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Cryoprotection with L- and *meso*-Trehalose: Stereochemical Implications

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Two unnatural stereoisomers of α,α-trehalose (L- and meso*-trehalose) were synthesized and evaluated as cryoprotectants in order to determine the functional consequences of relative or absolute stereochemistry on their physicochemical properties. Adherent yeast cell cultures were frozen in 10% solutions of D-, L-, and* meso*-trehalose for periods of 7–28 days, then evaluated by a MTT viability assay. D- and L-trehalose were equally effective in maintaining high rates of cell survival, thus demonstrating the absence of chiral discrimination at the carbohydrate–lipid interface, whereas* meso*-trehalose was inferior in cryoprotection efficacy. Differential scanning calorimetry revealed a difference in the glass transition temperatures (T_g) of D- and* meso*-trehalose of nearly 75 °C. This can be attributed to differences in conformational behavior, as portrayed by torsional energy maps for rotation about the glycosidic bonds of D- and* meso*-trehalose. We conclude that the biostabilizing properties of α,α-trehalose depend on relative stereochemical factors, but are independent of absolute stereochemical configuration.*

Introduction

The nonreducing disaccharide α,α-D-trehalose (**1**, D-Glc(α1→1)α-D-Glc) is well known for its outstanding ability to stabilize cell membranes and proteins against harsh environmental conditions such as subfreezing temperatures, desiccation, pressure, temperature shock, and oxidative stress.[1,2] The biostabilizing properties of **1** are remarkable when compared with those of other simple sugars, such as glucose and sucrose, and make trehalose the excipient of choice for applications involving cryopreservation and anhydrobiosis.[3,4] Numerous investigations have been conducted to understand the basis for membrane stabilization by D-trehalose (**1**) and other excipients, many of which have been summarized in recent reviews.[1,2] Crowe and co-workers have collected ample evidence that points to two dominant factors: 1) depression of the gel-phase transition temperature (T_m) of the cell membrane, which prevents cracking and leakage at low temperatures, and 2) formation of a vitrified state that inhibits membrane fusion upon thawing or rehydration.[1,3,5] Phospholipid liposomes dried in the presence of **1** exhibit a depression in T_m of 10–20 °C relative to their hydrated state and can maintain a stable gel phase well below the freezing point of water.[5] With respect to vitrification, anhydrous **1** has a notably higher glass-transition temperature (T_g) than other sugars,[6] a factor that is particularly relevant for membrane stabilization in a freeze-dried state.[5,7] Although it has been argued that α,α-trehalose does not provide any special advantages for stabilizing membranes when optimized cryogenic conditions are applied,[1] no other sugar has demonstrated comparable efficacy in cryoprotection under standard (suboptimal) experimental conditions.

Several theories have been put forth to explain α,α-trehalose's mechanism of membrane stabilization. These include the "water-replacement" hypothesis, in which **1** is in intimate contact with the phospholipid membrane;[8] the "water-entrapment" hypothesis based on the preferential exclusion mechanism,[9] in which the role of **1** is to preserve the hydration layer; and the aforementioned vitrification hypothesis, in which **1** forms an amorphous glass to reduce physical perturbations to the membrane.[5] Support for the water-replacement hypothesis comes from differential scanning calorimetry (DSC) and IR spectroscopic studies at the trehalose–lipid interface,[8] and by changes in the Langmuir-domain structures of phospholipid monolayers when trehalose is added to the aqueous subphase.[10] Studies of trehalose–lipid mixtures in the anhydrous solid state also suggest that the mobility of the phospholipid head groups is hindered by their close association with trehalose.[11] Support for the water-entrapment hypothesis is provided by the observation that **1** has an anomalously large radius of hydration,[12] a factor that has also been used to explain trehalose's exceptional ability to preserve proteins under environmentally stressful conditions.[5,13] It should be mentioned that these hypotheses are not mutually exclusive; for example, a molecular-dynamics study simulating the interactions between **1**, water, and phospholipid membrane suggests that trehalose–lipid contact and water entrapment can occur simultaneously at the membrane surface.[14]

Although many cryoprotection studies with **1** and other naturally occurring disaccharides have been reported, the molecular features responsible for biostabilization have not yet been examined by systematic structural modification. For example, an intimate trehalose–lipid association implies the possible formation of diastereomeric complexes,[15] but the relative impor-

