ISOLATION ON TREHALAMINE, THE AGLYCON OF TREHAZOLIN, FROM MICROBIAL BROTHS AND CHARACTERIZATION OF TREHAZOLIN RELATED COMPOUNDS

Osamu Ando, Mutsuo Nakajima*, Kiyoshi Hamano, Kazuko Itoi, Shuji Takahashi, Yasuyuki Takamatsu, Akira Sato, Ryuzo Enokita†, Takao Okazaki†, Hideyuki Haruyama†† and Takeshi Kinoshita††

Fermentation Research Laboratories, Sankyo Co., Ltd.,
††Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd.,
1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140, Japan
†Tsukuba Research Laboratories, Sankyo Co., Ltd.,
Miyukigaoka 33, Tsukuba-shi, Ibaraki Prefecture, 305, Japan

(Received for publication December 18, 1992)

Trehalamine, (3a*R*,4*R*,5*S*,6*S*,6a*S*)-2-amino-4-(hydroxymethyl)-3a,5,6,6a-tetrahydro-4*H*-cyclopent[*d*]oxazole-4,5,6-triol (**1**) and D-glucose were obtained by acid hydrolysis of trehazolin (**3**), a trehalase inhibitor produced by actinomycetes. More vigorous hydrolytic treatment of trehazolin afforded an aminocyclitol, (1*R*,2*S*,3*R*,4*S*,5*R*)-5-amino-1-(hydroxymethyl)cyclopentane-1,2,3,4-tetraol (**2**). Trehalamine, the aglycon of trehazolin, was also found in the culture broths of two trehazolin producing strains, *Micromonospora* sp. SANK 62390 and *Amycolatopsis* sp. SANK 60791. These trehazolin related compounds trehalamine (**1**) and **2** were poor inhibitors of trehalase (**1**; IC_{50} 1.8×10^{-4} M, **2**; $>5.0\times10^{-4}$ M). On the other hand they inhibited more potently rat intestinal sucrase (**1**; IC_{50} 6.8×10^{-5} M) and sweet almond β-glucosidase (**2**; IC_{50} 5.6×10^{-6} M) than trehazolin.

In the previous paper, we reported the isolation and characterization of trehazolin (**3**), an inhibitor of trehalase produced by *Micromonospora* sp. SANK 62390[1]. Trehazolin showed a specific and potent inhibitory activity against trehalase and was shown to have a glucosyl moiety and a unique aglycon part in its structure. Since the aglycon portion of trehazolin seems to account for the trehalase inhibitory activity, it will be informative to assess the enzyme inhibitory properties of the aglycon to understand the nature of the activity of trehazolin. Furthermore, in respect to the microbial biosynthesis of the unique metabolite, it is of interest if the aglycon itself is present in the broths of the trehazolin producing microorganisms. Therefore, we attempted to characterize the aglycon and related compounds by hydrolysis of trehazolin and further to discover them from culture broths of trehazolin producers.

On the first attempt of acidic hydrolysis of trehazolin at 100°C, the sole product was a 5-membered

Fig. 1. The structures of trehalamine (**1**), **2**, and trehazolin (**3**).

Trehalamine (**1**) **2** Trehazolin (**3**)

aminocyclitol (**2**), which corresponds to a partial structure of the aglycon. Further efforts revealed that the aglycon (**1**), and D-glucose could be isolated under milder hydrolytic conditions. The aglycon (**1**) was named trehalamine when it was later found to be a natural product. In addition, formation of **2** from trehalamine on acidic treatment was confirmed. Through the search for these compounds in culture broths, we successfully isolated and purified trehalamine from culture broths of trehazolin producers, *Micromonospora* sp. SANK 62390 and *Amycolatopsis* sp. SANK 60791. Enzyme inhibitory activities of these compounds are also described.

Hydrolysis of Trehazolin

Fifty mg of trehazolin were dissolved in 5 ml of 4 N hydrochloric acid, and heated at 100°C for 24 hours in five fused ampuls. The solution was diluted in water and concentrated to dryness under reduced pressure. The residue was dissolved in water and evaporated again to remove hydrochloric acid. After this evaporation procedure was repeated three more times, the residue was dissolved in 20 ml of water, adjusted to pH 6.0 with aq NaOH solution, and applied to a column of Amberlite CG-50 (NH_4^+ type, 5 ml). After the column was washed with deionized water, the adsorbed material was eluted with 0.2 N NH_4OH. The fractions were monitored by TLC (see Physico-chemical Properties), and those showing a spot positive to ninhydrin reaction were combined, concentrated under reduced pressure, and lyophilized to yield 19 mg of pure **2** as a basic white powder.

