



Structural analysis of novel trehalose-based oligosaccharides from extremely stress-tolerant ascospores of *Neosartorya fischeri* (*Aspergillus fischeri*)

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ABSTRACT

Different fungi, including the genera *Neosartorya*, *Byssoschlamys* and *Talaromyces*, produce (asco)spores that survive pasteurization treatments and are regarded as the most stress-resistant eukaryotic cells. Here, the NMR analysis of a series of trehalose-based oligosaccharides, being compatible solutes that are accumulated to high levels in ascospores of the fungus *Neosartorya fischeri*, is presented. These oligosaccharides consist of an α,α -trehalose backbone, extended with one [α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp; isobemisiolose], two [α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp] or three [α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp] glucose units. The tetra- and pentasaccharide, dubbed neosartose and fischerose, respectively, have not been reported before to occur in nature.

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1. Introduction

Spores are an integral part of the life cycle of many species within the kingdom of fungi. They are the main vehicle for dispersion, via air, water or other means. In addition, they survive extended periods of time in a dormant state. Heat-tolerance above 70 °C is mostly observed within the fungal family of the *Trichocomaceae*.^{1–3} Stress-resistant ascospores are characterized by a thick cell wall (≥ 0.5 μ m) that is shed during germination and accumulate certain molecules, called compatible solutes, up to high concentrations. For instance, ascospores of the fungus *Talaromyces macrosporus* harbour 9–17% wet weight of the protective disaccharide trehalose (more precisely α,α -trehalose).⁴ *T. macrosporus*, *Neosartorya spinosa* and *Byssoschlamys spectabilis* form ascospores that survive more than 1 h at 85 °C and also ultra-high pressure

treatments at 600 MPa.^{5–14} This is comparable to the resistance of spores of *Bacillus subtilis* and explains why *Byssoschlamys*, *Neosartorya* and *Talaromyces* have caused a number of spoilage incidents after pasteurization.¹⁵

Stabilization is a process in which biomolecules and complexes thereof are protected by these compatible solutes. They include polyols, sugars, betaines and amino acids, which are compatible with cellular functioning even at high concentration. Compatible solutes protect cells against stresses such as desiccation and high temperature that have a disruptive effect on water–macromolecule interactions. In fungal cells, trehalose, and the polyols glycerol, erythritol, arabinitol, and mannitol are important compatible solutes. Glycerol is a predominant protector against osmotic stress,^{16,17} besides erythritol, arabinitol, and mannitol.^{18–20} Trehalose is regarded as a superb protective sugar.^{21–23} Its unique ($\alpha 1 \leftrightarrow 1\alpha$) glycosidic linkage makes trehalose non-reducing and therefore unreactive. These properties are important for successful stabilization. Trehalose protects complete micro-organisms, enzymes, membranes and DNA in vitro^{21,24–26} against heat, freezing, desiccation, radiation, and

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oxidative stress.^{24,27–32} In addition, chaotropicity-mediated stresses of substances such as hydrocarbons and solvents are alleviated by trehalose.³³ Protection of trehalose is thought to be based on its high glass transition temperature (T_g),^{34,35} the ability to replace water by its hydroxyl groups (water-replacement hypothesis)^{21,36} and its stabilizing effect on intermolecular interactions in biomolecules.^{26,37,38} In this context, also oligosaccharides have been described that contain a trehalose backbone extended with one or more monosaccharide units.^{39–45} These compounds are believed to play a role in osmoprotection in bacteria and insects,^{39,44,45} but further data on protective mechanisms are unknown.

Here, we describe the isolation and characterization of three trehalose-based oligosaccharides that are accumulated to high concentrations in ascospores of the fungus *Neosartorya fischeri*. Two of these compounds have not been described to occur in nature before. Their possible biological function in terms of prolonged stabilization of cells against stress has been reported elsewhere.⁴⁶

2. Experimental

2.1. Fungal ascospores

The ascospores of the fungi *N. fischeri* (CBS 317.89) and *T. macrosporus* used in this study were obtained from cultures as described before.⁴⁶ Cell-free extracts were prepared after breaking of the ascospores by means of the Qiagen tissue lyser.⁴⁶

2.2. Gas-liquid chromatography—mass spectrometry

Lyophilized cell-free extracts were treated with 1:1:5 hexamethyldisilazane:trimethylchlorosilane:pyridine for 30 min at rt. Trimethylsilylated monosaccharides, disaccharides and alditols were analyzed by gas-liquid chromatography—electron ionization mass spectrometry (GLC-EIMS) using a Fisons Instruments GC 8060/MD 800 system (Interscience, Breda, The Netherlands) equipped with an AT-1 column (30 m×0.25 mm, Alltech/Grace, Deerfield, IL) (temperature program: 200–280 °C at 4 °C/min, 15 min at 280 °C).

