Novel autophagy inducers lentztrehaloses A, B and C

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Trehalose has widespread use as a sweetener, humectant and stabilizer, and is now attracting attention as a promising candidate for the treatment of neurodegenerative diseases as it is an autophagy inducer and chemical chaperone. However, the bioavailability of trehalose is low because it is digested by the hydrolyzing enzyme trehalase, expressed in the intestine and kidney. Enzyme-stable analogs of trehalose would potentially solve this problem. We have previously reported an enzyme-stable analog of trehalose, lentztrehalose, and herein report two new analogs. The original lentztrehalose has been renamed lentztrehalose A and the analogs named lentztrehaloses B and C. Lentztrehalose B is a di-dehydroxylated analog and lentztrehalose C is a cyclized analog of lentztrehalose A. All the lentztrehaloses are only minimally hydrolyzed by mammalian trehalase. The production of the lentztrehaloses is high in rather dry conditions and low in wet conditions. Lentztrehalose B shows a moderate antioxidative activity. These facts suggest that the lentztrehaloses are produced as humectants or protectants for the producer microorganism under severe environmental conditions. All the lentztrehaloses induce autophagy in human cancer cells at a comparable level to trehalose. Considering the enzyme-stability, these lentztrehaloses can be regarded as promising new drug candidates for the treatment of neurodegenerative diseases and other autophagy-related diseases, such as diabetes, arteriosclerosis, cancer and heart disease.

**INTRODUCTION**

Trehalose is a unique disaccharide formed from two molecules of glucose linked by an α,1,1-glucoside bond. It is prevalently observed in plants, microorganisms and animals. Industrially, trehalose is widely used in foods, drugs and cosmetics as a sweetener, humectant or stabilizer. Trehalose is known to have several biological activities, including bone reinforcement, anti-tumor and anti-obesity activities. As it is a chemical chaperone, a neuroprotective effect is also expected and the application of trehalose in the treatment of neurodegenerative diseases has also been investigated. Decrease of aggregates, improved motor dysfunction and extended life span were reported in mice administered trehalose in a model of Huntington’s disease. In addition, the recent discovery of the autophagy-inducing activity of trehalose in cultured cells is of considerable interest.

Autophagy is a catabolic process that involves degradation of unnecessary or dysfunctional cellular components through the action of lysosomes. The accumulation of abnormal proteins in neurons is a common feature in neurodegenerative diseases, such as Alzheimer’s disease, amytrophic lateral sclerosis, Parkinson’s disease, Huntington’s disease and prion diseases, and which causes the progression of such diseases. The accumulated proteins can be decreased by autophagy, which leads to an increased survival of neural cells and improvement in the disease condition. Thus, autophagy-inducing agents are attracting attention as new drug candidates for such diseases. In the past few years, several groups have reported alleviation of disease progression after trehalose administration in mouse neurodegenerative disease models of prion, Alzheimer’s, amytrophic lateral sclerosis and Parkinson’s diseases. Unlike other autophagy inducers, such as rapamycin and verapamil, trehalose is a food constituent and thus attracts special attention as a safe candidate for the treatment of neurodegenerative diseases. There are, however, several problems with the therapeutic use of trehalose. Orally taken trehalose is digested by the hydrolyzing enzyme trehalase expressed in the intestine and kidney, making its bioavailability low. Hence, a large amount of trehalose would be needed for the treatment of disease. Applying the administration protocol used in mouse experiments to human use, more than 50 g of trehalose would be needed to be taken daily. The burst release of the resultant product, glucose, is also problematic and may cause, or exacerbate, diabetes, obesity and vascular disorders. The development of enzyme-stable analogs of trehalose would be helpful in solving this problem.