Since the aglycon of trehazolin was not detected in the reaction described above, acid hydrolysis of trehazolin under milder condition was attempted. A solution of trehazolin (200 mg) in water (40 ml) was adjusted to pH 2.0 by addition of 1 N hydrochloric acid and stirred at 84°C on an oil bath for 72 hours. The hydrolysate was concentrated to dryness under reduced pressure, and the residue dissolved in 10 ml of water. The solution was adjusted to pH 6.0 with aq NaOH solution, and loaded on a column of Amberlite CG-50 (NH_4^+ type, 40 ml). After the column was washed with water, adsorbed material was eluted with 0.5 N NH_4OH. The fractions were monitored by HPLC (see below), and fractions containing trehalamine were combined, concentrated under reduced pressure, and lyophilized to yield 52 mg of semi-purified trehalamine as a white powder. The powder was dissolved in hot MeOH and subjected to crystallization to yield 30 mg of colorless plates.

Glucose, which appeared in flowthrough fractions of Amberlite CG-50 column chromatography described above, was monitored by TLC on SiO_2 plate (Merck Art. 5715) with the developing solvent of CH_3CN - AcOH - H_2O (6 : 1 : 3) by visualization with sulfuric acid. Fractions containing glucose were combined, and concentrated to dryness under reduced pressure. The crude material was purified by preparative HPLC using a column of NH_2-4251-N (Senshu Scientific) with the solvent of 84% CH_3CN - H_2O and detection of refractive index. Effluent containing glucose (retention time from 18 to 21 minutes) was collected, concentrated, and lyophilized to yield 39.4 mg of glucose.

In order to characterize the process of hydrolysis, formation of **2** by acidic treatment of trehalamine was confirmed. Powder of trehalamine (16 mg) was dissolved in 2 ml of 6 N HCl, and heated at 100°C for 24 hours in a fused ampul. The solution was diluted with water, and hydrochloric acid was distilled off by evaporation. After the procedure was repeated for three additional times, the residue was dissolved in 20 ml of water, adjusted to pH 6.0 with aq NaOH solution, and applied to a column of Amberlite CG-50 (NH_4^+ type, 20 ml). After the column was washed with water, adsorbed material was eluted with 0.2 N NH_4OH. Fractions containing **2** were collected, and concentrated under reduced pressure. The concentrate

(5 ml) was applied to a column of Dowex 1-X2 (OH^- type, 50 ml), washed with deionized water, and elution was carried out with 20% MeOH - H_2O. The ninhydrin active fractions were combined, concentrated, and lyophilized to yield pure powder of **2** (6.2 mg).

Fermentation of *Amycolatopsis* sp. SANK 60791 and Purification of Trehalamine (**1**)

For the isolation of trehalamine from microbial broths, a sample of trehalamine obtained by acid hydrolysis was used as a reference compound. The strain *Amycolatopsis* sp. SANK 60791 was found as a trehazolin producer following *Micromonospora* sp. SANK 62390[1]. A loopful of *Amycolatopsis* sp. SANK 60791 was inoculated from a slant into two baffled 500-ml Erlenmeyer flask containing 80 ml of seed medium composed of glucose 1%, sucrose 1%, glycerol 1%, oatmeal 0.5%, soybean meal 2%, Casamino acid 0.5%, pressed yeast 1%, $CaCO_3$ 0.1%, Disfoam CB-442 0.01%, and adjusted to pH 7.0 before autoclave. The culture was incubated with a rotary shaker at the speed of 210 rpm at 28°C for 96 hours. Seventy five ml of the culture were transferred to each of two 30-liter jar fermentors containing 15 liters of production medium consisting of glucose 2%, soluble starch 1%, pressed yeast 0.9%, Polypepton 0.5%, NaCl 0.5%, $CaCO_3$ 0.3%, and CB-442 0.01%, and adjusted to pH 7.2 before autoclave. The organism was cultured with agitation rate of 100 ~ 400 rpm to keep solubilized oxygen concentration as 2 ppm and with aeration at a rate of 7.5 liters/minute at 28°C for 144 hours.

