ELSEVIER

Carbohydrate Research 260 (1994) 323–328

CARBOHYDRATE RESEARCH

Note

Gram-scale synthesis of α,α-trehalose 6-monophosphate and α,α-trehalose 6,6′-diphosphate

Tor E.C.L. Rønnow, Morten Meldal, Klaus Bock *

Carlsberg Laboratory, Department of Chemistry, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark

(Received January 21st, 1994; accepted February 23rd, 1994)

α,α-Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a naturally occurring, nonreducing disaccharide with important biological properties. α,α-Trehalose stabilizes proteins, lipid membranes, and other biomolecules in so-called anhydrobiotic organisms which can survive almost complete desiccation for several years, but the molecular basis for this phenomenon is not yet known in detail [1–4]. We have initiated studies on this unique property of α,α-trehalose and report herein the synthesis of some α,α-trehalose derivatives, namely α,α-trehalose 6-monophosphate and α,α-trehalose 6,6′-diphosphate. These compounds are furthermore of biochemical interest, because α,α-trehalose 6-phosphate is an intermediate in the biosynthetic pathway of α,α-trehalose, in which α,α-trehalose 6-phosphate phosphatase (EC 3.1.3.1) hydrolyzes α,α-trehalose 6-phosphate to trehalose and inorganic phosphate. α,α-Trehalose 6-phosphate has recently been isolated and purified from *Saccharomyces cerevisiae* [5], and has been found to play an important role in the regulation of the first steps of the yeast glycolysis, mainly through the inhibition of hexokinase II [6].

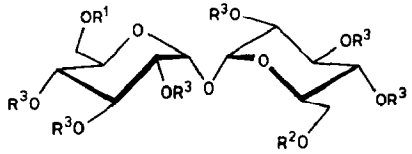
Recently, a simple large-scale synthesis of D-mannose 6-phosphate [7] was described and the present work describes analogous methods to afford the α,α-trehalose phosphates. The methods employ trimethylsilylation and selective methanolysis of primary trimethylsilyloxy groups [8].

α,α-Trehalose dihydrate **1** was converted into its octakis-*O*-trimethylsilyl derivative **2** by modification of a previously described procedure [8]. The present large-scale synthesis afforded 41.91 g of crystalline product in 94% yield from 20 g of dihydrate **1**, using much reduced amounts of silylating reagents and, in particular, of the solvent (pyridine) than previously described [8,9]. Compound **2** was subjected to selective methanolysis with potassium carbonate in methanol afford-

* Corresponding author.

SSDI 0008-6215(94)00082-Q

ing either 2,3,4,2′,3′,4′,6′-heptakis-*O*-trimethylsilyl-α,α-trehalose (**3**) or 2,3,4,2′,3′,4′-hexakis-*O*-trimethylsilyl-α,α-trehalose (**4**) depending on the reaction conditions [8,10]. Methanolysis at 0–4°C for ca. 20 min afforded the monohydroxylated compound **3**, in mixture with unreacted **2** and dihydroxylated compound **4**. Compound **3** was obtained pure in 36% yield after purification by silica gel chromatography. In our hands, **3** could not be crystallized [8] and was hence phosphorylated directly as a syrup (vide infra). Compound **4** was obtained by methanolysis at 0–4°C for 3 h. The yield was 87% after chromatographic purification.



1 $R^1 = R^2 = R^3 = H$
2 $R^1 = R^2 = R^3 = Me_3Si$
3 $R^1 = H, R^2 = R^3 = Me_3Si$
4 $R^1 = R^2 = H, R^3 = Me_3Si$
5 $R^1 = POCl_2, R^2 = R^3 = Me_3Si$
6 $R^1 = PO(OH)_2, R^2 = R^3 = H$
7 $R^1 = R^2 = POCl_2, R^3 = Me_3Si$
8 $R^1 = R^2 = PO(OH)_2, R^3 = H$

Phosphorylation of **3** with phosphorus oxychloride and *N*-ethylmorpholine gave, after work-up, the hygroscopic and unstable dichlorophosphate **5** which was characterized by NMR spectroscopy. The ^{13}C NMR spectrum (Table 1) revealed an unsymmetrical compound and the doublet (J_{C-P} 6.3 Hz) at δ 70.8 (C-6) clearly indicated phosphorylation in one glucopyranosyl ring. The ^1H NMR spectrum showed two anomeric signals at δ 4.98 and 4.92, and the phosphorylated ring had a signal for H-5 at δ 4.08 (in the nonphosphorylated ring H-5 resonates at δ 3.80), and signals for H-6 and (H-6)′ at δ 4.48 and 4.40 (vs. δ 3.71 when not phosphory-