For monosaccharide composition analysis, extracts and isolated carbohydrate samples were first subjected to methanolysis (1 M methanolic HCl, 24 h, 85 °C) to give methyl glycosides, which were trimethylsilylated as described above, and analyzed by GLC-EIMS using the same system (temperature program: 140–240 °C, 4 °C/min).⁴⁷

2.3. Thin-layer chromatography

Ascospore cell-free extracts and carbohydrate samples were spotted on TLC sheets (Merck Kieselgel 60 F254, 20×20 cm) and run using 2:1:1 *n*-butanol:acetic acid:water as mobile phase. Sugar-containing compounds were visualized by orcinol/sulfuric acid staining (100 mg orcinol monohydrate, 95 mL methanol, 5 mL sulfuric acid). Glucose, trehalose, raffinose, verbascose and stachyose were used as standards.

2.4. Gel-filtration chromatography

Oligosaccharides were purified by fractionation of extracts on Bio-Gel P-2 columns (50×2 cm and 100×1.5 cm, Bio-Rad Laboratories, Veenendaal, The Netherlands), using 10 mM NH_4HCO_3 as eluent at a flow rate of 15 mL/h. 5-Min fractions were collected and monitored by TLC; those containing oligosaccharide material of the same retention time were pooled and lyophilized.

2.5. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

MALDI-TOF-MS experiments were performed using an Axima™ mass spectrometer (Shimadzu Kratos Inc., Kyoto, Japan) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were recorded using the reflector mode at a resolution of 5000 FWHM and delayed extraction (450 ns). The accelerating voltage was 19 kV with a grid voltage of 75.2%. The mirror voltage ratio was 1.12, and the acquisition mass range was 200–1100 Da. Samples were prepared by mixing on the target 0.5 μL extract/carbohydrate solutions with 0.5 μL aqueous 10% 2,5-dihydroxybenzoic acid as matrix solution.

2.6. NMR spectroscopy

Resolution-enhanced 1D/2D 500-MHz ^1H NMR spectra were recorded in D_2O on a Bruker DRX-500 spectrometer (Wormer, The Netherlands) at a probe temperature of 300 K (27 °C). Prior to analysis, samples were exchanged twice in D_2O (99.9 atom% D, Cambridge Isotope Laboratories Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D_2O . Suppression of the HOD signal was achieved by applying a WEFT pulse sequence for 1D experiments and by a pre-saturation of 1 s during the relaxation delay in 2D experiments. The 2D TOCSY spectra were recorded using an MLEV-17 mixing sequence with spin-lock times of 20–200 ms. The 2D ROESY spectra were recorded using standard Bruker XWINNMR software with a mixing time of 200 ms. The carrier frequency was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance 2D ^{13}C – ^1H HSQC experiments were recorded without decoupling during acquisition of the ^1H FID. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for 1D spectra or by multiplication with a squared-bell function phase shifted by $\pi/(2.3)$ for 2D spectra, and when necessary, a fifth order polynomial baseline correction was performed. Chemical shifts (δ) are expressed in parts per million by reference to internal acetone (δ 2.225 for ^1H and δ 31.07 for ^{13}C). NMR data were processed using in-house developed software (J.A. van Kuik†, Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University).

3. Results

Washed and pelleted ascospores of *N. fischeri* and *T. macrosporus* from 40-day-old cultures were broken and cell-free extracts were used for isolation and analysis of carbohydrates. For inactivation of degrading enzymes, the cell-free extracts needed were heated at 95 °C for 30 min.

The presence of mannitol and trehalose in *N. fischeri* (molar ratio 1.0:0.5) and *T. macrosporus* (molar ratio 1.0:2.7) ascospores was determined by analysis of dried aliquots of cell-free extracts using trimethylsilylation/GLC-EIMS. The anomeric configuration of trehalose (i.e., $\alpha 1 \leftrightarrow 1\alpha$) was confirmed by ^1H NMR spectroscopy. No free glucose or other monosaccharides, nor other disaccharides and alditols were observed by GLC-EIMS. Note that the GLC conditions used cannot detect saccharides with a degree of polymerization (DP) over 2 (see below).