We previously reported such an enzyme-stable analog, lentztrehalose, isolated from a rare actinomycete Lentzea sp. MLA57-mF8. Lentztrehalose is only minimally hydrolyzed by a porcine kidney trehalase and thought to be stable in the mammalian body. This stability is likely the cause of the better or comparable levels of antitumor, bone reinforcement and suppression of obesity activities in mice administered lentztrehalose compared with trehalose, when lentztrehalose was administered at 1/4–1/2 the amount of trehalose.
In this study, we describe two new lentztrehalose analogs from the same actinomycete strain. Their structures, physicochemical properties and biological activities, including autophagy-inducing activities are reported herein.

RESULTS AND DISCUSSION
Isolation and properties of lentztrehaloses B and C
In this study, we describe two new analogs of lentztrehalose. Since the new analogs were isolated from the same actinomycete strain Lentzea sp. ML457-mF8 and highly relevant to lentztrehalose, we renamed lentztrehalose as lentztrehalose A and named the new analogs lentztrehaloses B and C (Figure 1a). The producing strain was previously cultured on steamed flattened barley and impurities hid the existence of lentztrehaloses B and C in the isolation process. The media was changed to steamed germinated brown rice, which has less impurities, and the new analogs were found in the more hydrophobic fractions during the isolation of lentztrehalose A. To obtain pure (≥95%) lentztrehaloses, the culture extract was sequentially subjected to chromatography with DIAION HP20, octadecyl silica (HPLC), polyamine (HPLC), silica gel, activated carbon and Sephadex LH-20 columns. Approximately 1.0, 1.0 and 0.6 g of lentztrehaloses A, B and C, respectively were purified from media made from 1.0 kg of germinated brown rice grain.

The physicochemical properties of lentztrehaloses B and C are shown in Table 1. The optical rotations of lentztrehaloses B and C were +177° and +160°, respectively. The molecular formulas of lentztrehaloses B and C were determined to be C_{17}H_{30}O_{11} and C_{17}H_{30}O_{12} by high resolution electrospray ionization (HRESI)-MS, elemental analysis and NMR data (Table 1 and Table 2, and Supplementary Figures 1–19). The sweetness of the lentztrehaloses was comparable to that of trehalose (Table 1) with lentztrehalose C the sweetest. Lentztrehalose B had some sharpness of taste, which made it less sweet. None of the lentztrehaloses showed toxicity against 36 strains of microbes from 16 species up to 128 μg ml⁻¹ and 53 strains of human and mouse cancer cell lines and three mouse splanchic primary culture cells up to 200 μg ml⁻¹. Acute toxicity was not observed in the ICR mouse up to 500 mg kg⁻¹ po administration and i.v. injection (data not shown).

Structure elucidation of lentztrehalose B
The structure determination was mainly carried out by comparison with the published data for lentztrehalose A.\(^1\) The \(^1\)H-NMR spectra indicated a two spin–two spin coupling system resulting from two hexoses between H-1’ (δ_H 5.11) and H-6’ (δ_H 3.65, 3.77), and H-1” (δ_H 5.09) and H-6” (δ_H 3.67, 3.79) as shown in Figure 1 and Supplementary Figures 4 and 7. The hexoses are connected with a 1,1-glycosidic linkage as determined by HMBC correlations, from H-1’ to C-1’” (δ_C 95.1) and H-1” to C-1’ (δ_C 95.0). These chemical shifts (Table 2) and the characteristic 1H coupling constants (all large except for \(^{3}J\_H1-H2=3.7\) Hz and \(^{3}J\_H11-H12=3.7\) Hz), NOEs (Supplementary Figures 8 and 9) and optical rotation listed above revealed a trehalose moiety. In lentztrehalose B, another \(^1\)H COSY correlation, from the oxymethylene at δ_H 4.17 and 4.37 (H-1) to the olefinic proton at δ_H 5.37 (H-2) was observed. Further connectivity was established by HMBC (Supplementary Figure 6). Long-range couplings were observed from H-5 (δ_H 1.74) and H-4 (δ_H 1.69) to C-3 (δ_C 137.6) and C-2 (δ_C 122.7); from H-5 to C-4 (δ_C 18.2); from H-4 to C-5 (δ_C 25.9); from H-1 to C-4’ (δ_C 79.0); and from H-4’ (δ_H 3.25) to C-1 (δ_C 70.1) established that lentztrehalose B possesses a 3-methyl-2-butenyl side chain attached to the C-4’ of trehalose. The structure of lentztrehalose B was elucidated to be 4-O-[(3-methyl-2-butenyl)] trehalose as shown in Figure 1.