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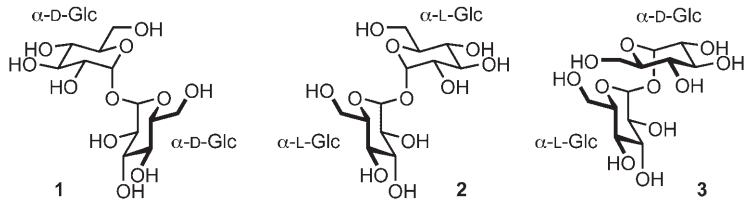
tance of chiral association has not been addressed. It has also been noted that α,α-trehalose is an unusually rigid molecule, adopting a double "*exo*-anomeric" conformation with a well-defined arrangement of its hydroxyl groups (Scheme 1).[16,17]



Scheme 1. D-, L-, and *meso*-trehalose (**1**–**3**), drawn in their double "*exo*-anomeric" conformations. The conformations of **1** and **2** are expected to be highly stable, whereas local steric interactions destabilize the staggered conformations across the glycosidic bond of *meso*-trehalose (**3**).

This conformational rigidity has been postulated to be an important factor in cryoprotection, but the effect of rational changes in the relative stereochemistry of α,α-trehalose have not been explored. In this regard, it is worth mentioning that conformational rigidity has also been implicated in the activity of α,α-trehalose 6,6′-dimycolate (cord factor), a toxic glycolipid produced by *Mycobacterium tuberculosis* known to inhibit the fusion of phospholipid vesicles.[18,19] The mycolate diesters of two unnatural trehalose stereoisomers (α,β- and β,β-trehalose) have been shown to be much less toxic than α,α-trehalose dimycolate, thus providing some evidence for the importance of relative stereochemistry in the disaccharide core.[19]

In this paper we address the importance of relative and absolute stereochemistry in biostabilization by α,α-trehalose by examining the cryoprotectant properties of two novel stereoisomers of **1**, L-trehalose (**2**; L-Glc(α1→1)α-L-Glc) and *meso*-trehalose (**3**; D-Glc(α1→1)α-L-Glc) (Scheme 1). L-Trehalose (**2**) is the enantiomer of **1** and has an identical conformational and physicochemical profile, but its association with phospholipid head groups may be considered as diastereomeric. *meso*-Trehalose (**3**) is expected to have a very different conformational behavior from that of **1** or **2**. While its *exo*-anomeric conformation is superficially similar, a more careful analysis reveals that the staggered conformations of **3** are destabilized by *syn*-pentane interactions; this implies greater conformational flexibility across the glycosidic linkage.[20] The cryoprotective efficacy of **2** will determine whether chiral association is a significant factor in membrane stabilization by α,α-trehalose, whereas studies involving **3** will address the importance of conformational rigidity, as modulated by a rational change in relative stereochemistry.

Results and Discussion

The cryoprotection efficacies of trehaloses **1**, **2**, and **3** were evaluated by freezing yeast cells in 10% aqueous solutions of each sugar at −20 °C, followed by storage at this temperature for up to 28 days. The survival rates were also compared with those of cells frozen in 10% D-sucrose, another nonreducing disaccharide commonly used in cryoprotection,[21] and of cells frozen with <2% D-sucrose (i.e., the residual sugar in the growth media). The latter control provided a baseline for the relative contribution of residual sucrose toward cell viability.

Significant differences in cryoprotection were observed even after just seven days of storage: the survival rates were highest for yeast cells previously frozen in either D-trehalose (**1**; 62.0±6.0%) or L-trehalose (**2**; 61.0±4.5%), whereas those previously frozen in *meso*-trehalose (**3**; 42.5±6.0%) or D-sucrose (41.0±5.0%) were less viable (Figure 1). Yeast cells frozen without additional sugar (control) experienced the lowest survival rates, as expected. Cell survival rates decreased after longer periods of storage, but with the same relative trend: after 28 days at −20 °C, the survival rates for yeast cells previously frozen in trehaloses **1**–**3** were 23, 22, and 17% respectively. A *t*-test analysis (d*f* = 19, $p < 0.01$) indicated no significant differences when comparing the mean values of D- and L-trehalose, or of *meso*-trehalose and D-sucrose, but a statistical difference was confirmed between D- and *meso*-trehalose (see Table 1).

