ORIGINAL ARTICLE

Structure and biological properties of lentztrehalose: a novel trehalose analog

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A new trehalose analog, lentztrehalose [4-*O*-(2,3-dihydroxy-3-methylbutyl)trehalose], was isolated from an actinomycete *Lentzea* sp. Lentztrehalose is only weakly hydrolyzed by the trehalose-hydrolyzing enzyme, trehalase, so can be regarded as an enzyme-stable analog of trehalose. Although lentztrehalose does not show apparent toxicity to mammalian cells and microbes, it has antitumor activity in mice bearing S-180 sarcoma and Ehrlich carcinoma cells. In ovariectomized mice, lentztrehalose displayed a bone reinforcement effect in the femur that was superior to trehalose and induced non-morbid suppression of weight gain comparable with trehalose. These results indicate that enzyme-stable analogs of trehalose, such as lentztrehalose, may be more beneficial for human health and thus have potential as substitutes for trehalose as a sweetener.

The Journal of Antibiotics (2014) 67, 319–322; doi:10.1038/ja.2013.143; published online 22 January 2014

Keywords: antitumor; *Lentzea*; lentztrehalose; trehalase; trehalose

INTRODUCTION

Trehalose, a disaccharide formed from two molecules of glucose linked by an α,α -1,1-glucoside bond, is a component of tissues in animals, plants and microorganisms. In many insects, trehalose is the main sugar present in the blood. Trehalose is used as an energy source because hydrolysis of trehalose by the catabolic enzyme, trehalase, produces glucose. Although mammals do not synthesize trehalose, trehalase is expressed in the kidneys and intestine for digestion of trehalose consumed in food.¹ Trehalose is used as an additive in many foods, drugs and cosmetics² as it has a high water retention capacity. Medically relevant biological properties of trehalose include suppression of bone loss,³ antitumor effects⁴ and mitigation of insulin resistance.⁵ The wide utility of trehalose means that investigating analogs with potentially improved properties deserves exploration.

During our regular screening for antitumor agents from soil microbes, an actinomycete strain *Lentzea* sp. ML457-mF8 was discovered, which produced pyrazomycins and staurosporine. Although no new pyrazomycin or staurosporine compounds were found, a new trehalose analog was isolated from this strain. This compound, 4-*O*-(2,3-dihydroxy-3-methylbutyl)trehalose, we named lentztrehalose.

RESULTS AND DISCUSSION

Isolation and structure determination of lentztrehalose

An actinomycete strain *Lentzea* sp. ML457-mF8 was found to produce pyrazomycins and staurosporine. To search further for new analogs and other metabolites from this strain, the culture extract was initially separated by HPLC using an octadecyl silica column. Some fractions with pyrazomycin activity were identified that contained a novel

compound with a MW of 444. This compound was then isolated by Sephadex LH-20 gel filtration. No further contamination of pyrazomycins or staurosporine was detected by TLC or HPLC. Approximately 1.0 g of the compound was obtained from 1 kg of wheat cultures of the actinomycete strain.

The actinomycete strain was isolated from a soil sample collected in Sado, Niigata Prefecture, Japan. The substrate hyphae are branched and have a pale to dull yellow color. The aerial mycelia are straight or flexuous, with fragmentation into ellipsoidal spores (0.4–0.5 μm by 1.1–1.5 μm). The spore surface is smooth and white. Whole-cell hydrolysates contained *meso*-A2pm. The partial 16S ribosomal RNA (rRNA) gene sequence (1474 bp, GeneBank/EMBL/DDBJ accession number AB838595) of the strain showed high homology with those of the genus *Lentzea* such as *L. waywayandensis* (JCM 9114^T, T: Type strain, 1456/1469 bp, 99.1%) and *L. albida* (IFO 16102^T, 1458/1472 bp, 99.0%). On the basis of taxonomic properties and the 16S rRNA sequence, the strain ML457-mF8 was determined to belong to the genus *Lentzea*. Therefore, the strain was designated as *Lentzea* sp. ML457-mF8. As the new compound is a trehalose derivative from *Lentzea*, we coined the name lentztrehalose. No other analogs of lentztrehalose from this strain have been found so far.

