3 and 4

Because of the fact that the protons in partial structure **3** was a singlet (s) in ^{1}H-NMR spectrum, **3** was considered to bond with either a hetero atom or a quarternary carbon atom. It was not conceivable that the both ends of 3 bonded with hetero atoms because of the fact of the observed ^{13}C-NMR chemical shift effect. Therefore, one end was considered to bond with a quarternary carbon atom (shown in Fig.4).

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Also, because of the ^{13}C-NMR chemical shift, one of the two carbon atoms at the both ends of the partial structure **2** was believed to bond with a quarternary carbon atom. Therefore, the following two partial structures were believed to exist (shown in Fig.4).

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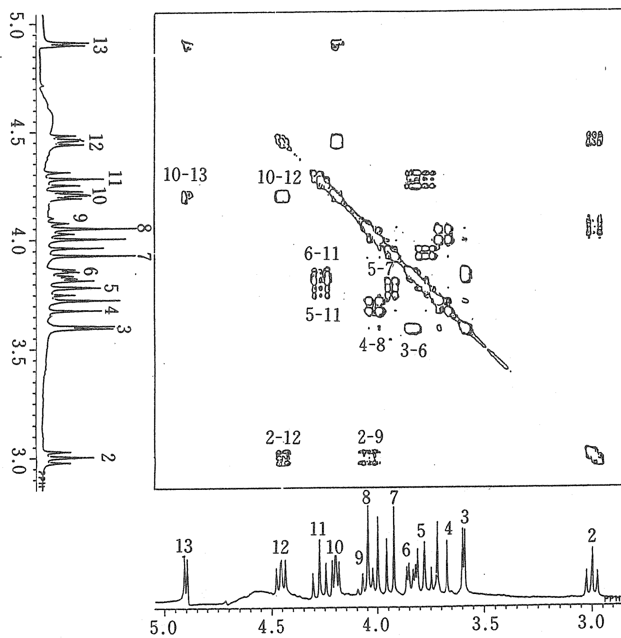


Fig. 2. ^{1}H-^{1}H COSY NMR spectrum.

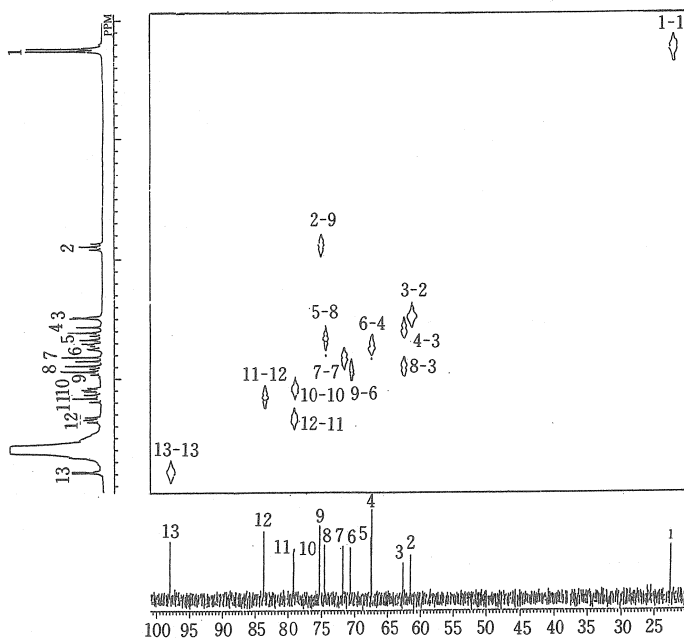
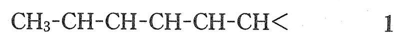


Fig. 3. ^{13}C-^{1}H COSY NMR spectrum.

Table 2. Chemical shift, conjugated bond to proton and atomic group

No. of carbon	Chemical shift* (δ)	Conjugated bond to proton	Atomic group
1	22.3	1	CH_3
2	61.1	3	CH
3	62.3	4, 8	CH_2
4	67.1	6	CH
5	67.3	-	C
6	70.3	9	CH
7	71.3	7	CH
8	74.1	5	CH
9	74.9	2	CH
10	78.7	10	CH
11	78.8	12	CH
12	83.3	11	CH
13	97.6	13	CH

* Chemical shifts are ppm (δ) downfield from internal TMS in DMSO-d_6.