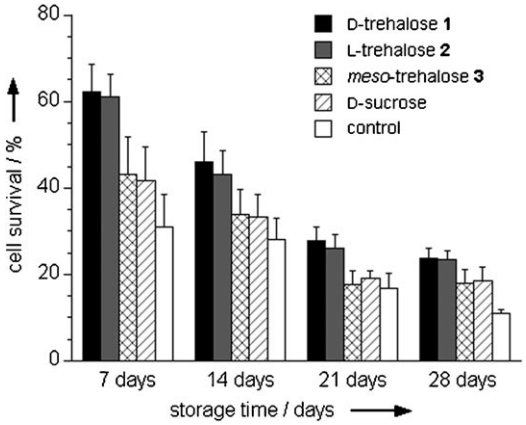


Figure 1. Viability of yeast cells after storage at −20 °C under various conditions for 7 to 28 days. Error bars represent one standard deviation ($N = 20$). Control wells (dilution without added sugar) contain less than 2% D-sucrose.

To determine the effect of exogenous trehalose on the preservation of yeast cells already equipped with endogenous cryoprotectants, similar studies were performed on cells that had been subjected to a mild cold-shock treatment prior to freezing. This preconditioning induces a systemic adaptive response and stimulates the production of a large assortment of proteins, including trehalose synthetase (Tps1, Tps2) and several temperature-shock proteins, followed by the accumulation of intracellular trehalose.[22] It should be mentioned that the additional stabilization does not involve the uptake of exogenous trehalose, as the cell-survival rates were unaffected by the presence or absence of extracellular sugar during the precooling stage, prior to freezing (see the Supporting Information).

Cryoprotection studies preceded by a cold-shock treatment were conducted by refrigerating the yeast cells at 10 °C for 3 h, followed by freezing them in 10% sugar solutions (**1**, **2**, **3**, or D-sucrose) at −20 °C for up to 28 days, as before. The cold

Table 1. *t*-Tests comparing mean survival rates ($df = 19$, $p < 0.01$). Values in bold indicate a significant difference between means ($\Delta t > 2.539$).

t-tests	7 days	14 days	21 days	28 days
No cold shock				
D-, L-trehalose (**1** vs. **2**)	0.616	1.542	1.583	0.324
D-, *meso*-trehalose (**1** vs. **3**)	**7.835**	**6.214**	**10.054**	**6.404**
meso-trehalose, D-sucrose	0.573	0.275	1.949	0.705
With cold shock				
D-, L-trehalose (**1** vs. **2**)	1.199	1.415	1.585	0.807
D-, *meso*-trehalose (**1** vs. **3**)	**17.076**	**21.933**	**36.626**	**45.344**
meso-trehalose, D-sucrose	2.406	**4.737**	**3.086**	**2.898**
Controls (cold shock vs. none)	0.645	2.230	0.306	**13.257**

shock had a significant impact on cell viability, with survival rates as high as 80% after 7 days in frozen D-trehalose solution (see Figure 2). Again, no differences in viability were found for cells protected by D- or L-trehalose, whereas those frozen in *meso*-trehalose had significantly lower survival rates; a difference between *meso*-trehalose and D-sucrose after 14 days was also observed (see Table 1). The contrast in cryoprotective efficacy increased with storage time: after 28 days, the survival rates of cells thawed from solutions of **1** and **2** remained high (~60%), whereas the viability of cells thawed from **3** or sucrose was reduced by a factor of nearly three. It is interesting to note that the differences between control experiments without and with cold shock (cf. Figures 1 and 2, respectively) are insignificant except at the 28-day-storage period (Table 1). This suggests that, while intracellular cryoprotectants have some beneficial effect, it is the exogenous cryoprotectants that are of primary importance, at least in the case of yeast cells.