The culture broth (25 liters) was filtered with the aid of diatomaceous earth. The filtrate (23 liters) was adjusted to pH 6.0 by hydrochloric acid, and applied to an Amberlite IRC-50 column (NH_4^+ type, 3 liters). The column was washed with deionized water, and adsorbed material was eluted with 0.5 N NH_4OH. The fractions in which trehalamine was detected by HPLC assay were combined, concentrated under reduced pressure to yield 200 ml of solution. The concentrate was loaded on a column of Dowex 1-X2 (OH^- type, 450 ml) equilibrated with deionized water, and elution was performed with deionized water. Active fractions were combined, concentrated under reduced pressure, and lyophilized to give 16.6 mg of pure trehalamine as a white powder.

Fermentation of *Micromonospora* sp. SANK 62390 and Purification of Trehalamine (**1**)

To a slant culture of *Micromonospora* sp. SANK 62390 10 ml of physiological saline was added, and the culture was homogenized with a Potter-Elvehjem homogenizer. One milliliter of the suspension was inoculated into each of two baffled 2-liter Erlenmeyer flasks containing 500 ml of seed medium which consisted of glucose 2%, yeast extract 0.5%, Polypepton 0.5%, $CaCO_3$ 0.1%, and CB-442 0.01% and was adjusted to pH 7.2 before autoclave. The organism was incubated by a rotary shaker with rotation at a rate of 210 rpm at 28°C for 96 hours as the first seed culture. Six hundred ml of the first seed culture were transferred to a 60-liter jar fermentor containing 30 liters of the seed medium. The second seed culture was accomplished with an agitation rate of 165 rpm and an aeration rate of 15 liters/minute at 28°C for 48 hours. Fifteen liters of the second seed culture was transferred to a 600-liter fermentation tank containing 300 liters of production medium composed of glucose 8%, of which solution was autoclaved separately and mixed later, Lustergen FK 2%, pressed yeast 1.8%, Polypepton 1%, meat extract 1%, NaCl 0.5%, $CaCO_3$ 0.3%, K_2HPO_4 0.25%, and CB-442 0.02% and adjusted to pH 7.2 before sterilization. The culture was incubated with agitation rate of 82 ~ 142 rpm to maintain solubilized oxygen concentration of 2 ppm and aeration rate of 150 liters/minute at 28°C for 144 hours.

Twenty liters of the whole filtrate (290 liters) obtained by filtration of the culture broth (300 liters) with diatomaceous earth were used to isolate trehalamine. The filtrate was adjusted to pH 6.0 by hydrochloric

acid, and basic compounds were adsorbed on a column of Amberlite IRC-50 (NH_4^+ type, 3 liters), and eluted with 0.5 N NH_4OH after washing with deionized water. Fractions containing trehalamine were combined, concentrated under reduced pressure, and the concentrate was applied to a column of Dowex 1-X2 (OH^- type, 500 ml) and chromatographed with deionized water. Active portion was concentrated under reduced pressure, and lyophilized to yield 9.6 mg of semi-purified trehalamine. The powder was purified again with a column of Dowex 1-X2 (OH^- type, 100 ml), and through concentration and lyophilization, 4.8 mg of pure trehalamine were furnished as a white powder.

Table 1. Cultural characteristics of strains SANK 62390 and SANK 60791.