Table 1
^{13}C NMR chemical shift data (δ, ppm) for compounds **5–8**

	5 [a,c]	**6** [b,c]	**7** [a]	**8** [b]
C-1	94.84	94.18	94.94	94.26
C-1′	94.44	94.14		
C-2	72.80	71.70	72.44	71.58
C-2′	72.49	71.75		
C-3	73.40	73.18	73.15	73.10
C-3′	73.31	73.26		
C-4	71.34	69.93	71.23	69.86
C-4′	71.52	70.45		
C-5	70.82	71.60	71.04	71.60
C-5′	73.49	72.95		
C-6	70.84 (6.3) [d]	65.59 (3.4) [d]	70.63 (8.0) [d]	65.59 (3.0) [d]
C-6′	61.94	61.29		

The solvents used were: [a] $CDCl_3$; [b] D_2O. [c] The prime (′) refers to the nonphosphorylated ring. [d] ^2J coupling to phosphorus (Hz, doublet).

Table 2
^{1}H NMR chemical shift data (δ, ppm) and coupling constants (J, Hz) for compounds **6–8**

	5 [a,c]	**6** [b,c]	**7** [a]	**8** [b]
H-1	4.98 (d)	5.10 (d)	4.95 (d)	5.01 (d)
H-1′	4.92 (d)	5.10 (d)		
H-2	3.45 (dd)	3.58 (dd)	3.47 (dd)	3.48 (dd)
H-2′	3.42 (dd)	3.55 (dd)		
H-3	3.94 (t)	3.78 (t)	3.92 (t)	3.67 (t)
H-3′	3.90 (t)	3.76 (t)		
H-4	3.48 (t)	3.44 (t)	3.49 (t)	3.35 (t)
H-4′	3.48 (t)	3.36 (t)		
H-5	4.08 (m)	3.87 (m)	4.07 (m)	3.78 (m)
H-5′	3.80 (dt)	3.73 (m)		
H-6	4.48 (m)	4.48 (m)	4.47 (dd)	4.02 (m)
H-6′	3.71 (m)	4.14–4.05		
H-6′	4.40 (m)	3.73 (m)	4.41 (dd)	4.02 (m)
H-6″	3.71 (m)	4.14–4.05		
$J_{1,2}$	3.6	3.9	3.6	3.6
$J_{1',2'}$	3.6	3.9		
$J_{2,3}$	9.8	9.2	9.7	9.9
$J_{2',3'}$	9.8	9.2		
$J_{3,4}$	9.8	9.2	9.7	9.5
$J_{3',4'}$	9.8	9.2		
$J_{4,5}$	9.8	9.2	9.7	9.5
$J_{4',5'}$	9.8	9.2		

The solvents used were: [a] $CDCl_3$; [b] D_2O. [c] The prime (′) refers to the nonphosphorylated ring.

lated). The dichlorophosphate **5** was directly hydrolyzed to give α,α-trehalose 6-monophosphate (**6**) in 98% overall yield. The compound was very hygroscopic and was characterized by its ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2). The ^{13}C NMR spectrum showed anomeric signals at δ 94.18 and 94.14, respectively, and the characteristic doublet ($J_{C\text{-}P}$ 3.4 Hz) at δ 65.6 for the phosphorylated C-6 (compared with δ 61.3 for the nonphosphorylated C-6′). The ^{1}H NMR spectrum showed only one anomeric signal at δ 5.10, but phosphorylation was apparent from the asymmetric character of the spectrum and from the signals at δ 4.14–4.05 [H-6,H-6′] and δ 3.87 (H-5).

Phosphorylation of **4** with 3 equiv of phosphorus oxychloride afforded the likewise hygroscopic and unstable bis-dichlorophosphate **7** which was characterized by NMR spectroscopy. The ^{13}C NMR spectrum showed a symmetrical compound with the signal for C-6 and C-6′ at δ 70.6 (*d*, $J_{C\text{-}P}$ 8 Hz), clearly indicating phosphorylation, and the ^{1}H NMR spectrum showed signals at δ 4.41 and 4.47 for the secondary protons and at δ 4.07 for H-5 and H-5′. Hydrolysis afforded the symmetrical 6,6′-diphosphate **8** in 98% overall yield. The diphosphate was characterized by its ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2). The ^{13}C NMR spectrum showed the expected doublet at δ 65.6 (C-6, C-6′, $J_{C\text{-}P}$ 3 Hz), and the ^{1}H NMR spectrum showed signals at δ 3.78 for H-5 and H-5′ and signals at δ 4.02 for the secondary protons, all of which indicate 6,6′-di-*O*-phosphorylation.