The structure of lentztrehalose is shown in Figure 1a and the physicochemical properties of the compound are summarized in Table 1. The molecular formula was determined to be $\text{C}_{17}\text{H}_{32}\text{O}_{13}$ by HR ESI-MS, elemental analysis, and NMR data (Supplementary Figures 1–5). Analysis of the ^1H – ^1H COSY spectrum revealed two sets of spin systems (from 1'-H to 6'-H₂ and 1''-H to 6''-H₂), indicating the presence of two hexose moieties. The coupling constants (Table 2) indicated that the sugars are glucopyranoses.

Figure 1 (a) Structure of lentztrehalose. (b) ¹H-¹H COSY and HMBC correlations of lentztrehalose.

Table 1 Physicochemical properties of lentztrehalose

Appearance	White solid
Optical rotation	$[\alpha]_D^{22} +137$ (c 0.94, MeOH)
UV λ_{max}	End absorption
IR (KBr) ν_{max} (cm ⁻¹)	3400, 2935, 1653, 1375, 1150, 1079, 1047, 991, 802, 610
Molecular formula	C ₁₇ H ₃₂ O ₁₃
HR ESI-MS (m/z)	
Calculated for	467.1735
C ₁₇ H ₃₂ O ₁₃ Na	
Found	467.1739 (M + Na) ⁺
Elemental analysis	
Calculated for	C 42.5% H 7.5%
C ₁₇ H ₃₂ O ₁₃ · 2H ₂ O	
Found	C 42.5% H 7.7%
Sweetness score ^a	5.9 ± 3.8 (water 0, sucrose 10, trehalose 6.8 ± 3.2)

^aArbitrary score measured by a sensory test. Ten panelists tasted 10 μ l of 500 mg ml⁻¹ blinded solution of lentztrehalose, sucrose, trehalose and water. The sweetness of lentztrehalose and trehalose was scored on a scale where water and sucrose were scored as 0 and 10, respectively. Mean $\pm$ s.d.

The presence of a 2,3-dihydroxy-3-methylbutoxy moiety was established from the HMBC spectrum (Figure 1b). In the HMBC spectrum, cross-peaks were observed from the methylene protons (H-1) to an oxymethine carbon (C-4', δ_C 80.4). An HMBC correlation was also observed between H-4' and a methylene carbon (C-1, δ_C 74.7), indicating the linkage of C-1 in the 2,3-dihydroxy-3-methylbutoxy group moiety and C-4' through an oxygen atom. The configuration of the glucose moiety was determined by the mutarotase-glucose oxidase method.⁶ After acid hydrolysis of the compound, the reaction mixture formed a red pigment, indicating the presence of D-glucose. The optical rotation values of the compound were also consistent with D-(+)-glucose. The presence of a 1,1-glycosidic linkage was established from the HMBC correlation between C-1' and C-1'' as shown in Figure 1b. The coupling constants