Methanolysis, which converts oligosaccharides into their monomeric components (methyl glycosides), of cell-free extracts of *T. macrosporus*, followed by trimethylsilylation/GLC-EIMS revealed only the presence of glucose derived from trehalose. In contrast, cell-free extracts of *N. fischeri* showed a very high ratio of glucose compared to mannitol (25:1), much higher than would be expected if only trehalose was present. This suggested the presence of

glucose-containing oligosaccharides with a higher DP than trehalose. TLC (not shown) and MALDI-TOF-MS performed with the cell-free extract of *N. fischeri* ascospores indicated the presence of tri-, tetra- and pentasaccharides, as summarized in Table 1. Also the occurrence of a very small amount of hexasaccharide is suggested to be present (only MALDI-TOF-MS).

Fractionation of the oligosaccharides in the cell-free extract of *N. fischeri* ascospores on Bio-Gel P-2 yielded four fractions, denoted DP2 to DP5 (range 0.5–1 mg), corresponding with di- up to pentasaccharides (MALDI-TOF-MS analysis). Monosaccharide composition analysis demonstrated the presence of only glucose. The four fractions were further analyzed by 1D and 2D (TOCSY, HSQC, ROESY) NMR spectroscopy.

3.1. NMR analysis of DP2

The 1D ^1H NMR spectrum of disaccharide DP2, presented in Fig. 1a, corresponds with that of reference trehalose (B-A) (Fig. 2a).⁴⁸ It contains one anomeric doublet coinciding for the two H-1 atoms of the symmetric α -D-Glcp residues at δ 5.182 ($^3J_{1,2}$ 4.0 Hz). The complete assignment of the ^1H and ^{13}C signals are included in Tables 2 and 3, respectively.

3.2. NMR analysis of DP3

The 1D ^1H NMR spectrum of trisaccharide DP3 is depicted in Fig. 1b. The ^1H and ^{13}C NMR data are in agreement with those of reference α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp (D-B-A) (Fig. 2b). The anomeric protons of the two Glc residues (A and B) of trehalose overlap at δ 5.199 ($^3J_{1,2}$ 3.9 Hz) and there is an extra anomeric signal at δ 4.960 ($^3J_{1,2}$ 3.9 Hz), stemming from an (α 1 \rightarrow 6)-linked Glc residue (labelled D).⁴⁹ The peak area ratio of the two anomeric signals is 2:1, in accordance with a trisaccharide. For a complete assignment of the ^1H NMR spectrum, see Table 2. The ^{13}C NMR data are presented in Table 3. The 6-substitution of the B residue is indicated by the ^1H δ values of B H-4 (3.54 ppm), B H-5 (4.02 ppm) and B H-6a (3.97 ppm), and by the ^{13}C δ value of B C-6 (65.6 ppm; downfield shift of $\Delta\delta$ 4.4 ppm, as compared to C-6 of trehalose).

3.3. NMR analysis of DP4

The 1D ^1H NMR spectrum of tetrasaccharide DP4 (D-C-B-A) (Fig. 1c) showed α -anomeric signals at δ 5.202 (B H-1)/5.199 (A H-1) ($^3J_{1,2}$ 3.8 Hz), δ 4.973 (C H-1, $^3J_{1,2}$ 4.0 Hz) and δ 4.961 (D H-1, $^3J_{1,2}$ 4.0 Hz) in a peak area ratio of 2:1:1. The nearly coinciding A and B signals correspond with the two H-1 atoms of an α , α -trehalose moiety and the C and D signals to H-1 atoms of two (α 1 \rightarrow 6)-linked Glc residues.⁴⁹ The splitting of the four H-1 signals suggests already an asymmetrical structure for the tetrasaccharide. 2D TOCSY, HSQC and ROESY measurements (Fig. 3) gave all ^1H and ^{13}C chemical shifts