To evaluate the impacts of the relative and absolute stereochemistries of the α,α-trehaloses on glass stability, a factor that has been used to support the vitrification hypothesis in cryoprotection,[5–7] DSC studies were conducted on amorphous, anhydrous samples of enantiopure **1**, racemic DL-trehalose (**1**/**2**), and *meso*-trehalose (**3**; see Figure 3 and the Supporting Information). The midpoint T_g value of anhydrous **1** was determined to be 114.6 °C, which is comparable to earlier measurements performed under similar conditions.[23] The T_g of racemic DL-trehalose was also measured and found to be only slightly lower than that of enantiopure **1** (111.8 °C); this demonstrates that glass stability is not significantly affected by enantiomeric purity. The insensitivity of T_g to chiral purity has also been observed with other materials; for example, enantiopure polylactide and its racemic blend have been shown to have nearly identical T_g values, although the T_m and crystallization temperature for the latter are significantly higher.[24]

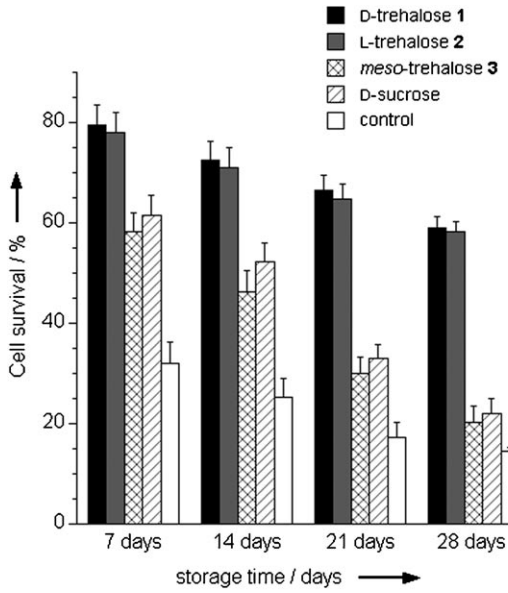


Figure 2. Viability of yeast cells after cold-shock treatment, followed by storage at −20 °C under various conditions for 7 to 28 days. Error bars represent one standard deviation ($N = 20$). Control wells (dilution without added sugar) contain less than 2% D-sucrose.

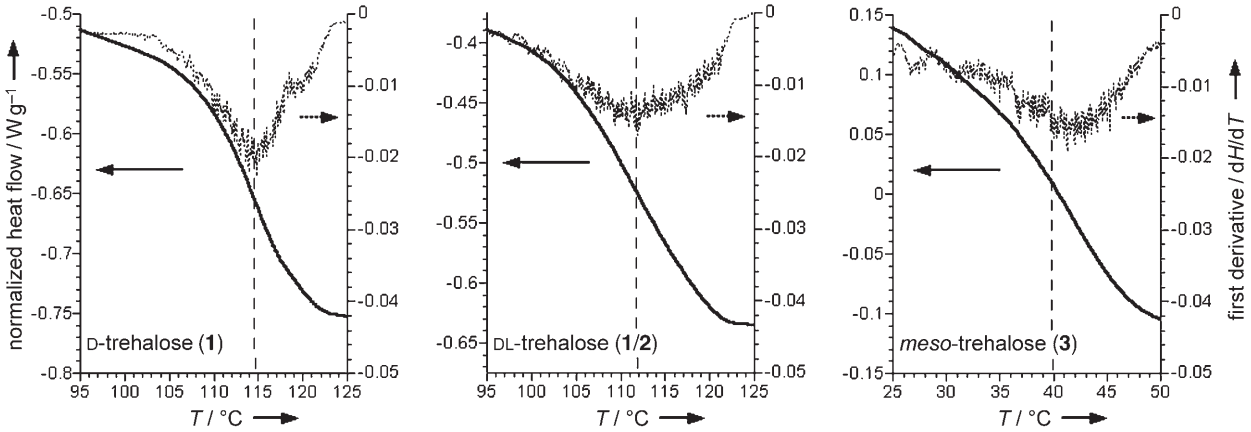


Figure 3. DSC measurements of D-trehalose (**1**), DL-trehalose (**1**/**2**), and *meso*-trehalose (**3**) in their anhydrous glassy states. The maximum change in heat flow (dH/dT, ····) is coincident with the calculated midpoint T_g (-----).

The midpoint T_g of anhydrous *meso*-trehalose (**3**) was determined to be 39.9 °C, a drop of nearly 75 °C in comparison with D-trehalose. The glass-transition temperature of **3** is also much lower than that of anhydrous sucrose, which has a recorded T_g of 65 °C.[23] The low glass-transition temperature is in accordance with the assertion that the α-D- and α-L-glucose rings of **3** are sterically mismatched and are unable to adopt stable conformations across the glycosidic bond, thus increasing the molecule's configurational entropy with a subsequent effect on T_g. This structural mismatch might also have a destabilizing impact on conformations amenable to glass formation through hydrogen bonding with nearby water molecules, with the rapid reorientation of the glucose rings likely to increase the lability of the hydration shell. The latter is supported by a recent molecular-dynamics study by Jeong and co-workers that indicated that the hydration shell around D-trehalose is more stable and longer lasting than that of other disaccharides, including several 1,1-linked stereoisomers.[16d] Their study correlates conformational rigidity of the excipient with a lower energy of hydration and a reduction in motional diffusion, which has a direct impact on the vitrification process.