Table 2 ¹H and ¹³C NMR data for lentztrehalose in methanol-d₄

Position	δ_C^a	δ_H^b
1	74.7 (t)	4.00 (1H, dd, $J = 10.6, 2.8$ Hz) 3.65 (1H, m)
2	78.2 (d)	3.54 (1H, dd, $J = 8.0, 2.8$ Hz)
3	72.8 (s)	
4	26.4 (q)	1.18 (3H, s)
5	25.4 (q)	1.16 (3H, s)
1'	95.1 (d)	5.10 (1H, d, $J = 3.8$ Hz)
2'	73.3 (d)	3.50 (1H, dd, $J = 9.6, 3.8$ Hz)
3'	74.4 (d)	3.90 (1H, t, $J = 9.6$ Hz)
4'	80.4 (d)	3.28 (1H, t, $J = 9.6$ Hz)
5'	72.7 (d)	3.85 (1H, br dt, $J = 9.6, 3.3$ Hz)
6'	62.1 (t)	3.78 (1H, m), 3.75 (1H, m)
1''	95.0 (d)	5.09 (1H, d, $J = 3.8$ Hz)
2''	73.2 (d)	3.45 (1H, dd, $J = 9.6, 3.8$ Hz)
3''	74.5 (d)	3.77 (1H, m)
4''	71.9 (d)	3.31 (1H, t, $J = 9.5$ Hz)
5''	73.8 (d)	3.79 (1H, m)
6''	62.6 (t)	3.78 (1H, m), 3.67 (1H, dd, $J = 12.0, 4.7$ Hz)

^aRecorded at 150 MHz.

^bRecorded at 600 MHz.

between H-1' and H-2'' ($J = 3.9$ Hz) and between H-1'' and H-2'' ($J = 3.9$ Hz) indicated the presence of α -glycosidic linkages.

Naturally occurring trehalose-containing molecules are relatively uncommon. To date, only 40 such compounds are registered in the database, Dictionary of Natural Products. The 2,3-dihydroxy-3-methylbutoxy moiety is also rare. Among 60 compounds containing this moiety registered in the database, five are derived from fungi and the others from plants. Therefore, we believe that lentztrehalose is the first compound to contain a 2,3-dihydroxy-3-methylbutoxy moiety isolated from an actinomycete strain.

The sweetness of lentztrehalose is comparable to trehalose as shown in Table 1.

Biological activity of lentztrehalose

Lentztrehalose was not observed to have antimicrobial activity against 39 species, and 106 strains of bacteria and fungi. The MIC value was 64 μ g ml⁻¹ against 13 strains of microbes, including *Haemophilus influenza* and *Cryptococcus neoformans* and against other microbes even higher MIC values were observed. In addition, no cytotoxicity was observed with lentztrehalose at concentrations up to 200 μ g ml⁻¹ in 52 strains of human cancer cells (data not shown).

We initially considered the possibility that lentztrehalose might be a trehalase inhibitor, but lentztrehalose failed to inhibit porcine kidney trehalase up to 8 mg ml⁻¹ (data not shown). However, lentztrehalose was only weakly hydrolyzed by trehalase and should be more enzyme stable than trehalose in mammals (Figure 2).

Several biological activities of trehalose in mammals have been reported. Ukawa *et al.*⁴ reported that oral administration of trehalose 25 mg kg⁻¹ per day or higher markedly suppressed S-180 sarcoma tumor growth in the ICR mouse. Contrary to this report, we did not observe tumor suppression with trehalose in a similar model (Figure 3a). The antitumor effect of trehalose has been speculated to be caused by modification of the intestinal immune environment.⁴ Thus, the observed discrepancy in results may be due to differences in food and rearing conditions that may influence the immune system. Alternatively, the difference might be due to variations in the properties of S-180 cells because the cell line used in our experiment does not form a big tumor, such as seen with Ukawa

Figure 2 Hydrolysis of trehalose and lentztrehalose by porcine kidney trehalase. Trehalose or lentztrehalose was reacted with porcine kidney trehalase and the concentrations of released glucose were measured by a hexokinase assay. Mean $\pm$ s.e. from triplicate determinations.

Figure 3 Antitumor effect of lentztrehalose. (a) Tumor growth in ICR mice ($n=5$) subcutaneously inoculated with S-180 sarcoma cells. Trehalose and lentztrehalose were administered p.o. or i.v. as indicated. Error bars indicate s.e. (b) Survival of ICR mice ($n=7$) bearing Ehrlich ascites carcinoma cells. Lentztrehalose was administered at 50 mg kg^{-1} , p.o. Arrowheads indicate the days of sample administration.