(Tables 2 and 3). Starting points for the interpretation of the TOCSY spectra were the anomeric signals of the various residues. Comparison of TOCSY spectra with increasing mixing times (20, 40, 80, 200 ms) allowed the assignment of the chemical shifts belonging to the same spin system. Connectivities from H-1 to H-2,3,4,5,6a,6b were traced for residues A, B, C and D (Fig. 3) but due to overlap, some uncertainties could not be resolved on the basis of the TOCSY data alone. Additional assignments and confirmation of the assignments were obtained from ROESY cross-peaks and by correlating the ^1H resonances to the corresponding ^{13}C resonances in the HSQC spectrum. The ^1H chemical shifts patterns of residues A (H-1, δ 5.199) and B (H-1, δ 5.202) reflect a trehalose moiety of which one of the Glc residues (in this case B) is substituted at O-6, as suggested by the δ values of B H-4, B H-5 and B H-6a. The TOCSY C H-1 track (H-1, δ 4.973) showed the complete scalar coupling network H-1,2,3,4,5,6a,6b of a -(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- residue. The ^{13}C chemical shifts of B C-6 (δ 65.9) and C C-6 (δ 65.9), as deduced from the HSQC spectrum, suggested 6-substituted Glc residues (downfield shift of $\Delta\delta$ 4.7 ppm, as compared to C-6 of trehalose). The ^1H and ^{13}C chemical shifts patterns of residue D (H-1, δ 4.961) are characteristic for a terminal α -D-Glcp-(1 \rightarrow 6)- residue.⁴⁹ ROESY analysis gave rise to intra-residual cross-peaks for H-1 to H-2 of all four Glc units, in accordance with their α -configurations. Inter-residual cross-peaks were observed for D H-1:C H-6a, D H-1:C H-6b, C H-1:B H-6a, C H-1:B H-6b affording the sequence D(1 \rightarrow 6)C(1 \rightarrow 6)B. In case of the B(1 \leftrightarrow 1)A segment, inter-residual cross-peaks were detected for A H-1:B H-5 and B H-1:A H-5. The latter observation, also seen for DP5 (see below), fits earlier NMR and MM3 studies on trehalose itself, supporting low flexibility in the α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp part of all elongated compounds.⁵⁰ Taking all data together, the structure of the tetrasaccharide is α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp (Fig. 2c). No indications were found for the presence of the symmetric variant.

3.4. NMR analysis of DP5

The 1D ^1H NMR spectrum of pentasaccharide DP5 (D-C'-C-B-A) (Fig. 1d) showed trehalose H-1 signals at δ 5.201 (B H-1)/5.199 (A H-1) ($^3J_{1,2}$ 3.8 Hz). Furthermore, three additional α -anomeric signals were observed at δ 4.975 (C and C' H-1, $^3J_{1,2}$ 3.7 Hz) and δ 4.964 (D H-1, $^3J_{1,2}$ 3.8 Hz). The five signals occurred in the peak area ratio 2:2:1. The increase of the signal at δ 4.975, as compared to the spectrum of DP4, suggested the presence of an extra -(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- residue. 2D TOCSY, HSQC and ROESY measurements gave all ^1H and ^{13}C chemical shifts (Tables 2 and 3). TOCSY spectra with increasing mixing times (20, 40, 80, 200 ms), combined with additional information from ROESY and HSQC experiments, allowed the assignments of the sequential order of the chemical shifts belonging to the same spin system for the residues A, B, C, C' and D. The δ -values of the complete scalar coupling networks H-1,2,3,4,5,6a,6b on the TOCSY A/B H-1 track (H-1, δ 5.20) corresponded with those found for the trehalose moiety in the tetrasaccharide. The mostly overlapping TOCSY C/C' H-1 track (H-1's, δ 4.975) revealed the complete scalar coupling network H-1,2,3,4,5,6a,6b of -(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- residues. The ^{13}C chemical shifts of B C-6 (δ 65.9) and C/C' C-6 (δ 65.9), as observed in the HSQC spectrum, confirm the presence of three 6-substituted Glc units (downfield shift of $\Delta\delta$ 4.7 ppm, as compared to C-6 of trehalose). Finally, as for DP4, the ^1H and ^{13}C chemical shifts patterns of residue D are in accordance with a terminal α -D-Glcp-(1 \rightarrow 6)- residue. In a similar way, as explained for DP4, ROESY analysis led in the case of DP5 to the following monosaccharide sequence D(1 \rightarrow 6)C'(1 \rightarrow 6)C(1 \rightarrow 6)B(1 \leftrightarrow 1)A, affording the structure α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp (Fig. 2d).

Table 1
MALDI-TOF-MS data of saccharides in cell-free extracts of *N. fischeri* (Nf) and *T. macrosporus* (Tm) ascospores

Saccharide ^a	M (Da)	[M+Na] ⁺	[M+K] ⁺	Nf	Tm
DP2 (trehalose)	342	365	381	+++	+++
DP3 (trisaccharide)	504	527	543	++	–
DP4 (tetrasaccharide)	666	689	705	+	–
DP5 (pentasaccharide)	828	851	867	+	–
DP6 (hexasaccharide)	990	1013	1029	+/-	–

^a The relative amounts of the 5 compounds are indicated by–(absent), +/- (very minor), + (minor), ++ (intermediate), and +++ (major).