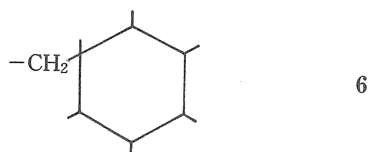


Fig. 4. Partial structure.

2) Determination on number of "-OH" and "-NH-" groups

It was investigated as to whether or not hydrogen atoms bonded with nitrogen and oxygen atoms in the ^{13}C-NMR spectrum by using a double insulator tube of ϕ 10 and ϕ 15 (shown in Fig.5, Table 3). The result showed that there were seven carbon atoms (an odd number) bonding with X (X is either a nitrogen or an oxygen atom). Therefore, it was considered that there was one nitrogen atom contained in X and it was inferred that XH were all -OH and the remaining hetero atoms were one of -N- and two of -O-.

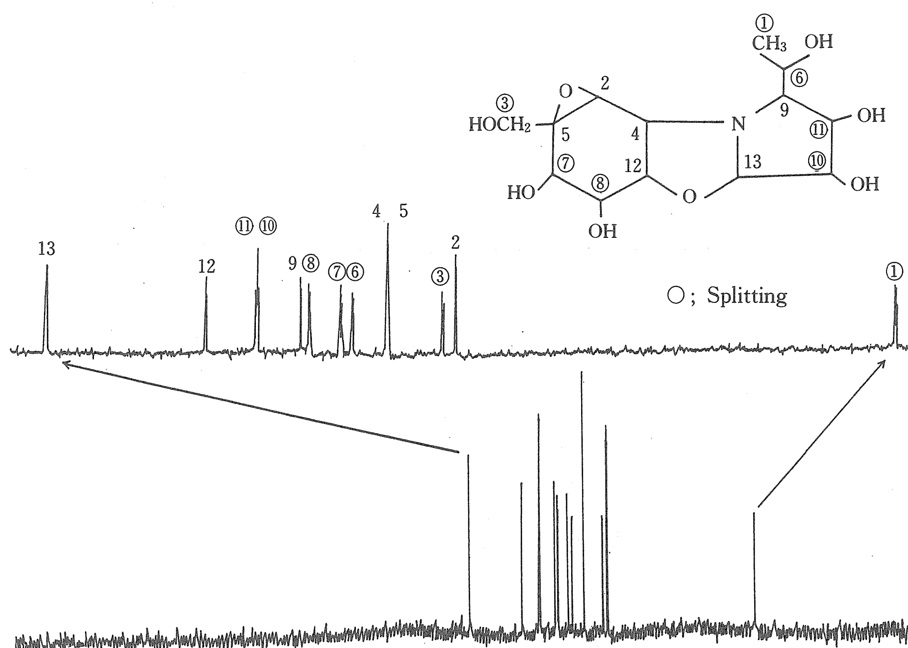
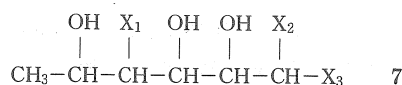


Fig. 5. Bond relationship of $-N<$ and -O-.

Table 3. Bond relationship of -N< and -O-

Inferential structure formula	Nitrogen atom	Oxygen atom	Oxygen atom
9	1, 4, 2	3, 6	6, 7
10	1, 5, 2	3, 4	6, 7
11	1, 6, 2	4, 5	3, 7
12	1, 7, 2	4, 5	3, 6
13	4, 5, 2	1, 3	6, 7
14	6, 7, 2	1, 3	4, 5

The abbreviation of the groups were the same as legend in Fig. 7.



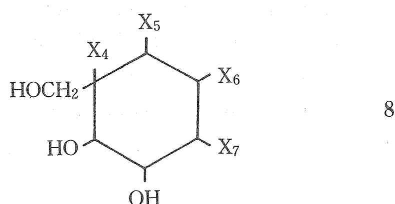


Fig. 6. Partial structures.

3) Inference of structure formula

To assemble a structure formula by combining the partial structures 7 and 8 (shown in Fig.6), any one of X1 ~ X3 in 7 must be a nitrogen atom.