Figure 1 Structure of lentztrehaloses. (a) Structure of lentztrehaloses. TRH: trehalose, LTA, B, C: lentztrehaloses A, B, C. (b) \(^1\)H-\(^1\)H COSY and HMBC correlations of lentztrehaloses B and C.
Table 1: Physicochemical properties of lentztrehaloses B and C

|                          | Lentztrehalose B | Lentztrehalose C |
|--------------------------|------------------|------------------|
| Appearance               | White solid      | White solid      |
| Optical rotation (°)     | [α]D25 +177 (c 1.06, MeOH) | [α]D25 +160 (c 1.03, MeOH) |
| UV λmax (MeOH) (cm⁻¹)   | End absorption   | End absorption   |
| IR (KBr) max (cm⁻¹)     | 3410, 2931, 1674, 1449, 1378, 1203, 1191, 1153 | 3422, 2935, 1635, 1457, 1146, 1105, 1053, 995, 839, 801, 613 |
| Molecular formula        | C₁₇H₃₀O₁₁     | C₁₇H₃₀O₁₂     |
| HRESI-MS (m/z)           | 433.1680 (C₁₇H₃₀O₁₁+Na⁺) | 449.1629 (C₁₇H₃₀O₁₂+Na⁺) |
| Elemental analysis       |                  |                  |
| Calculated               | C 47.7% H 7.5%  | C 45.9% H 7.3%  |
| Found                    | 433.1683 (M+Na) | 449.1629 (M+Na) |
| ORAC value (μmol)        | 207.3           | 8.1             |
| TE (g⁻¹)                 |                  |                  |
| Mean ± s.d.              | 5.5 ± 4.3       | 8.2 ± 3.9       |

Abbreviation: ORAC, oxygen radical absorbance capacity.

*Arbitrary score measured by a sensory test.

Ten panelists tasted 10 μl of 500 mg ml⁻¹ blotted solutions of the lentztrehaloses, sucrose, trehalose and water. The sweetness of the lentztrehaloses and trehalose was scored on a scale where water and sucrose were scored as 0 and 10, respectively. The scores of lentztrehalose A and trehalose measured at the same time were 6.2 ± 2.5 and 6.8 ± 2.2, respectively.