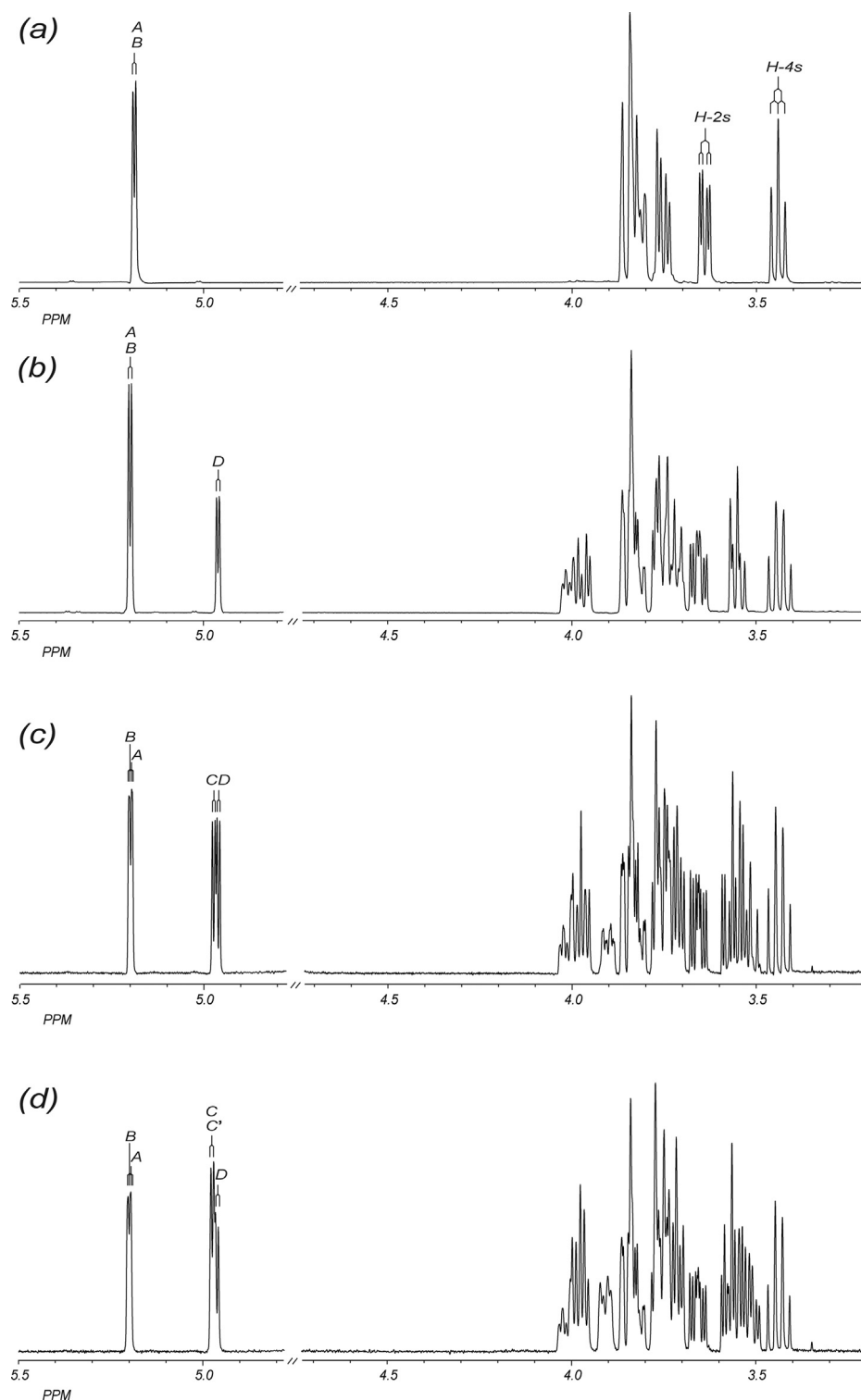


Fig. 1. 1D 500-MHz ^1H NMR spectra in D_2O at 300 K of (a) $\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\alpha\text{-D-Glcp}$ (B–A); (b) $\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\alpha\text{-D-Glcp}$ (D–B–A); (c) $\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\alpha\text{-D-Glcp}$ (D–C–B–A); and (d) $\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\alpha\text{-D-Glcp}$ (D–C'–C–B–A) isolated from a cell-free extract of *N. fischeri* ascospores.