The disparate conformational behaviors of **1** and **3** were confirmed by MM2* calculations of the torsional strain energies across the glycosidic bonds, as defined by the dihedral angles Φ and Ψ. A conformational energy map of **1** reveals a single, localized minimum centered at $(\Phi,\Psi)=(65^\circ,65^\circ)$, comparable to that described in previous conformational studies of D-trehalose (Figure 4 A).[16] In comparison, *meso*-trehalose (**3**) exhibited two shallow minima at (70°,75°) and (105°,110°) that were separated by an energy barrier of less than 3 kcal mol^{-1} (Figure 4 B). *meso*-Trehalose (**3**) is clearly much less confined in Φ,Ψ space than **1**; this supports the postulated role of conformational rigidity in the glass stability of D-trehalose.

The excellent biostabilizing properties of L-trehalose (**2**) further support the importance of conformational rigidity in forming a vitrified state during cryoprotection. The essentially identical activities of **1** and **2** are not surprising from a purely physicochemical perspective, but it is intriguing to consider that their cryoprotectant properties seem unaffected by potential diastereomeric differences at the carbohydrate–lipid interface. Although the water-replacement hypothesis assumes the first layer of trehalose to be in physical contact with the phospholipid layer, the above results do not suggest the formation of a well-defined complex between α,α-trehalose and the chiral lipid head groups.

The apparent insignificance of molecular chirality in trehalose's cryoprotective activity does not necessarily rule out the water-replacement hypothesis, as other studies have also indicated that chiral recognition is not a prerequisite for supramolecular function at the interface of biological membranes. The mirror-image versions of some channel-forming oligopeptides (comprising solely D-amino acids) have been shown to possess identical hemolytic activity as their natural enantiomers, and exhibit similar degrees of cell specificity.[25] If one considers chiral interactions at biological interfaces in a broader context, there are numerous cases in which the enantiospecificity of biomolecular associations can be considered as a matter of degree.[26] Pheromone receptor signaling is often modulated by the enantiomeric excess of the ligand (an observation that has been documented for insects[27] and more recently for mammals[28]), and in the particular case of carbohydrates, it is known that the mammalian taste bud receptor does not differentiate D-sugars from their L-enantiomers, but registers each as being equally sweet.[29]

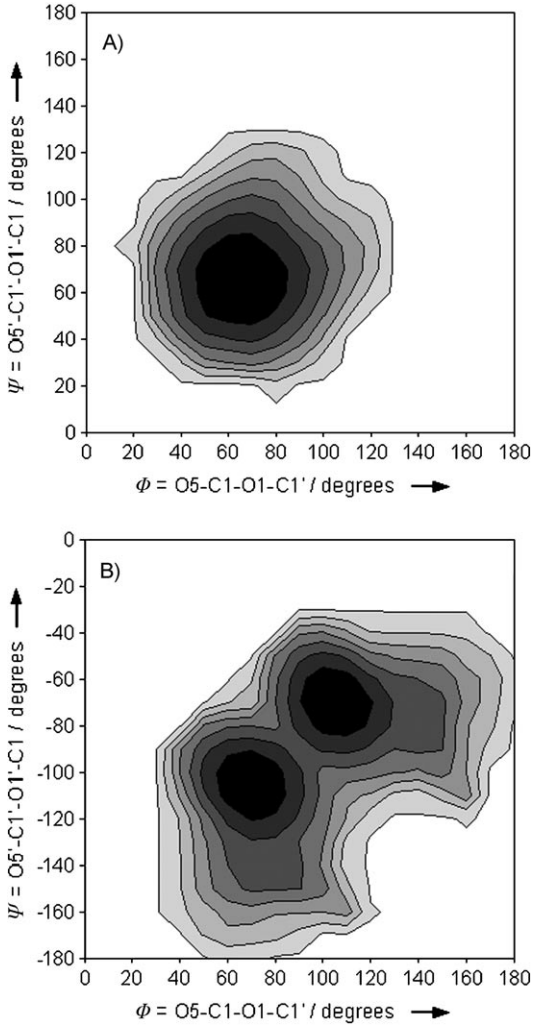


Figure 4. Conformational free-energy plots of A) D-trehalose (**1**) and B) *meso*-trehalose (**3**). Contours are graded in 1 kcal mol^{-1} increments, starting from the global energy minimum and up to 7 kcal mol^{-1}.