Medium		SANK 62390	SANK 60791
Yeast extract-malt extract agar (ISP 2)	G:	Good, flat, dark brownish gray	Good, wrinkled, dull yellow
	AM:	Poor, rudimentary, brownish white	Poor, rudimentary, white
	R:	Brownish black	Dull yellowish orange
	SP:	None	None
Oatmeal agar (ISP 3)	G:	Good, flat, orange	Moderate, flat, pale yellowish brown
	AM:	Poor, rudimentary, white	Poor, rudimentary, white
	R:	Orange	Pale yellow
	SP:	None	None
Inorganic salts-starch agar (ISP 4)	G:	Good, flat, light orange	Moderate, flat, pale yellowish brown
	AM:	None	Poor, rudimentary, pale yellow
	R:	Gray	Pale yellowish brown
	SP:	None	None
Glycerol-asparagine agar (ISP 5)	G:	Moderate, flat, light orange	Good, flat, brownish white
	AM:	None	Poor, rudimentary, white
	R:	Light brownish gray	Pale yellowish brown
	SP:	None	None
Peptone-yeast extract-iron agar (ISP 6)	G:	Good, flat, orange	Moderate, flat, pale yellowish orange
	AM:	Poor, white	None
	R:	Light yellowish orange	Pale yellowish brown
	SP:	None	None
Tyrosine agar (ISP 7)	G:	Moderate, flat, dull orange	Abundant, wrinkled, brownish white
	AM:	Poor, rudimentary, white	Poor, pale orange
	R:	Brownish white	Pale orange
	SP:	None	None
Sucrose-nitrate agar	G:	Moderate, flat, pale orange	Good, flat, yellowish gray
	AM:	None	Moderate, white
	R:	Pale orange	Yellowish gray
	SP:	None	None
Glucose-asparagine agar	G:	Moderate, flat, yellowish orange	Moderate, flat, pale orange
	AM:	None	Poor, yellowish gray
	R:	Pale orange	Pale brown
	SP:	None	None
Nutrient agar	G:	Moderate, flat, yellow orange	Good, flat, pale yellowish brown
	AM:	None	Poor, rudimentary, white
	R:	Dull yellowish orange	Pale yellow
	SP:	None	None
Water agar	G:	Moderate, flat, pale yellowish orange	Moderate, flat, yellowish gray
	AM:	None	Moderate, white
	R:	Gray	Yellowish gray
	SP:	None	None
Potato extract-carrot extract agar	G:	Good, flat, yellowish gray	Moderate, flat, yellowish gray
	AM:	Poor, rudimentary, white	Poor, white
	R:	Brownish white	Yellowish gray
	SP:	None	None

G: growth, AM: aerial mycelium, R: reverse, SP: soluble pigment.

Taxonomy of Producing Organisms

Strain SANK 62390 grew well or moderately on agar media employed. The strain produced single spores with smooth surface (Plate 1) and spherical in shape only on substrate mycelium. No special morphological organs such as whirls, sclerotia, sporangia were observed on the media tested. The cultural characteristics on various agar media at 28°C for 14 days are shown in Table 1. The color of the vegetative mycelium was light orange, orange to dark brownish gray. The strain formed aerial mycelium rudimentarily with brownish white in color. The strain grew within the temperature range of 17 to 42°C.

In Table 2 the physiological properties are summarized. Starch hydrolysis, gelatin liquefaction and action on milk were detected, while nitrate reduction and melanin formation could not be demonstrated. D-Glucose, D-fructose, L-arabinose, D-xylose, inositol and raffinose were utilized as a sole carbon source but not L-rhamnose, sucrose, and D-mannitol.

meso-Diaminopimelic acid, arabinose, and xylose were found in the cell wall, indicating that the cell wall type was type II and the whole-cell sugar pattern was type D. Mycolic acid was not detected. A type PII phospholipid pattern was found. The type of the acyl group was glycolyl type, and major menaquinones were MK-10 (H_6), MK-10 (H_4), and MK-10 (H_8). From these results, the strain was considered to be a member of the genus *Micromonospora* and named as *Micromonospora* sp. SANK 62390.

Strain SANK 60791 grew relatively well on various agar media tested. The strain exhibited occasional fragmentation of both the aerial mycelium and the substrate mycelium at the later stage of the cultivation. Aerial mycelium broke down into chains of rod shaped elements with smooth surface. Nocardioid zig-zag formation of aerial mycelium or substrate mycelium was not observed.

The cultural characteristics of the strain are summarized in Table 1. Substrate mycelium developed well with brownish white, pale yellowish brown to dull yellow in color. Aerial mycelium was formed poorly or rudimentarily with white, pale yellow to light orange in color. Gelatin liquefaction, nitrate reduction and milk coagulation were positive. Other physiological properties are listed in Table 2. The strain SANK 60791 utilized D-glucose, D-fructose, L-rhamnose, D-xylose, sucrose, raffinose and D-mannitol, but not inositol. Utilization of L-arabinose, was weakly demonstrated.

meso-Diaminopimelic acid in the cell wall was detected. Arabinose and galactose were present in

Plate 1. Scanning electron micrograph of strain SANK 62390 on potato extract - carrot extract agar at 28°C for 7 days.

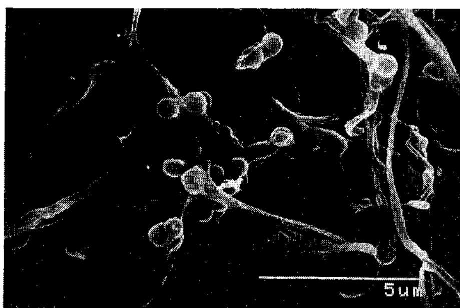


Table 2. Physiological properties of strains SANK 62390 and SANK 60791.