4. Discussion

This report on the isolation and characterization of oligosaccharides, which are abundant compatible solutes in the ascospores of the fungus *N. fischeri*, has led to the finding of a series of elongated trehaloses, of which to the best of our knowledge the

tetrasaccharide $\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\alpha\text{-D-Glcp}$ and the pentasaccharide $\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\alpha\text{-D-Glcp}$ have not been described before to occur in nature. The identified trisaccharide $\alpha\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-(1}\rightarrow\text{1)-}\alpha\text{-D-Glcp}$, named isobemisirose, has been isolated earlier from the silverleaf whitefly *Bemisia argentifolii*,

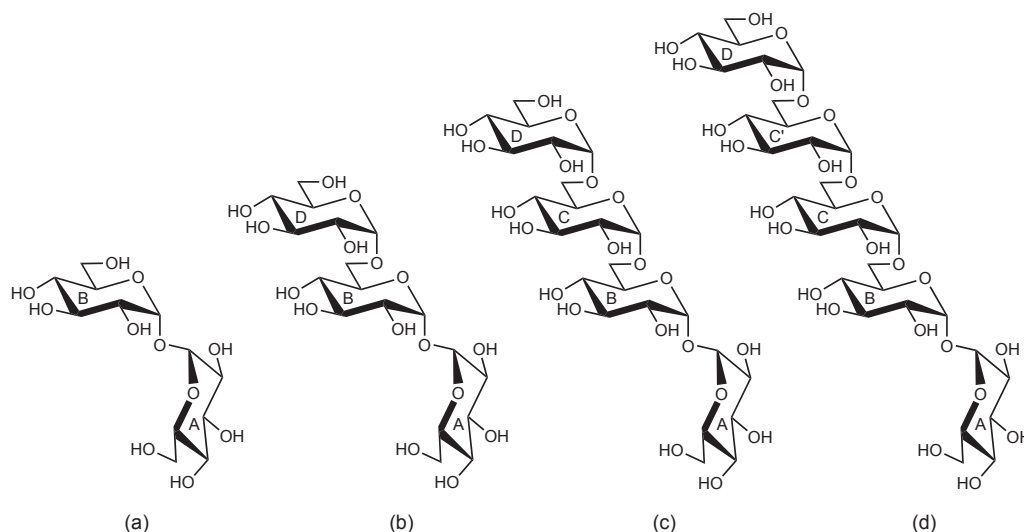


Fig. 2. Structures of (a) α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp; (b) α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp; (c) α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp; and (d) α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp.

and demonstrated to occur also in other whitefly species as well as in several aphid species.³⁹ In relation to isobemisirose, we propose the names neosartose and fischerose for the novel tetra- and pentasaccharide, respectively. Interestingly, the elongation of trehalose with (α 1 \rightarrow 6)-linked Glc residues takes place at only one

site of the non-reducing disaccharide. MALDI-TOF-MS even suggested the occurrence of a hexasaccharide.

Earlier reports have appeared describing trehalose-based oligosaccharides distinct from isobemisirose, neosartose and fischerose. *B. argentifolii* not only synthesizes isobemisirose in the body (primarily in the hemolymph), but also produces α -D-Glcp-(1 \rightarrow 4)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp, named bemisirose, in the honeydew.⁴⁰ The latter trisaccharide is also formed by the bacterium *Mycobacterium smegmatis*,⁴¹ together with five other trehalose-based

Table 2

¹H chemical shifts of Glc residues of di- (DP2; B-A), tri- (DP3; D-B-A), tetra- (DP4, D-C-B-A) and penta- (DP5; D-C'-C-B-A) saccharides, isolated from ascospores of *N. fischeri* (internal standard acetone, δ 2.225)