Collectively, these studies and ours indicate that molecular chirality can be a surprisingly open-ended parameter at the chemistry–biology interface, and suggest the likelihood of identifying other situations in which the biological role of the carbohydrate is independent of its absolute stereochemistry. Investigations into the chirality–function relationship might prove insightful for defining key factors in "soft" biological interactions, such as those mediated by carbohydrates, that are often weak yet appear to be structure-specific. Mirror-image molecules are ideal for investigating such relationships because their chemical properties (as defined by relative stereochemistry and conformational behavior) are identical to those of the naturally occurring enantiomer, yet are cleanly segregat-

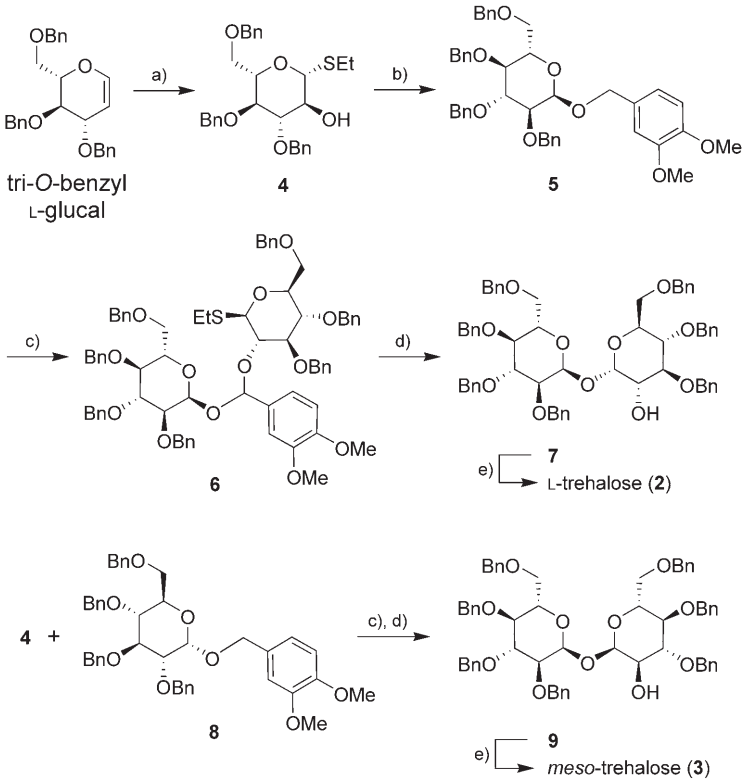
ed from enantioselective processes. This is a noteworthy distinction from typical structure–function relationship studies, whose interpretations can be complicated by changes in chemical behavior that often accompany modification of local structure.

Conclusion

The cryoprotective efficacy of L-trehalose (**2**) is identical to that of D-trehalose (**1**), whereas that of *meso*-trehalose (**3**) is inferior, similar to that of other disaccharides. The dramatic differences in T_g of **1** versus **3** support the importance of conformational rigidity in the vitrification of α,α-trehalose. On the other hand, the cryoprotective properties of **2** demonstrate that chiral association is not a distinguishing factor in membrane stabilization. Mirror-image carbohydrates might be useful for addressing the relative importance of chiral recognition in other carbohydrate-mediated functions that depend on weak but structure-specific associations.