In conclusion, convenient syntheses of α,α-trehalose 6-monophosphate (**6**) and

α,α-trehalose 6,6′-diphosphate (**8**) have been described, taking advantage of the selective deprotection of per-trimethylsilylated α,α-trehalose and underscoring the scope of the trimethylsilyl group as a protecting group in the phosphorylation reaction [7]. The syntheses reported herein of the α,α-trehalose phosphates are simpler, faster, and more reliable than those previously described [5,11,12].

1. Experimental

General methods.—Analytical grade solvents were dried over molecular sieves (pyridine was distilled from KOH and CH_2Cl_2 from P_2O_5). Chlorotrimethylsilane, *N*-ethylmorpholine, hexamethyldisilazane, and P_2O_5 were purchased from Fluka and α,α-trehalose dihydrate from Sigma. Optical rotations were recorded on a Perkin–Elmer 241 polarimeter, TLC was performed on Merck coated aluminium foil plates F_{254}, and vacuum liquid chromatography (VLC) was performed on silica Gel 60 H (E. Merck). ^1H NMR spectra were recorded on a Bruker AM500 instrument. Samples containing trimethylsilyl groups were dissolved in alumina-purified $CDCl_3$. ^{13}C NMR spectra were recorded at 300 K in $CDCl_3$ relative to $CDCl_3$ (δ 77.0) or in D_2O with an external reference of 1,4-dioxane (δ 67.4). ^1H NMR spectra were recorded at 300 K in $CDCl_3$ relative to Me_4Si (δ 0.000), or in D_2O relative to acetone (δ 2.225). Assignment of ^1H NMR spectra was achieved by homonuclear correlated 2D-spectroscopy and of ^{13}C NMR spectra by heteronuclear correlated 2D-spectroscopy.

2,3,4,6,2′,3′,4′,6′-Octakis-O-(trimethylsilyl)-α,α-trehalose (**2**).—A mixture of chlorotrimethylsilane (200 mL, 1.58 mol) and hexamethyl disilazane (80 mL, 0.38 mol) was carefully added to a vigorously stirred solution of α,α-trehalose dihydrate (**1**, 20.0 g, 48.3 mmol) in pyridine (300 mL) at 0°C. The temperature was slowly raised to 20°C and the solution was stirred overnight. Solvent and excess reagents were evaporated under reduced pressure at 50°C. The resulting syrup was taken up in pentane (200 mL), washed with water, dried ($MgSO_4$), and concentrated to afford a syrup, which was crystallized from MeOH; yield, 41.91 g (94%); mp 80–82°C; $[\alpha]_D^{22}$ +93.4° (*c* 1.8, $CHCl_3$); lit. [8] mp 80–82°C, $[\alpha]_D$ +94° (*c* 1.5, $CHCl_3$). The ^1H and ^{13}C NMR data were identical with those published [8].

2,3,4,2′,3′,4′,6′-Heptakis-O-(trimethylsilyl)-α,α-trehalose (**3**).—Compound **2** (10 g, 10.9 mmol) was kept in a solution of K_2CO_3 in MeOH (220 mL, 4.5 g/L) at 0–4°C for 20 min. After neutralization with AcOH (20 mL), the mixture was concentrated to 100 mL and then worked up as previously described [8]. The yield of **3** after purification by silica gel chromatography was 3.1 g (36%); $[\alpha]_D^{22}$ +113° (*c* 2.5, petroleum ether); lit. [8] $[\alpha]_D$ +114.5°. Attempts to crystallize the **3** from 9:1 MeCN–Et_2O [8] were unsuccessful. The ^1H and ^{13}C NMR data were identical with those published [8].

2,3,4,2′,3′,4′-Hexakis-O-(trimethylsilyl)-α,α-trehalose (**4**).—Compound **2** (10 g, 10.9 mmol) was dissolved in a solution of K_2CO_3 in MeOH (66 mL, 4.5 g/L) and MeOH at 0°C. The mixture was stirred at 0–4°C for 3 h, then neutralized with AcOH (6 mL). The mixture was concentrated under reduced pressure to 100 mL, and then worked up as previously described [8]. A sample (4 g) of the crude, syrupy

product (8.23 g) was purified by silica gel chromatography to afford the title compound **4** as a colourless, amorphous solid (3.7 g, 87%); mp 114–115°C; $[\alpha]_D^{22}$ +99.5° (*c* 2.7, $CHCl_3$); lit. [8] mp 116–118°C, $[\alpha]_D$ + 100°. The ^1H and ^{13}C NMR data were identical with those published [8].