*et al.*⁴ In our study, a higher concentration of trehalose might be required for tumor suppression. Lentztrehalose, conversely, showed potent suppression of tumor growth (Figure 3a). This effect was observed from a dose of 12.5 mg kg^{-1} per day (p.o.) and was stable at

Figure 4 Bone reinforcement and anti-obesity effects of lentztrehalose in a postmenopausal mouse model. Female ICR mice (20 weeks old) were separated into intact, sham-operated and ovariectomized (OVX) groups. Intact, sham and H_2O groups were given distilled water while the lentztrehalose (LT) and trehalose (TH) groups were offered water containing 0.6 mg ml^{-1} lentztrehalose and 1.2 mg ml^{-1} trehalose, respectively as the daily drink. (a) Dry weight of a femur at 5 weeks after ovariectomy. Error bars indicate s.e. (b) Body weights of mice during the experiment. Mean $\pm$ s.e.

50 mg kg^{-1} per day (p.o. and i.v.). We also evaluated the antitumor effect of lentztrehalose in several other mouse models. Lentztrehalose was observed to prolong the survival of ICR mice intraperitoneally harboring Ehrlich ascites carcinoma by $\sim 14.7\%$ (Figure 3b).

The effect on bone reinforcement is one of the best studied biological activities of trehalose.^{3,7} We performed a similar experiment to Nishizaki *et al.*³ using ovariectomized mice as a postmenopausal osteoporosis model. Ovariectomized mice (Figure 4a, OVX H_2O) lost $\sim 5\%$ of femur weight compared with that of

sham-operated mice. Mice provided with drinking water containing up to 1.2 mg ml^{-1} trehalose (Figure 4a, OVX TH) did not show an obvious increase in bone weight. However, mice provided with 0.6 mg ml^{-1} lentztrehalose (OVX LT) were observed to have an increase in femur weight of 3–4%. Such increases in femur weight were also observed in mice treated with 50 mg kg^{-1} lentztrehalose (p.o.) in the antitumor experiments (data not shown).

Obesity is another major problem in postmenopausal women. In ovariectomized mice, a 3.5% (1 week) to 7.5% (5 week) weight gain was observed compared with sham-operated controls (Figure 4b). Lentztrehalose and trehalose induced a weight reduction in ovariectomized mice to a level comparable with sham-operated mice. Toxic effects such as vomiting, diarrhea or reduction of food and drink consumption were not observed in the bone reinforcement experiment and neither compound induced weight reduction in 5-week-old mice used in the antitumor experiment (data not shown). Therefore, the weight reduction observed with the middle-aged mouse administered lentztrehalose and trehalose was not morbid. Arai *et al.*⁵ has reported that trehalose prevents adipocyte hypertrophy. The weight loss seen with lentztrehalose is likely to be induced by the same mechanism. Therefore, lentztrehalose may also be expected to prevent obesity in middle-aged or older people if used as a diet sweetener.

The improved biological activities that we have observed with lentztrehalose compared with trehalose could be because of the higher enzyme stability of lentztrehalose. Although trehalose analogs of a similar size have been reported recently,^{8,9} comparative activity with trehalose was not reported. Thus, we conclude that lentztrehalose is the first potential substitute for trehalose with improved biological activity.

MATERIALS AND METHODS

Taxonomy of the actinomycete strain

The morphological properties of strain ML457-mF8 grown on ISP media No. 2, 3 and 5¹⁰ were observed with a scanning electron microscope Hitachi S-500 (Hitachi, Tokyo, Japan). For chemotaxonomic studies, cells were grown in YD medium containing 1% yeast extract and 1% glucose (pH 7.0) on a rotary shaker at 28 °C for 4 days and freeze dried. Isomers of diaminopimelic acid in whole-cell hydrolysate were analyzed by the method of Stanek and Roberts.¹¹ Analysis of 16S rRNA was performed as previously described.¹²

Isolation and structural determination of lentztrehalose

Strain ML457-mF8 was cultured in steamed wheat at 30 °C for 2 weeks. The whole culture was extracted with 70% ethanol and separated by sequential chromatography with several cycles of octadecyl silica HPLC (hydrosphere C18, YMC, Kyoto, Japan) and a gel filtration using Sephadex LH-20 (GE Healthcare Bio-Science AB, Uppsala, Sweden). The elution solvents were 0.1% trifluoroacetic acid for HPLC and MeOH for gel filtration.