Properties	SANK 62390	SANK 60791
Hydrolysis of starch	Positive	Negative
Liquefaction of gelatin	Positive	Positive
Reduction of nitrate	Negative	Positive
Coagulation of milk	Positive	Positive
Peptonization of milk	Positive	Negative
Sodium chloride resistance (medium 1[a])	2%	3%
Decomposition of:		
Casein	Positive	Negative
Tyrosine	Negative	Positive
Xanthine	Negative	Negative
Production of melanoid pigment:		
Medium 2[b]	Negative	Negative
Medium 3[c]	Negative	Negative
Medium 4[d]	Negative	Negative

[a] Medium 1: Yeast extract - malt extract agar (ISP 2).
[b] Medium 2: Tryptone - yeast extract broth (ISP 1).
[c] Medium 3: Peptone - yeast extract - iron agar (ISP 6).
[d] Medium 4: Tyrosine agar (ISP 7).

Table 3. Physico-chemical properties of trehalamine (1) and the aminocyclitol 2.

	1	2
Nature	Colorless plates	White powder
MP	163~165°C	
$[\alpha]_D^{25}$	+13.5° (*c* 0.74, H_2O)	−3.7° (*c* 0.51, H_2O)
Molecular formula	$C_7H_{12}N_2O_5$	$C_6H_{13}NO_5$
FAB-MS (*m/z*)	205 $(M+H)^+$	180 $(M+H)^+$
HRFAB-MS (*m/z*)	Obsd 205.0842 Calcd for $C_7H_{13}N_2O_5$ $(M+H)^+$ 205.0825	Obsd 180.0889 Calcd for $C_6H_{14}NO_5$ $(M+H)^+$ 180.0872
Analysis	Calcd for $C_7H_{12}N_2O_5$: C 41.18, H 5.85, N 13.80 Found: C 41.14, H 5.92, N 13.72	
UV (H_2O)	No characteristic maxima above 210 nm	No characteristic maxima above 210 nm
IR ν_{max} (KBr) cm^{-1}	3484, 3425, 3356, 3278, 1702, 1685, 1654, 1413, 1051	3358, 1582, 1474, 1380, 1040
Solubility	Soluble in water Insoluble in acetone and chloroform	Soluble in water Insoluble in acetone and chloroform
TLC (SiO_2)[a]	0.48	0.39
Color reaction	Positive to Hanessian dip	Positive to ninhydrin and Hanessian dip
HPLC	Retention time 8.4 minutes[b]	

[a] SiO_2 plate (Merck Art. 5715), with developing solvent of CH_3CN-AcOH-H_2O (6:1:3).
[b] Column Asahipak ES-502C (Asahi Chemical Industry), solvent 20 mM ammonium acetate (pH 8.5) with 50 mM NaCl, flow rate 1.0 ml/minute, detection UV at 210 nm.

Table 4. ^1H and ^{13}C NMR parameters of trehalamine (1) in D_2O.

Position	^1H (ppm)	Multiplicity	(*J*=Hz)	^{13}C (ppm)
1				85.6
2	3.81	d	(4.4)	83.0 or 83.3
3	4.07	dd	(2.4, 4.4)	83.0 or 83.3
4	4.89	dd	(8.8, 2.4)	90.4
5	4.22	d	(8.8)	76.7
6	3.57	d	(12.0)	64.9
	3.65	d	(12.0)	
7*				164.9

* Position of the carbon in the oxazole ring attached to the amino group was numbered as 7 in this table.

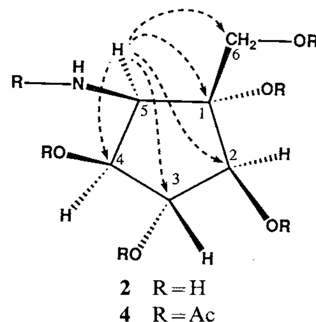
the whole-cell as the major sugar component. No mycolic acid was detected. Phospholipid pattern was PII. Acyl type and the major menaquinones of the strain were shown to be glycolyl type and MK-9 (H_4), respectively. Based on the taxonomic properties described above, the strain was considered to be a member of the genus *Amycolatopsis* and identified as *Amycolatopsis* sp. SANK 60791.