Structure elucidation of lentztrehalose C

The ¹H-¹H COSY and TOCSY spectra also indicated a two spin-spin coupling system from H-1 (δ₇ 5.14) to H-6 (δ₇ 3.64, 3.73) and from H-1” (δ₇ 5.10) to H-6” (δ₇ 3.67, 3.79) derived from two hexoses coupled via a 1,1-glycosidic linkage as determined by HMBMC in the same manner as for lentztrehalose B (Supplementary Figure 13 and 15–17). The chemical shifts of lentztrehalose C were slightly different from those of lentztrehalose A in C-2’ to C-4’, but the ¹H coupling constants (Table 2), which were closely related to those of A, and the optical rotation indicated that lentztrehalose C also possesses a trehalose moiety. In the HMBMC spectrum, cross-peaks were observed from H-5 (δ₇ 1.37) and H-4 (δ₇ 1.16) to C-3 (δ₂C 78.7) and C-2 (δ₂C 79.1); from H-5 to C-4 (δ₂C 23.4); from H-4 to C-5 (δ₂C 25.0); from H-1 (δ₂C 3.63, 3.86) to C-4’ (δ₂C 79.0); and from H-4’ (δ₇ 3.17) to C-1 (δ₂C 72.4) established that lentztrehalose C also possesses a 2,3-dihydroxy-3-methylbutoxy chain attached to the C-4’ of trehalose, the same as lentztrehalose A. Newly appeared long-range correlations were observed from the H-3’ (δ₇ 3.92) of trehalose to the oxymethylene at C-2 (δ₂C 79.1) and from H-2 (δ₇ 3.79) to C-3’ (δ₂C 76.1). These correlations established the presence of a six-membered ring structure between C-2 in the 2,3-dihydroxy-3-methylbutoxy moiety and the C-3’ of trehalose with an ether linkage as shown in Figure 1. The NOEs observed between H-2 and H-3’ showed the 2-hydroxy-2-propyl group of C-2 was in an equatorial orientation in the additional six-membered ring. The structure of lentztrehalose C was elucidated to be 3,4-O-[(1R,1)-(2-hydroxy-2-propyl)-1,2-ethanediyl] trehalose as shown in Figure 1. Although ~60–70 natural compounds other than lentztrehalose A are known to have a 2,3-dihydroxy-3-methylbutoxy moiety, none of these compounds are reported to have a cyclized analog as observed for lentztrehalose C.

Production of lentztrehaloses

The side chain of lentztrehalose B is likely to be derived from dimethylallyl pyrophosphate, which is made in most organisms by the mevalonate and non-mevalonate pathways. Because both trehalose and the side chain moiety are prevalent in many organisms, we considered that lentztrehaloses might be common compounds and searched for other lentztrehalose producing actinomycetes. Although more than 50 species of actinomycetes were cultured under the same conditions and the metabolites analyzed, no other species produced any of the lentztrehaloses A, B or C (data not shown). We theorized that there is a unique enzyme required to transfer dimethylallyl to the C-4 of trehalose that is found in the producing strain.

During the exploration of the optimal culture conditions for a high yield of lentztrehaloses, it was observed that the producing strain preferred rather dry conditions and excess humidity suppressed the production of lentztrehaloses (Figure 2). In a germinated brown rice medium (rice grain:water = 3:5), trehalose, and lentztrehaloses A, B and C were produced at approximately 5.0-, 2.0-, 1.5- and 0.8-mg per 1.0 g rice grain, respectively after 4 weeks’ culture (Figure 2, 1×). When the water content was reduced by half, the culture seemed dry at 4 weeks and the yields of trehalose and the lentztrehaloses were not much changed (Figure 2, 1/2×). Contrarily, in the porridge-like medium, the yields of trehalose and the lentztrehaloses were greatly reduced (Figure 2, <1×). We next examined the addition of trehalose to the culture, because trehalose might be the direct material used for the synthesis of the lentztrehaloses and thus its addition increases the yield (Figure 2, 3% trehalose bars). The addition of trehalose slightly increased the yield of lentztrehalose A and decreased the yield of lentztrehalose B in 1× and 1/2× cultures. Thus, trehalose does not appear to be an efficient material for the synthesis of lentztrehaloses under these conditions. In 2× culture, however, additional trehalose increased the yield of lentztrehalose A to a level comparable to drier conditions. The biosynthesis of the trehalose moiety appears to be the rate limiting step for lentztrehalose production in wet environmental conditions and the transfer of the side chain component seems independent of it. The higher yield in dry conditions suggests that the lentztrehaloses, as well as trehalose, are produced as humectants or protectants for the producing microorganisms.