Residue	H	DP2	DP3	DP4	DP5
A: -(1 \leftrightarrow 1)- α -D-Glcp	H-1	5.182	5.199	5.199	5.199
	H-2	3.62	3.65	3.65	3.65
	H-3	3.84	3.84	3.84	3.84
	H-4	3.436	3.447	3.448	3.448
	H-5	3.82	3.82	3.82	3.83
	H-6a	3.85	3.84	3.84	3.84
B: [-(1 \rightarrow 6)]- α -D-Glcp-(1 \leftrightarrow 1)-	H-1	5.182	5.199	5.202	5.201
	H-2	3.62	3.67	3.67	3.67
	H-3	3.84	3.85	3.85	3.85
	H-4	3.436	3.54	3.54	3.55
	H-5	3.82	4.02	4.02	4.02
	H-6a	3.85	3.97	3.97	3.98
C: -(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)-	H-1			4.973	4.975
	H-2			3.58	3.58
	H-3			3.71	3.72
	H-4			3.53	3.53
	H-5			3.90	3.92
	H-6a			3.97	3.98
C': -(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)-	H-1				4.975
	H-2				3.58
	H-3				3.72
	H-4				3.53
	H-5				3.92
	H-6a				3.98
D: α -D-Glcp-(1 \rightarrow 6)-	H-1		4.960	4.961	4.964
	H-2		3.55	3.55	3.55
	H-3		3.73	3.73	3.73
	H-4		3.425	3.428	3.428
	H-5		3.72	3.72	3.72
	H-6a		3.84	3.84	3.84
	H-6b		3.76	3.76	3.76

Table 3

¹³C chemical shifts of Glc residues of di- (DP2; B-A), tri- (DP3; D-B-A), tetra- (DP4, D-C-B-A) and penta- (DP5; D-C'-C-B-A) saccharides, isolated from ascospores of *N. fischeri* (internal standard acetone, δ 31.07)

Residue	C	DP2	DP3	DP4	DP5
A: -(1 \leftrightarrow 1)- α -D-Glcp	C-1	93.8	93.8	93.8	93.8
	C-2	71.7	71.4	71.3	71.5
	C-3	73.2	73.2	73.2	73.1
	C-4	70.3	70.1	70.1	70.0
	C-5	72.8	72.8	72.8	72.8
	C-6	61.2	60.8	60.7	60.7
B: [-(1 \rightarrow 6)]- α -D-Glcp-(1 \leftrightarrow 1)-	C-1	93.8	93.8	93.8	93.8
	C-2	71.7	71.4	71.3	71.5
	C-3	73.2	73.2	73.2	73.1
	C-4	70.3	70.1	70.1	70.0
	C-5	72.8	71.0	71.0	71.0
	C-6	61.2	65.6	65.9	65.9
C: -(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)-	C-1			98.2	98.2
	C-2			72.0	72.1
	C-3			72.5	72.5
	C-4			70.1	70.0
	C-5			70.8	70.8
	C-6			65.9	65.9
C': -(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)-	C-1				98.2
	C-2				72.1
	C-3				72.5
	C-4				70.0
	C-5				70.8
	C-6				65.9
D: α -D-Glcp-(1 \rightarrow 6)-	C-1		98.2	98.2	98.2
	C-2		72.0	72.0	72.1
	C-3		73.7	73.8	73.8
	C-4		70.1	70.1	70.0
	C-5		73.8	73.8	73.8
	C-6		60.7	60.7	60.7