α,α-Trehalose 6-monophosphate (**6**).—A mixture of **3** (2.48 g, 3.31 mmol), *N*-ethylmorpholine (0.98 mL, 7.73 mmol), dry CH_2Cl_2 (20 mL), and $POCl_3$ (0.66 mL, 7.08 mmol) was stirred overnight at room temperature under Ar. After work-up as described below, the syrupy, hygroscopic dichlorophosphate **5** was characterized by ^1H and ^{13}C NMR spectroscopy, then hydrolyzed and lyophilized as described below for **8**, to afford the title compound **6** as a very hygroscopic, colourless syrup (1.37 g, 98%). The ^1H and ^{13}C NMR data for **5** and **6** are presented in Tables 1 and 2. Compound **6** contained less than 1% of a secondary phosphorylation product, according to ^1H NMR (vide infra). Due to the previously observed [11,13] difficulty of crystallizing and characterizing the barium or cyclohexylamine salts of the product, no attempts to prepare these salts were made.

α,α-Trehalose 6,6'-diphosphate (**8**).—A mixture of **4** (2.0 g, 2.58 mmol), *N*-ethyl morpholine (1.08 mL, 8.56 mmol), dry CH_2Cl_2 (20 mL), and $POCl_3$ (0.73 mL, 7.83 mmol) was stirred under Ar overnight [7]. The CH_2Cl_2 (10 mL) was removed by evaporation and the mixture was diluted with hexane (15 mL). After stirring at 0°C for 10 min, the resulting precipitate was removed by filtration. The filtrate was cooled to −5°C and extracted with 1% HCl (5 mL) and ice (10 mL), then three times with water (3 × 1 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated to give the bis-dichlorophosphate **7** as a colourless syrup, which was characterized by ^1H and ^{13}C NMR spectroscopy. Water (10 mL) was added and a white precipitate was formed. The mixture was left rotating on an evaporator for 30 min (at room temperature), whereupon the water was removed under reduced pressure (bath temperature raised to 45°C). Two successive additions and evaporations of water (2 × 10 mL) followed by lyophilization afforded the title compound **8** as a very hygroscopic, colourless syrup (1.28 g, 98%). The ^1H and ^{13}C NMR data for **7** and **8** are presented in Tables 1 and 2. According to ^1H NMR, **8** contained less than 2% of a secondary phosphorylation product, as suggested by a small doublet at δ 79.0 ($J_{C,P}$ 3 Hz) in the ^{13}C NMR spectrum. Due to the previously observed difficulty of crystallizing and characterizing the barium or cyclohexylamine salts of **8** [11], no attempts to prepare these salts were made.

Acknowledgment

The Danish Technical Research Council (STVF) is thanked for a grant (TECLR).

References

[1] B. Roser, *Trends Food Sci. Technol.*, (1991) 166–169.
[2] J.H. Crowe, F.A. Hoekstra, and L.M. Crowe, *Ann. Rev. Physiol.*, 54 (1992) 579–599.

[3] J.H. Crowe, L.M. Crowe, and D. Chapman, *Science*, 223 (1984) 701–703.
[4] K. Fukase, *Tetrahedron Lett.*, 33 (1992) 7165–7168.
[5] C.R.M. Meleiro, J.T. Silva, A.D. Panek, and V.M.F. Paschoalin, *Anal. Biochem.*, 213 (1993) 171–172.
[6] M.A. Blaquez, R. Laguanas, C. Gancedo, and J.M. Gancedo, *FEBS Lett.*, 329 (1993) 51–54.
[7] M. Meldal, M.K. Christensen, and K. Bock, *Carbohydr. Res.*, 235 (1992), 115–127.
[8] A.K. Datta, K. Takayama, M.A. Nashed, and L. Anderson, *Carbohydr. Res.*, 218 (1991) 95–109.
[9] R. Toubiana, B.C. Das, J. Defaye, B. Mompon, and M.J. Toubiana, *Carbohydr. Res.*, 44 (1975) 308–312.
[10] A. Ahibo-Coffy, H. Aurelle, C. Lacave, J.C. Prome, G. Rizo, and A. Savagnac, *Chem. Phys. Lipids*, 22 (1978) 185–195.
[11] D.L. MacDonald and R.Y.K. Wong, *Biochem. Biophys. Acta*, 86 (1964) 390–392.
[12] B. Helferich, A. Lowa, W. Nippe, and H. Riedel, *Hoppe-Seyler's Z. Physiol. Chem.*, 128 (1923) 141–153.
[13] R. Robison and W.T.J. Morgan, *Biochem. J.*, 22 (1928) 1277–1288.