Enzyme stability of lentztrehaloses B and C

Unlike trehalose, all of the lentztrehaloses were minimally hydrolyzed by porcine kidney trehalase (Figure 3a). In our previous report, lentztrehalose A slightly released glucose when a large amount was reacted with porcine kidney trehalase. This effect may have been caused by an impurity in the previous lot of lentztrehalose A, which might have contained trehalose or other oligo- or poly-saccharides. These impurities released glucose after reaction with trehalase or other glycosidases present as contaminants. In the more recent purification process, several more chromatography steps using silica, polyamine and activated carbon columns were employed to reduce contamination with other sugars. Therefore, it is concluded that pure lentztrehaloses are trehalase-stable. While lentztrehaloses A and C did not suppress the activity of trehalase up to 10 μM, lentztrehalose B weakly suppressed it at a high concentration (Figure 3b). The mechanism, however, is unclear and lentztrehalose B is not considered to be a specialized inhibitor of trehalase.

Antioxidative activity of lentztrehalose B

As a part of the investigation into the properties of the lentztrehaloses, we examined the antioxidative activity using an oxygen radical absorbance capacity (ORAC) activity assay. In the assay, fluorescein
| Position | $\delta_C$ (multiplicity) | $\delta_H$ (J, in Hz) NOE | HMBC | Position | $\delta_C$ (multiplicity) | $\delta_H$ (J, in Hz) NOE | HMBC | Position | $\delta_C$ (multiplicity) | $\delta_H$ (J, in Hz) NOE | HMBC |
|----------|--------------------------|---------------------------|------|----------|--------------------------|---------------------------|------|----------|--------------------------|---------------------------|------|
| 1        | 70.1 (t)                 | 4.17 (1H, dd, 7.1, 11.2)  | 4'   | 1        | 72.4 (t)                 | 3.63 (1H, dd, 1.6, 11.9)  | 4'   | 1        | 74.7 (t)                 | 4.00 (1H, dd, 2.8, 10.6)  | 3.65 m |
| 2        | 122.7 (d)                | 5.37 (1H, m)              | 1, 5 | 4        | 2                    | 3.79 (1H, dd, 1.6, 8.5)  | 1, 3, 4, 5 | 2        | 78.2 (d)                 | 3.54 (1H, dd, 2.8, 8.0)  |       |
| 3        | 137.6 (s)                | 4                        |     | 3        | 2                    | 3.79 (1H, dd, 1.6, 8.5)  | 1, 3, 4, 5 | 3        | 72.8 (s)                 | 1.18 (3H, s)              |       |
| 4        | 18.2 (q)                 | 1.69 (3H, s)              | 2, 3, 5 | 4        | 2                    | 3.79 (1H, dd, 1.6, 8.5)  | 1, 3, 4, 5 | 4        | 26.4 (q)                 | 1.16 (3H, s)              |       |
| 5        | 25.9 (q)                 | 1.74 (3H, s)              | 2, 3, 4 | 5        | 2                    | 3.79 (1H, dd, 1.6, 8.5)  | 1, 3, 4, 5 | 5        | 25.4 (q)                 | 1.16 (3H, s)              |       |