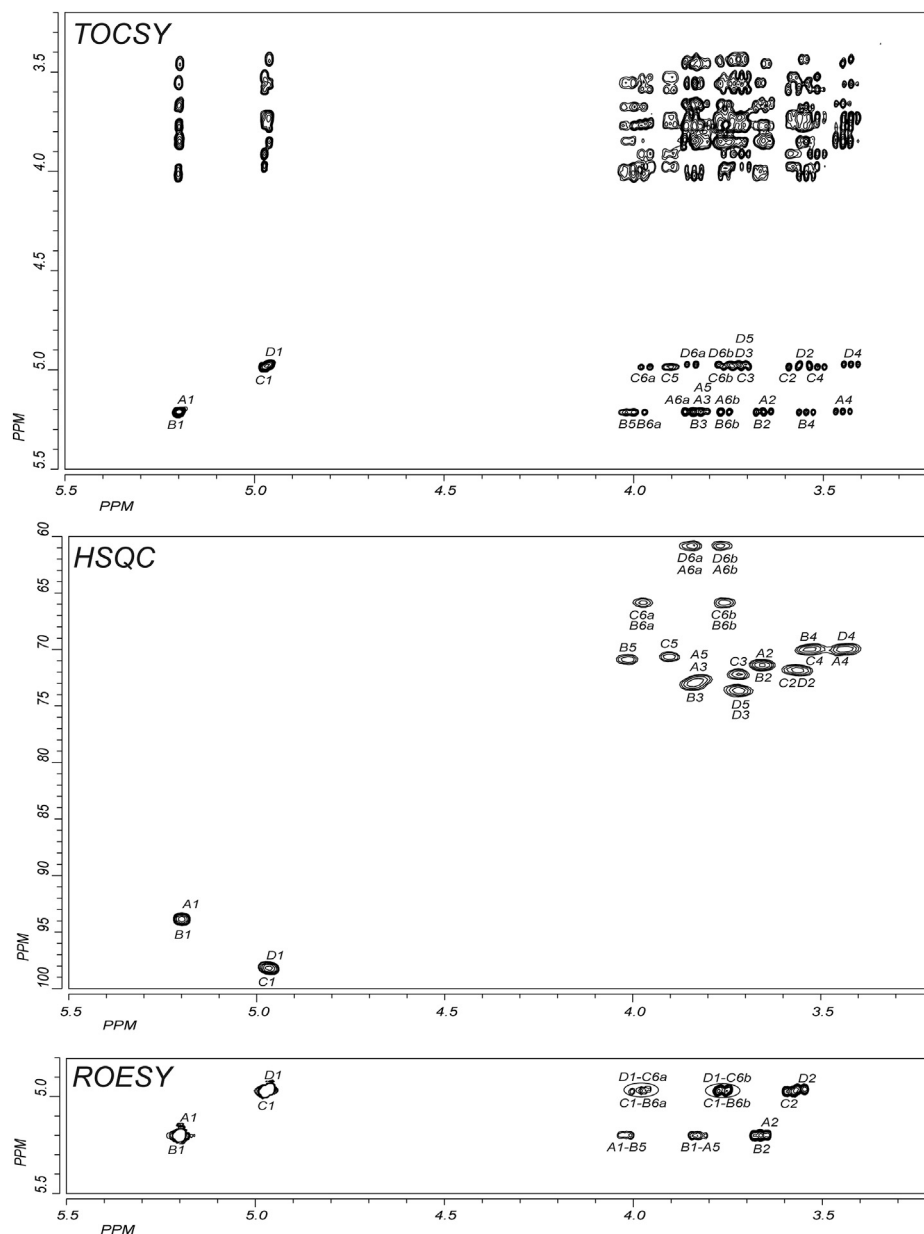


Fig. 3. TOCSY (200 ms), HSQC, and ROESY (200 ms; relevant part) spectra of the tetrasaccharide α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp (D-C-B-A). In the ROESY spectrum, the cross-peaks confirming the glycosidic linkages are indicated with circles.

oligosaccharides (elongations with Glc and/or Gal units), including β -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp and β -D-Glcp-(1 \rightarrow 6)- β -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp, the β -variants of isobemisirose and neosartose. The trisaccharide β -D-Glcp-(1 \rightarrow 6)- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp has also been found in a cell free extract of the yeast *Saccharomyces cerevisiae*.⁴² Furthermore, the bacterium *Sinorhizobium meliloti* has been reported to produce, besides trehalose, a number of trehalose-based oligosaccharides, but here with (α 1 \rightarrow 2)-linked Glc residues, [α -D-Glcp-(1 \rightarrow 2)]₁₋₄- α -D-Glcp-(1 \leftrightarrow 1)- α -D-Glcp.^{43–45} It should be noted that incubation of trehalose with glucose in the presence of α -glucosidase from *Saccharomyces* sp. or of glucoamylase from *Rhizopus niveus* yielded isobemisirose,⁵¹ whereas incubation of trehalose with maltotetraose in the presence of α -glucosidase from *Aspergillus niger* resulted in transglucosylation, yielding isobemisirose, neosartose, and the symmetric α -isomaltosyl α -isomaltoside.⁵²

These observations illustrate that trehalose-based oligosaccharides have evolved in different kingdoms and may have important and variable functions including storage of nutrients and stabilization of biomolecules against different stressors. It is of interest to study why the more elaborated trehalose-based oligosaccharides are accumulated (in situ biosynthesis with glucosyltransferases) and whether they have superb protective properties on its own, compared to trehalose.

5. Conclusions

Ascospores of heat-resistant fungi belonging to the genus *Neosartorya* accumulate, besides trehalose, mannitol and a mixture of trehalose-based oligosaccharides.⁴⁶ We were able to isolate and characterize the DP3, DP4, and DP5 components of the oligosaccharide mixture, i.e. isobemisirose, neosartose, and fischerose,

showing the extension of non-reducing trehalose on one side with ($\alpha 1 \rightarrow 6$)-linked glucose residues as a recurrent theme. A similar extension of non-reducing sucrose with ($\alpha 1 \rightarrow 6$)-linked galactose residues is known from plants: raffinose, stachyose, and verbascose.

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