7 and 8

Since X2 and X3 are equivalent, either X1 or X2 is a nitrogen atom. Under the assumption that X1 or X2 bonds with conjuated X such as X4 and X5 or X6 and X7 and does not bond with a non-conjugated hetero atoms such as X4 and X6 or X5 and X7, structure formulas, **9**, **10**, **11**, **12**, **13** and **14** are assemble (shown in Fig.7).

9, **10**, **11**, **12**, **13** and **14**

Though it is considered that there are $\delta c83.3$ of epoxy ring in **9**, **10** and **13** and $\delta c83.3$ bonding with the nitrogen atom in **12** and **14**, and these inferential structure formulas are not valid one. In **11**, all twelve carbon atoms except methyl group bond directly with either nit-

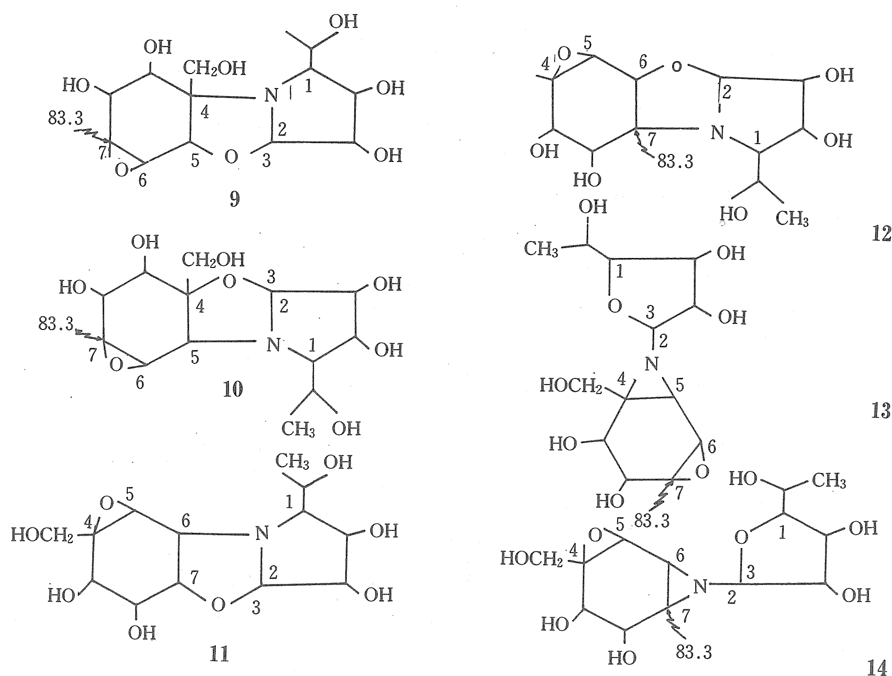


Fig. 7. Inferential structure formulas.

rogen or oxygen atoms, as shown by the chemical shifts such as glucose etc. Carbon atoms at positions 4 and 5 in the six membered ring are δc67.1 and δc61.1, respectively. It is reasonable to believe these chemical shifts are moving somewhat to upfield, these may be valid that the inferential structure formula includes an epoxy ring. When standard *J* values are compared with the actual measured *J* value of the sample (shown in Fig.8) , it was found that these values agreed well with each other. From the above mentioned finding, it appears that structure formula **11** (shown in Fig.7) is the most valid. Meanwhile, a significant absorption of a hydroxyl group on the IR spectra was observed, and the IR absorption on 2,965 cm^{-1} was somewhat weak and so probably is on a lower wavenumber side. Therefore, it is imposible to judge the presence or absence of an epoxy group from the IR spectra.

3.4 Effects of AI-2020 on Blood Glucose Levels in Mice Dosed with Starch Orally

As shown in Table 4, the blood glucose levels in mice increased and these levels lasted at least 4 h after dosing starch at a dose of 1 g per 1 kg body weight. The concurrent dosing of 1 g/kg of starch and 10mg, 20mg or 40mg/kg of AI-2020 inhibited the blood glucose levels in mice. Thus, hyperglycemia caused by ingestion of starch was reduced to 60% of the control group with the oral dosing AI-2020 to the mice.