| 1'       | 95.0 (d)                 | 5.11 (1H, d, 3.7)         | 2', 5', 5, 1' | 1'       | 94.6 (d)               | 5.14 (1H, d, 3.9)         | 2', 5', 5, 1' | 1'       | 95.1 (d)                 | 5.10 (1H, d, 3.8)         |       |
| 2'       | 73.5 (d)                 | 3.48 (1H, dd, 3.7, 9.6)   | 1', 4' 3' | 2'       | 71.8 (d)               | 3.44 (1H, dd, 3.9, 9.4)   | 1', 4' 3' | 2'       | 73.3 (d)                 | 3.50 (1H, dd, 3.8, 9.6)   |       |
| 3'       | 74.8 (d)                 | 3.88 (1H, d, 9.5)         | 5' 2', 4' | 3'       | 76.1 (d)               | 3.92 (1H, dd, 8.5, 9.2)   | 2, 1', 2, 1', 2, 4' | 3'       | 74.4 (d)                 | 3.90 (1H, t, 9.6)         |       |
| 4'       | 79.0 (d)                 | 3.25 (1H, dd, 8.9, 10.0)  | 1, 2, 1, 3', 5, 6' | 4'       | 79.0 (d)               | 3.17 (1H, dd, 8.3, 9.9)   | 1, 2', 6', 1, 4', 5, 6' | 4'       | 80.4 (d)                 | 3.28 (1H, t, 9.6)         |       |
| 5'       | 72.9 (d)                 | 3.81 (1H, dd, 2.1, 4.8, 10.1) | 5' 2' 2, 2, 10, 2, 2, | 5'       | 72.2 (d)               | 3.81 (1H, dd, 2.2, 5.2)   | 1, 2, 10, 2, 2, 2, 5, 2, 10, 2 | 5'       | 72.7 (d)                 | 3.85 (1H, br dt, 3.3, 9.6) |       |
| 6'       | 62.3 (t)                 | 3.65 (1H, dd, 4.8, 11.9)  | 6' 2', 6, 2, 6, 2, 6, 2, | 6'       | 62.5 (t)               | 3.64 (1H, dd, 5.2, 12.1)  | 4', 5' | 6'       | 62.1 (t)                 | 3.75 (1H, m)              |       |
| 1'       | 95.1 (d)                 | 5.09 (1H, d, 3.7)         | 2', 5', 1', 2', 5' | 1'       | 94.8 (d)               | 5.10 (1H, d, 3.7)         | 1', 2', 5', 1', 2', 5', 1' | 1'       | 95.0 (d)                 | 5.09 (1H, d, 3.8)         |       |
| 2'       | 73.3 (d)                 | 3.45 (1H, dd, 3.7, 9.6)   | 1', 4' 3' | 2'       | 73.2 (d)               | 3.48 (1H, dd, 3.7, 9.8)   | 1', 4' 3' | 2'       | 73.2 (d)                 | 3.45 (1H, dd, 3.8, 9.6)   |       |
| 3'       | 74.6 (d)                 | 3.78 (1H, t, 9.4)         | 3', 4', 3' | 3'       | 74.5 (d)               | 3.82 (1H, t, 9.3)         | 1', 2', 4', 3' | 3'       | 74.5 (d)                 | 3.77 (1H, m)              |       |
| 4'       | 72.0 (d)                 | 3.32 (1H, dd, 9.1, 9.9)   | 2', 3', 5', 6' | 4'       | 72.0 (d)               | 3.32 (1H, t, 9.2)         | 2', 6', 3', 5', 6' | 4'       | 71.9 (d)                 | 3.31 (1H, t, 9.5)         |       |
| 5'       | 73.8 (d)                 | 3.81 (1H, m)              | 4' 5' 3' | 5'       | 73.8 (d)               | 3.80 (1H, m)              | 4' 5' | 5'       | 73.8 (d)                 | 3.79 (1H, m)              |       |
| 6'       | 62.7 (t)                 | 3.67 (1H, dd, 5.5, 12.1)  | 5', 6' 6, 5', 6, 6 | 6'       | 62.7 (t)               | 3.67 (1H, dd, 5.2, 11.8)  | 4', 5' | 6'       | 62.6 (t)                 | 3.67 (1H, dd, 4.7, 12.0)  |       |