Experimental Section

Synthesis: L-Trehalose (**2**) and *meso*-trehalose (**3**) were prepared by using a modified version of the efficient synthetic route reported by Bertozzi and co-workers (see Scheme 2).[30,31] Dimethyldioxirane (DMDO) oxidation of 3,4,6-tri-*O*-benzyl-L-glucal (prepared in gram quantities by using a protocol recently developed in our laboratories[32]) followed by nucleophilic ring opening with EtSLi produced β-L-thioglucoside (**4**) in 70% yield. One equivalent of **4** was converted to 3,4-dimethoxybenzyl L-glucoside (**5**) and oxidized by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to a quinone methide, followed by condensation with a second equivalent of **4** to form mixed acetal **6**. Thioglycoside activation with dimethyl(methylthio)sulfonium triflate (DMTST) produced 1,1′-α,α-linked disaccharide **7**, which was globally deprotected to L-trehalose (**2**) in 41% overall yield from **4**. To make the achiral *meso* derivative, dimethoxybenzyl D-glucoside (**8**) was prepared from the corresponding glycal, oxidized, and coupled with L-thioglucoside (**4**) in the above manner to produce α,α-linked disaccharide **9**, and deprotected to *meso*-trehalose (**3**) in similar overall yields.



Scheme 2. Synthesis of L-trehalose (**2**) and *meso*-trehalose (**3**). Reagents and conditions: a) i. DMDO, CH_2Cl_2/acetone, −55 °C; ii. EtSH, *n*BuLi, THF, 0 °C (70% over two steps); b) i. NaH, BnBr, DMF; ii. 3,4-dimethoxybenzyl alcohol, MeOTf, CH_2Cl_2 (62% over two steps); c) **4**, DDQ, 4 Å molecular sieves, CH_2Cl_2; d) 2,6-di-*t*Bu-4-methylpyridine, 4 Å molecular sieves, MeOTf, $ClCH_2CH_2Cl$, 40 °C (68% over two steps); e) Pd/C, H_2, MeOH, RT (95%).

Cryoprotection assays: Quantitative cell-viability studies were conducted with commercial baker's yeast (Kroger) under the conditions described by Diniz-Mendes et al.,[33] followed by a colorimetric MTT assay.[34] Dry yeast cells were reactivated in a 4% sucrose solution and incubated for three days at 37 °C, then plated near the end of their logarithmic-growth phase. In a typical experiment, 20 wells of a standard microtiter plate were filled with yeast suspension (10 μL, 1.3×10^{10} cells per mL) and a 20% solution comprised of **1**, **2**, **3**, D-sucrose, or simply water (control; 10 μL). The wells were transferred to a −20 °C freezer and left for 7, 14, 21, or 28 days, then thawed at RT. As a variant of the above experiment, plated yeast cells were also subjected to a mild cold shock at 10 °C for 3 h prior to freezing (see Discussion). Thawed cells were treated with a freshly prepared 0.5% MTT solution (10 μL), incubated at 37 °C for 4 h, then fixed with a 20% solution of sodium dodecyl sulfate in DMF/H_2O (1:1, 20 μL) adjusted to pH 4.7. The fixed cells were placed in a dark cupboard at RT for 12 h to fully solubilize the purple formazan, which was measured by using a microplate reader at $\lambda_{abs}=575$ nm (VERAmax, Molecular Devices). Cell survival rates were established relative to MTT oxidation levels by yeast cell cultures with the same initial population density, prior to freezing.

Differential scanning calorimetry: Anhydrous samples of trehalose were prepared by lyophilization of aqueous solutions in glass vials, followed by heating in vacuo at 60 °C for 24 h in a drying pistol containing P_2O_5. Calorimetry was conducted on glassy samples in hermetically sealed aluminum pans at heating and cooling rates of 20 °C min^{-1} (TA Instruments, DSC Q10). Samples were subjected to two heating cycles, and annealed briefly at temperatures at least 25 °C above the first observable transition. Midpoint T_g values were calculated by using an accompanying software package and are based on changes in heat flow during the second heating cycle.

Computational methods: Molecular models of D- and *meso*-trehalose (**1** and **3**) were examined by using Macromodel version 7.0 (Schrödinger Inc.),[35] at a Silicon Graphics workstation. The two torsional angles defining the conformations of the glycosidic bonds are described as $\Phi=$O5–C1–O1–C1′ and $\Psi=$O5′–C1′–O1′–C1. Conformational analysis of **1** and **3** was performed by constraining Φ and Ψ in 10° increments, followed by full optimization by using MM2*.[36] Relative changes in torsional strain energies were plotted by using MatLab 7.0.1 (MathWorks Inc.).

Acknowledgements

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Keywords: carbohydrates · conformation analysis · cryoprotection · membranes · structure–activity relationships

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