3.5 Effects of AI-2020 on STZ-diabetic and Hyperlipidemic Rats

Triglyceride, total cholesterol, free fatty acid, β-lipoprotein and blood glucose levels obtained after AI-2020 mixed with feed had been given to STZ-diabetic rats for 3 weeks are

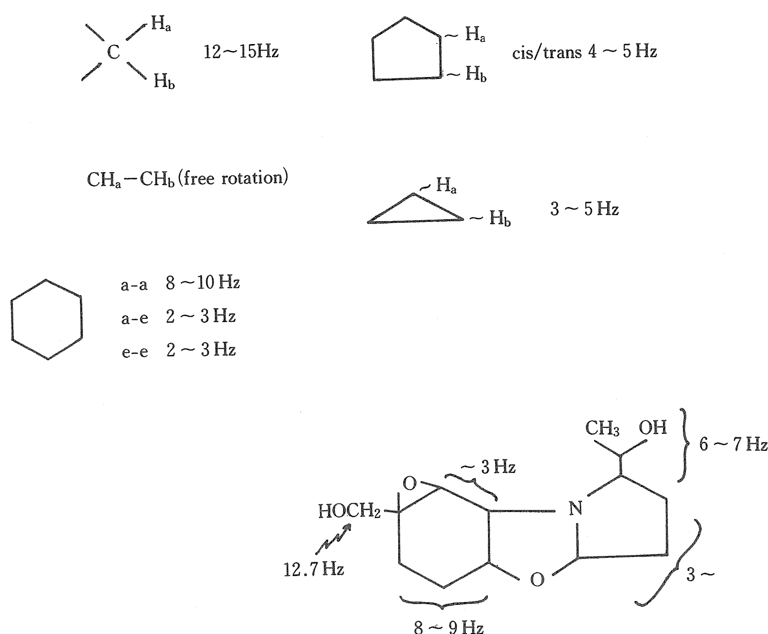


Fig. 8. Standard *J* values and *J* values of sample.

shown in Table 5. The results indicate that the blood glucose levels and lipidemic parameters in AI-2020 dosed groups were significantly reduced in a dose-dependent manner compared with the control group.

4 Disscussion

Three thousand seven hundred fifty strains of *Actinomycetes* like microorganisms isolated from seawater of the Pacific Ocean and the Indian Ocean were cultured in solution and the culture broth was studied for AI producing capabilities (the primary screening test) . Futhermore, from the culture broth of 360 strains producing AI, the crude AI powder was isolated and purified by treatment with activated charcoal and Dowex 50W (H^+) column chromatography. From the acid hydrolysis of the crude AI, amino oligosaccharide was detected with the GC method and compared with known substances (the secondary screening

Table 4. Effect of AI-2020 on blood glucose level of fasting mice after oral administration of starch

Dose \ Time (h)	0	1	2	3	4
Control with starch	43.3±5.8	105.0±3.3	102.6±5.4	94.5±6.1	87.5±6.6
AI (10mg) + starch	53.7±9.8	83.0±7.0	91.0±5.3	98.2±6.3	88.6±7.0
t-value	3.16*	9.85***	5.31***	1.46	0.40
AI (20mg) starch	48.7±12.6	76.8±6.6	78.8±2.6	75.4±5.3	77.3±6.7
t-value	1.35	13.24***	13.76***	8.19***	3.76**
AI (40mg) starch	46.8±5.2	73.0±7.1	73.2±4.3	72.0±1.8	68.7±14.7
t-value	1.56	14.16***	14.75***	12.26***	4.04***

Data represent means ± SD (n=12).
Significantly different (*p <0.05, **p <0.01, ***p <0.001).