$^1$H (600 MHz) and $^{13}$C (150 MHz). Chemical shifts in p.p.m. from TMS as internal standard.

$^a$Literature data.
were checked by western blotting in a preliminary experiment (Supplementary Figure 20). Immediately after proLC3 is translated, the C-terminal fragment is cleaved to yield a cytosolic form LC3-I. LC3-I is further converted to an autophagosome-associated form LC3-II.8 Except for two strains whose LC3 expression levels were constitutively high, other strains showed a clear increase in the expression level of LC3-II. We selected two highly sensitive cell lines, melanoma cell Mewo and ovarian cancer cell OVK18, for evaluating lentztrehaloses. As reported in the previous study,7 trehalose required a concentration of approximately 100 mM to induce a clear increase in LC3 expression (Figure 5a). The lentztrehaloses showed a similar pattern and treatment with only 100 mM could clearly increase the expression level of LC3. As seen in Mewo cells treated with 0.1–10 mM lentztrehalose C, a concentration-dependent increase in LC3 expression was sometimes observed. It was, however, not always reproducible. The induction of autophagy was also evaluated by using another marker Cyto-ID.17 This green fluorescent reagent turns bright in vesicles produced during autophagy. Although the fluorescence was observed in a few spots in the control cells, it spread over the whole cell, except for the nucleus, in Mewo and OVK18 cells treated with 100 mM trehalose or lentztrehalose (Figure 5b). These data indicated that lentztrehalose can induce autophagy in human cells at a comparable level to trehalose. Considering the activity of lentztrehalose C, the hydroxyl groups on C-3′ and C-4′ in trehalose can be modified while keeping the autophagy-inducing activity and it should be possible to synthesize trehalose analogs with better activity and bioavailability.

CONCLUSION

We identified two new analogs of lentztrehalose and determined their structures. Lentztrehaloses A and C are probably formed from prenyl ether lentztrehalose B through an epoxide that is hydrolyzed to give isomer A or intramolecular nucleophilic attack of C-3′-OH results in isomer C. It is not clear if both lentztrehaloses A and C are derived from a corresponding epoxide since the configuration of lentztrehalose A at C-2 is not yet determined. The chemical synthesis of lentztrehalose A is now under development at our institute and the configuration at C-2 will be determined in the near future and the structural relevance of lentztrehaloses A and C will be discussed in due course. All three lentztrehaloses, A, B and C, were trehalase-stable and showed a level of autophagy-inducing activity comparable to trehalose. As more stable analogs of trehalose and with comparable autophagy induction activities, lentztrehaloses are promising drug candidates for the treatment of neurodegenerative diseases and diabetes, arteriosclerosis, cancer and heart disease, which may be cured or alleviated by the induction of autophagy.8 In addition to its autophagy-inducing activity, lentztrehalose B has antioxidative activity. Antioxidants are being studied as possible drugs for amyotrophic lateral sclerosis treatment18 and the curative effects of lentztrehalose B for the treatment of amyotrophic lateral sclerosis is of particular interest for future studies.

MATERIALS AND METHODS

Isolation and structure determination of lentztrehaloses B and C

The previously reported methods for the production and isolation of lentztrehalose A were modified.15 Strain ML457-mF8 was cultured in a steamed germinated brown rice medium (IRits, Tokyo, Japan, rice grain:water = 3:5, autoclaved at 121 °C for 20 min) for 3 weeks. The whole culture was extracted with twice the volume of MeOH and EtOH, sequentially. The extract was evaporated and separated with a DIAION HP20 (Mitsubishi Chemical, Tokyo, Japan) column in a batch process using water and 50% MeOH. Although
lentztrehalose A was purified from the water fraction, B and C were isolated from the 50% MeOH fraction. Each fraction was separated by sequential chromatography with an octadecyl silica (Hydrosphere C18, YMC, Kyoto, Japan) and polyamine (YMC-Pack Polyamine II, YMC) HPLC, silica gel (Wakosil C-300, Wako Pure Chemical Industries, Ltd, Osaka, Japan), activated carbon (Wako) and Sephadex LH-20 (GE Healthcare Japan, Hino, Tokyo, Japan) open columns. The elution solvents used were water and MeOH for octadecyl silica HPLC, acetonitrile and water for polyamine HPLC, BuOH:MeOH:water = 4:1:2 mixture for the silica column and MeOH for the activated carbon and LH-20 columns. For the purity or yield check, lentztrehaloses were separated by HPLC with a hydrophilic interaction chromatography column (XBridge Amide, Waters, Milford, MA, USA) by using a linear gradient of 90–50% acetonitrile and detected by an evaporative light scattering detector system (ELSD 2000ES, Alltech, Deerfield, IL, USA).