Both the strains SANK 62390 and SANK 60791

Table 5. ^1H and ^{13}C NMR parameters of 2 in D_2O.

Position	^1H (ppm)	Multiplicity	(*J*=Hz)	^{13}C (ppm)
1				79.4
2	3.61	d	(7.0)	81.7
3	3.78	dd	(7.0, 6.4)	81.5
4	3.90	dd	(6.4, 7.1)	73.6
5	3.12	d	(7.1)	57.8
6	3.56	d	(11.9)	61.0
	3.61	d	(11.9)	

Fig. 2. The selected long range ^1H-^{13}C couplings in 2 by HMBC spectrum.



2 R=H
4 R=Ac

The couplings between 5-H and carbons were indicated by dotted arrows directed from the proton to the carbons.

have been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaraki Prefecture, Japan, under the accession numbers FERM BP-3521 and FERM BP-3513, respectively.

Table 6. IC_{50} (M) of trehalamine, the aminocyclitol **2**, and trehazolin against silkworm trehalase, rat sucrase, and sweet almond β-glucosidase.

Enzyme	Trehalamine (**1**)	**2**	Trehazolin (**3**)
Silkworm trehalase	1.8×10^{-4}	$>5.0\times10^{-4}$	5.2×10^{-8}
Rat sucrase	6.8×10^{-5}	$>5.0\times10^{-4}$	1.7×10^{-4}
Sweet almond β-glucosidase	$>5.0\times10^{-4}$	5.6×10^{-6}	$>5.0\times10^{-4}$

Physico-chemical Properties

All physical and chemical properties were identical for the both samples of trehalamine prepared by hydrolysis of **3** and fermentation. Physico-chemical properties of trehalamine and the aminocyclitol **2** were listed in Table 3. The ^1H and ^{13}C NMR data of trehalamine and **2** in D_2O are shown in Tables 4 and 5 under the numbering system indicated in Fig. 2.

Structural Studies

The molecular formula of trehalamine was established as $C_7H_{12}N_2O_5$ by high resolution FAB-MS, and its structure was confirmed by the comparison of ^1H and ^{13}C NMR spectra of trehalamine (Table 4) with those of trehazolin.

High resolution FAB-MS of **2** elucidated the molecular formula of $C_6H_{13}NO_5$. In ^1H and ^{13}C NMR spectrum, a coupled four spin system and an AB quartet signal were easily identified, and these were connected by the ^1H-^{13}C long range couplings as shown in Fig. 2. These results supported the aminocyclitol structure of **2**. The presence of an amino and five hydroxy groups in **2** estimated from its molecular formula was confirmed by the hexaacetyl derivative **4**. In the ^1H NMR spectrum the all non-labile protons were shifted to the lower field due to the acetylation, and an amide proton coupled with 5-H at 5.33 ppm was observed at 5.86 ppm. On the irradiation of the NH proton, the NOEs were observed at 6-Ha, 6-Hb and 3-H. These data were in good agreement with the stereochemistry shown in Fig. 2. Thus, the structure of **2** was established as shown in Fig. 1.

Structure and purity of glucose obtained by the hydrolysis were confirmed by ^1H NMR and HPLC. Since the specific rotation in H_2O (c 2.0) of +45.8° was nearly identical with the value reported for authentic D-glucose ($[\alpha]_D$ +52.6°)[2], the absolute configuration of the glucose was determined as D-form. The stereochemistry was also confirmed by susceptibility of the isolated glucose to glucose oxidase, which strictly distinguishes D- and L-glucose.

Inhibition of Glycosidases

Inhibitory activities of trehalamine (**1**), **2**, and trehazolin (**3**) against silkworm trehalase, rat sucrase, and sweet almond β-glucosidase are summarized in Table 6. Trehalamine inhibited trehalase, although inhibition was much weaker than that by trehazolin. However, trehalamine inhibited sucrase at lower concentration than trehazolin (IC_{50}, **1**; 6.8×10^{-5}, **3**; 1.7×10^{-4} M). The aminocyclitol **2** exhibited considerable activity against β-glucosidase (IC_{50} 1.4×10^{-6} M), whereas trehalase was inhibited only 24% at the concentration of 5.6×10^{-4} M.