Tabel 5. Serum lipids, glucose in STZ diabetic rats treated with AI-2020 for 3 weeks

Dose \ Item	TG (mg/dl)	NEFA (mEq/1)	TCH (mg/dl)	β-LP (mg/dl)	Glucose (mg/dl)
Control (STZ; 45mg/kg)	203.1±7.8	0.604±0.073	156.7±18.7	253.3±19.5	380.3±6.7
AI (10mg/kg)	141.2±9.6	0.360±0.021	136.0±8.6	225.2±7.7	275.3±5.0
t-value	17.34***	11.13***	3.48**	4.64***	43.51***
AI (20mg/kg)	118.0±8.0	0.275±0.019	117.1±5.3	206.2±5.3	230.1±4.8
t-value	26.38***	15.11***	7.06***	8.07***	63.13***
AI (40mg/kg)	75.4±4.0	0.202±0.048	102.6±7.8	177.3±6.8	179.9±7.4
t-value	50.46***	15.94***	9.25***	12.75***	69.54***

Data represent means ± SD (n=6).
Significantly different (**p <0.01, ***p <0.001)

test). From the results, the new amino oligosaccharide derivative was detected in an AI-producing strain, *Streptomyces* sp. No.2020, and its structure formula was identified by conducting the structure elucidation with the use of such instrumental analyses as elementary analysis, MS, NMR and IR.

The production of blood glucose and its adjustment after food ingestion are made by a variety of mechanisms, but its maladjustment causes adult dieases such as diabetes mellitus, obesity and hyperlipidemia. In general, it is well known that AI given with meals inhibits the digestion of the starch in the meal and the starch is eliminated intact as a fecal matter from the system. In this starch load test in mice, AI-2020 (10, 20 and 40 mg/kg) orally dosed mice demonstrated dose-dependent significant hyperglycemic activities, and so AI-2020 was thought to be effective for the prevention of obesity. Furthermore, it was revealed that AI-2020 ameliorated prognosis of STZ-induced experimental diabetes mellitus rats. In this experiment, AI-2020 given orally (100, 200 and 400 mg/kg) not only decreased blood glucose levels but also improved the secondary hyperlipidemia caused by the onset of diabetes mellitus in STZ-dosed diabetes mellitus rats.

In the meantime, food fibers now becoming the object of public atention as functional foods, can be expected to improve tolerance to glucose and to save insulin secretion in patients with diabetes mellitus. The mode of action is thought to depend principally on the inhibition of increase in blood glucose levels by delaying absorption of nutrients from the intestine as a result of extended the residing time of food in the stomach. Futhermore, upon digestion and absorption in the small intestine, some nutrients may be adsorbed by such gelatinized fibers and excreted in feces and so digestion and absorption would be distrupted. In addition, easy diet restriction due to a feeding of gastric fullness, accelerated secrection of bile acid, improvement in serum lipids due to inhibition of absorption in the intestine, and effects on absorption of nutrients tract hormones would be contributing factors. From this point of view, it is considered that AI may have physiological activities with a basis of mode similar to the action of food fibers. It is also known that serum cholesterol is reduced in a way similar to that of food fibers such as guar gum, glucomannans, pectin etc. Antihyperlipidemic activities of AI may be due to various reasons such as mediation by bile acid, increased excretion of lipids into feces, and inhibition of absorption of cholesterol. As discussed above, AI will be useful in therapeutic remedies of diabetes mellitus and obesty with resect to excretion of daily urinary glucose, and reduction of blood glucose and serum lipids, especially that of cholesterol. But, large amount of AI bring about inevitable nausea, vomiting and diarrhea, and possibily side effects such as flatulence, hyperperistalis, increase in the amount of feces etc. Therefore, it is necessary to conduct a longterm observation on the possible effects on human bodies.

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海洋性放線菌由来のα-アミラーゼ阻害物質の構造解析と *in vivo*における血糖・脂質低下作用

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海洋微生物が生産するα-アミラーゼ阻害物質検索過程のなかで，太平洋，インド洋海域の海水から分離した菌株 *Streptomyces* sp. がα-アミラーゼ活性阻害効果を示すことを見い出した。活性炭とDowex50W カラムクロマトグラフィーにより新規α-アミラーゼ阻害物質 AI-2020を単離精製した。阻害物質 AI-2020は配糖体であった。AI-2020の基本骨格構造は，MS スペクトルと元素分析より分子式$C_{13}H_{21}NO_8$であり，また，IR，^1H-^1H, ^{13}C-^1H COSY NMR スペクトル等の機器分析により新規アミノオリゴ糖誘導体であった。AI-2020は，マウスにおける糖質負荷後の血糖値上昇を抑制し，ストレプトゾトシン糖尿病ラットでは血中脂質低下効果を示した。