The structure and physicochemical properties of lentztrehalose were determined by spectrometric analyses performed as previously described¹² and using the mutarotase–glucose oxidase method.⁶ The novelty of lentztrehalose was confirmed with the databases Dictionary of Natural Products on DVD (Chapman & Hall/CRC Press, Boca Raton, FL, USA) and SciFinder (Chemical Abstracts Service, Columbus, OH, USA).

Trehalase assay

Porcine kidney trehalase was purchased from Sigma-Aldrich (St Louis, MO, USA). Trehalase (>98%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Lentztrehalose or trehalose was reacted with 0.24 u ml^{-1} trehalase in 135 mM citrate buffer (pH 7.5) at 37 °C for 20 min. The reaction was stopped by addition of an equal volume of 500 mM Tris–HCl (pH 7.5).

The released glucose was reacted with a threefold volume of Glucose Assay Reagent (Sigma-Aldrich, G3293) at room temperature for 15 min and the $A_{340\text{nm}}$ was measured.

Antitumor assay

The antitumor effect of lentztrehalose in ICR mice (Charles River, Yokohama, Japan) bearing S-180 sarcoma cells was evaluated according to the protocol of Ukawa *et al.*⁴ Briefly, ICR mice (5 weeks old, ♀, $n=5$) were subcutaneously inoculated with 4×10^5 cells per 100 μl saline. Lentztrehalose ($12.5\text{--}50 \text{ mg kg}^{-1}$) and trehalose (50 mg per kg) were orally or intravenously administered at days –1, 1–4, and 6–11 after cellular inoculation. The tumor size was measured with a caliper and calculated from the formula $1/2(\text{length} \times \text{width}^2)$. To evaluate the survival benefit of lentztrehalose in Ehrlich ascites carcinoma cell-bearing mice, ICR mice (5-week-old, ♀, $n=7$) were intraperitoneally inoculated with 2×10^6 cells per 250 μl saline. Lentztrehalose (50 mg kg^{-1}) was orally administered three times a week.

Bone reinforcement assay

The bone reinforcement effect in ovariectomized mice was examined by the modified method of Nishizaki *et al.*³ The ovaries of 20-week-old female ICR mice were surgically removed. The mice were kept at 23 °C and given lentztrehalose- or trehalose-containing water as the daily drink. Intact mice were not operated upon. An abdominal incision was made in the sham group of mice and the ovaries removed once and immediately replaced at the original location followed by lesion closure.

Mice were killed at 5 weeks after the ovariectomy and both hind legs were removed. Muscle was scraped away from the femurs as much as possible with surgical scissors. The femurs were then reacted with an enzyme solution containing 0.5 mg ml^{-1} collagenase Type I (Life Technologies, Carlsbad, CA, USA) and 3.3 mg ml^{-1} dispase (Life Technologies) in phosphate buffered saline (pH 7.5) (DS Pharma Biomedical, Tokyo, Japan) at 37 °C for 10 h. The femurs were washed with water and acetone, and weighed after desiccation.

ACKNOWLEDGEMENTS

We appreciate the help of Prof Sachi SriKantha, Gifu University, for his critical reading of this manuscript. We acknowledge the assistance of Dr M Igarashi, Dr R Sawa, Ms Y Kubota, Ms H Soutome, Mr K Inoue, Ms C Hayashi, Dr Y Takahashi and Dr K Yamazaki at the Institute of Microbial Chemistry for their support for this study and helpful discussion. This study was partially supported by the Japan Society for the Promotion of Science.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)