The structure and physicochemical properties of lentztrehaloses B and C were determined by spectrometric analyses performed as previously described using the equipment as follows. Optical rotations were recorded on a P-1030 polarimeter (JASCO Inc., Tokyo, Japan). UV spectra were obtained on a U2800 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan). $^3$H (600 MHz) and $^{13}$C (150 MHz) NMR spectra were measured in methanol-d$_4$ with a JNM-ECA600 spectrometer (JEOL Resonance Inc., Tokyo, Japan) using tetramethysilane as an internal reference. HRESI-MS spectra were obtained on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) or T-100LC TOF-MS (JEOL Ltd, Akishima, Tokyo, Japan). IR spectra were recorded on a FT/IR-4100 Fourier transform infrared spectrometer (JASCO). The novelty of the lentztrehaloses 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**

Trehalase digestion experiments were performed as previously described. The pH of 135-mM citrate buffer, however, was misdescribed as 7.5 in the previous report, the correct value is 5.7.

**ORAC assay**

The ORAC assay was performed using a OxiSelect Oxygen Radical Antioxidant Capacity Activity Assay kit (Cell Biolabs, Inc., San Diego, CA, USA) following the manufacturer’s protocol. The fluorescence was detected with a fluorescence plate reader ARVO (PerkinElmer, Waltham, MA) using the filters Ex. 485 nm and Em. 535 nm.

**Detection of autophagy**

Human melanoma cell Mewo (JCRB0066) was obtained from the Health Science Research Resources Bank (Sennan, Japan). Human ovarian cancer cell OVK18 was provided by the RIKEN BRC (Tsukuba, Japan) through the National Bio-Resource Project of the MEXT, Japan. The cells were plated on 96-well microplates at 1.6 × 10^4 cells per 0.2 ml per well in a Dulbecco’s Modified Eagle Medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO, USA) and 1% penicillin–streptomycin–glutamine solution (Thermo Fisher Scientific). The cells were cultured at 37°C for 72 h, and a half volume of medium was picked up to dissolve trehalose or the lentztrehaloses and put back in the original well to treat the cells with the sugars. After culturing for 24 h, the medium was removed and...
cells were washed with phosphate-buffered saline (DS Pharma Biomedical, Suita, Osaka, Japan) once and lysed with a 2 × SDS-PAGE sample buffer (4% SDS, 0.125 M Tris-HCl (pH 6.8), 10% sucrose). The protein concentration was measured by BCA assay (Thermo Fisher Scientific) and 2.5-μg protein/sample was applied on western blotting. Western blotting was basically performed as previously described\textsuperscript{20–22} using anti-LC3 antibody (Medical & Biological

![Figure 4](image)

**Figure 4** Antioxidative activity of lentztrehalose B. (a) Antioxidative activities of the lentztrehaloses were evaluated by oxygen radical absorbance capacity activity assay. TRH: trehalose, LTA, B, C: lentztrehaloses A, B, C. (b) Lentztrehalose B was dissolved at 0.5 mM in water or 15% H$_2$O$_2$, and allowed to stand for 6 days at room temperature. The mass spectrum was analyzed with a time-of-flight MS.
Figure 5 Autophagy inducing activity of lentztrehaloses. (a) Human melanoma cell Mewo and ovarian cancer cell OVK18 were treated with each concentration of trehalose (TRH) or lentztrehaloses (LTA, B and C) for 24 h and the expression level of autophagy markers LC3-I and II were observed by western blotting. The expression of β-tubulin was used as a control. The same 100-mM TRH sample was loaded on the different gels as a control. (b) Mewo and OVK18 cells were treated with 100-mM TRH or lentztrehaloses for 24 h and then autophagy marker dye Cyto-ID for 30 min. The vesicles formed during the autophagic process were stained brightly. PC, phase contrast.

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