Discussion

The structure of trehazolin resembles that of trehalose, the authentic substrate of trehalase, including

the 1α-configuration of the D-glucose moiety. The unique aglycon part with the cyclopentane-fused oxazoline ring evokes interest both in respect to the enzyme inhibitory activity and microbial biosynthesis of the new pseudodisaccharide.

From the structural resemblance and the potent inhibitory activity, trehalamine, the aglycon of trehazolin, is assumed to be a pseudosugar corresponding to glucose, and essential element to the enzyme inhibition. Actually, although trehalamine and the aminocyclitol **2** lost the potent inhibitory activity against silkworm trehalase, both compounds inhibited other glucosidases more potently than trehazolin. The results suggest that trehalamine would be a useful material for the design of new potent glycosidase inhibitors. In view of its inhibitory activity on β-glucosidase, the aminocyclitol **2** can also be a good building block. In fact, it has been reported that five- and six-membered aminocyclitols of natural origin such as mannostatins A and B[3], validamine[4,5], valienamine[5,6], and valiolamine[5] possess inhibitory activities against glycosidases.

We discovered trehazolin aglycon in the culture broths of trehazolin producers. Coproduction of **1** and trehazolin means they have close relationships in the biosynthetic pathway, although their precursor-product relationship remains to be determined.

Another trehalase inhibitor, trehalostatin, was recently reported[7], and assigned to be the epimer of trehazolin at the 5-position of the tetrahydrocyclopent[*d*]oxazole ring. Nevertheless, from physical and chemical properties trehazolin and trehalostatin seem to be the same compound. In the vast number of microbial metabolites there is only one group of compounds which have similar structures to trehazolin and its aglycon, trehalamine. Allosamidin and related compounds have been reported by SAKUDA *et al.*[8~10] as chitinase inhibitors produced by *Streptomyces* sp. Both trehalamine and allosamizoline[8,9], the aglycon of allosamidins, have the common tetrahydrocyclopent[*d*]oxazole nucleus. According to biosynthetic studies[11] allosamizoline is synthesized from D-glucose *via* *N*-acetylglucosamine. Considering the configuration of hydroxyl groups of the cyclopentane ring of trehalamine, **2**, and trehazolin, it can be hypothesized that this portion is derived from D-glucose by carbon-carbon bond formation between C1 and C5 atoms, although there are differences in the stereochemistry, position of the hydroxymethyl group, and the mode of substitution on the amino moiety between trehazolin and allosamidin.

As described above, trehalamine, **2**, and D-glucose were obtained from culture broths and/or by hydrolysis of trehazolin. The present results provided the targets of total syntheses aimed at the determination of the relative and absolute configuration of trehazolin[12]. At Sankyo, KOBAYASHI *et al.* achieved a chiral synthesis of trehalamine and **2** starting from D-glucose[13], and the synthetic samples of trehalamine, **2**, and its hexaacetate **4** were identical with the corresponding natural compounds in physico-chemical properties including specific rotation. These results definitely determined the absolute configuration of trehalamine and **2** as illustrated in Fig. 1. Accordingly, the absolute configuration of trehazolin, which was further confirmed by the total syntheses of trehazolin by KOBAYASHI *et al.*[13], was established as 1-deoxy-1{(3a*R*,4*R*,5*S*,6*S*,6a*S*)-4-hydroxymethyl-4,5,6-trihydroxy-3a,5,6,6a-tetrahydro-4*H*-cyclopent[*d*]oxazol-2-yl}amino-α-D-glucopyranose.

Experimental

Taxonomic Studies

The producing organisms, strain SANK 62390 and strain SANK 60791, were isolated from soil samples collected at Nikko, Tochigi Prefecture, and Tsushima island, Nagasaki Prefecture, respectively.

Methods and media described by the International Streptomyces Project (ISP)[14] and WAKSMAN[15] were used to determine most of the cultural and physiological characteristics. Purified cell wall and whole-cell hydrolysates were analyzed by the methods of BECKER *et al.*[16], LECHEVALIER *et al.*[17] and HASEGAWA *et al.*[18]. Phospholipid, acyl type in the cell wall, menaquinones, and mycolic acids were analyzed by the methods of LECHEVALIER *et al.*[19], UCHIDA and AIDA[20], COLLINS *et al.*[21], and HECHT and CAUSEY[22], respectively.

General

^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX400 spectrometer. Chemical shifts are expressed in δ values (ppm) with tetramethylsilane as external standards for the spectra in D_2O and

tetramethylsilane as internal standards for those in $CDCl_3$. Mass spectra were measured on a JEOL JMS-SX/SX102A mass spectrometer, and IR spectra on a JASCO FT/IR8300 spectrophotometer.

(1*R*,2*S*,3*R*,4*S*,5*R*)-1-Acetoxymethyl-5-acetylamino-1,2,3,4-tetraacetoxycyclopentane (**4**)

A suspension of **2** (30 mg) in pyridine (1.5 ml) containing 4-dimethylaminopyridine (3.75 mg) and acetic anhydride (400 μl) was kept at room temperature overnight. Ice water was added to terminate the reaction, and the solution was adjusted to pH 2.0 by adding 1 N hydrochloric acid and extracted with EtOAc. The organic layer was washed with saturated aq NaCl solution and 0.1 M Na_2HPO_4 solution, dried, and concentrated to dryness under reduced pressure. Residual material was subjected to crystallization in EtOAc-*n*-hexane to yield 20 mg of the crystalline hexaacetate; $[\alpha]_D^{25}$ +5.9° (*c* 1.08, chloroform); FAB-MS: *m/z* 432 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ 5.81 (d, *J*=5.0 Hz, 2-H), 5.24, (t, *J*=5.0, 5.0 Hz, 3-H), 5.38 (dd, *J*=7.8, 5.0 Hz, 4-H), 5.33 (dd, *J*=9.3, 7.8 Hz, 5-H), 4.56 (d, *J*=12.2 Hz, 6-Ha), 4.63 (d, *J*=12.2 Hz, 6-Hb), 5.86 (d, *J*=9.3 Hz, NH), 2.0~2.2 (s×6, Ac); ^{13}C NMR ($CDCl_3$) δ 86.7 (C-1), 76.8 (C-2), 79.1 (C-3), 73.4 (C-4), 52.9 (C-5), 59.6 (C-6), 20~25 (q×6, Ac).

Determination of Glucose by Glucose Oxidase-Peroxidase Method

Concentration of D-glucose was determined by glucose oxidase coupled with oxidation of 4-aminoantipyrine by peroxidase[23] (glucose oxidase-peroxidase method as described below except that a disaccharide substrate, glucosidase, and bovine serum albumin were not included in the reaction mixture). The absorbance value for the glucose sample (0.1~4 mg in each well) was equal to that for authentic D-glucose, whereas authentic L-glucose caused no change in absorbance.

Enzyme Assays

Silkworm trehalase was prepared as described in the previous paper[1]. Rat intestinal brush border glucosidases were prepared by the method of KESSLER *et al.*[24] and was used as a sucrase. Sweet almond β-glucosidase was purchased from Sigma Chemical Company. All the enzyme activities were measured in 96-well plates. Linearities of the enzyme activities to amounts of the enzyme and reaction time were confirmed for all the assays described below.

Trehalase and sucrase activities were assayed by one-step method using glucose oxidase-peroxidase system[23,25]. Reactions were carried out in a total volume of 150 μl containing 20 mM citrate-40 mM Na_2HPO_4 buffer (pH 5.6 for trehalase assay and pH 6.2 for sucrase assay), 3 units of glucose oxidase (Boehringer Mannheim), 0.12 units of horseradish peroxidase (Boehringer Mannheim), 40 μg phenol (Wako Pure Chemical Industries), 20 μg 4-aminoantipyrine (Sigma), 200 μg bovine serum albumin (Sigma), 5 mM trehalose or 20 mM sucrose, various concentration of inhibitors, and the appropriate amounts of the enzymes. The reaction mixture was preincubated at 37°C for 5 minutes and the reaction was started by the addition of 20 μl of trehalase or sucrase. After incubation at 37°C for 20 minutes colorimetric change at 492 nm was measured by the microplate reader, MTP-22 (Corona Electric), and the amount of released glucose was calculated.

Beta-glucosidase activity was assayed in a total volume of 150 μl containing 20 mM citrate-40 mM Na_2HPO_4 buffer, pH 5.6, 200 μg bovine serum albumin, 3.3 mM *p*-nitrophenyl β-D-glucoside, various concentrations of inhibitors and appropriate amounts of the enzyme at 37°C for 10 minutes. At the end of the incubation, the reaction was stopped by adding 20 μl of 4 M glycine-NaOH buffer, pH 10.4, and nitrophenol released was measured at 405 nm.

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