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The preparation of the four monophosphates of α,α'-trehalose from trehalose and sodium phosphate

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Abstract

α,α'-Trehalose, when dried to a moisture content of ca. 5% from aqueous sodium phosphate buffer (with an initial pH $\leq$ 5.5), produces, after warming, a mixture containing the four isomeric monophosphate esters which can be isolated by anion-exchange chromatography. Their structures were deduced from their hydrolysis products (trehalose and inorganic orthophosphate) after treatment with alkaline phosphomonoesterase, and by ^{1}H and ^{13}C NMR spectroscopy which, in the case of the 6-isomer, gave spectra identical with those of authentic material. Under similar conditions, other saccharides, including glucose, sucrose, amylose, cyclodextrins, alditols and cyclitols (e.g., glycerol, mannitol, and inositol), also produce (as shown by chromatographic and enzymic analyses and, in some instances, from spectroscopic analysis of isolated products) their corresponding phosphate esters. The presence of higher phosphorylated species is indicated in chromatograms of the mixtures, especially when metaphosphate is used as the phosphorylating agent. The methodology described provides a simple and direct route to such phosphate esters.

1. Introduction

Orthophosphate esters are important biochemicals which can be isolated from their natural sources, but usually with some difficulty since the amounts present are small and the mixtures complex. Some can be prepared enzymically in vitro by using a suitable kinase.

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A number of chemical synthetic methods for preparing phosphates of saccharides have been described either by direct esterification [1] using "anhydrous" phosphoric acid or by indirect esterification following the selective exposure of hydroxyl groups and reaction with a suitable phosphorylating reagent [2]. The former chemical method can be difficult to control as there are possibilities of other reactions occurring, to produce, for example, cyclic phosphates or anhydrides, and products arising from fission of glycosidic and other acid-labile bonds when these are present in the molecule. The latter chemical method can present difficulties for other reasons, including the large number of synthetic steps which are usually necessary to arrive at the desired product.

A chemical method of introducing phosphomonoester functions directly into saccharides, which is sufficiently mild so that phosphorylation can be achieved with little or no formation of side reaction products, should therefore be useful. Such a method is described in detail here for the monophosphorylation of α,α'-trehalose, using sodium phosphate.

2. Experimental

General methods. — α,α'-Trehalose, α,α'-trehalose 6-phosphate, alkaline phosphatase, and Dowex AG50 resin were obtained from Sigma Chemical Co. Sodium phosphate salts, NaOAc, aq 50% NaOH, and NH_4OAc were AnalaR grade from BDH Ltd. AG1-X8 anion-exchange, AG50-X8 cation-exchange, and Chelex 100 resins together with Bio-Gel P2 were obtained from Bio-Rad Laboratories Ltd. Moisture contents were determined with a Mitsubishi Moisture Meter, model CA05 (Mitsubishi Chemical Industries, Tokyo, Japan).

^1H NMR spectra were recorded under ambient conditions for solutions of ammonium salts in D_2O at 500 MHz with acetone as internal standard on a Varian Unity spectrometer. Assignments were made on the basis of double-quantum filtered COSY spectra. ^1H-Decoupled ^{13}C NMR spectra were recorded, for potassium salts, at 125 MHz under ambient conditions in D_2O at a pD of 3.6 on the same instrument with acetone as internal reference. Phosphorus was determined by the method of Chen [3]. HPLC ion-exchange was performed using a Dionex BioLC system with pulsed amperometric detection on a PA 100 column (4 × 250 mm) and a mobile phase of 100 mM NaOH, and with an NaOAc gradient (100–160 mM over 25 min).

α-D-Glucopyranosyl α-D-glucopyranoside 6-phosphate (**2**), *α-D-glucopyranosyl α-D-glucopyranoside 2-phosphate* (**3**), *α-D-glucopyranosyl α-D-glucopyranoside 4-phosphate* (**4**), *and α-D-glucopyranosyl α-D-glucopyranoside 3-phosphate* (**5**). — *(a) From sodium orthophosphate buffer, pH 5.5.* α,α'-Trehalose dihydrate (**1**, 1.0 g) was dissolved in sodium phosphate buffer (100 mL, 0.1 M, pH 5.5), and the solution was frozen and lyophilised under ambient conditions. The preparation (which contained ca. 5% moisture relative to the disaccharide) was then heated at 80°C in a closed container for 7 days. Analysis of this mixture by Dionex HPLC (Fig. 1)

indicated the presence of at least four products. The straw-coloured residue was dissolved in water (100 mL) and applied to a column (20 × 1000 mm) of AG1-X8 ion-exchange resin (acetate form) which was then eluted with a linear gradient of 0.2–0.8 M NH_4OAc; fractions (4 mL) were collected and assayed for phosphorus (total and inorganic). The four organic phosphorus-containing fractions (which were eluted *before* inorganic orthophosphate — see Fig. 2) were each repeatedly lyophilised to constant weight in order to remove residual NH_4OAc. The products obtained as amorphous solids (of high purity by HPLC) were further purified (to remove traces of salts) by chromatography on a Bio-Gel P2 polyacrylamide column (10 × 200 mm), eluting with water. Following lyophilisation, they were obtained as white amorphous hygroscopic solids. The esters were also prepared as their potassium salts by passing aqueous solutions of the ammonium salts through a column of Dowex AG50-X8 (K^+ form).

The first component eluted (from the AG1 column) was the 6-phosphate **2** (63 mg, 5.2%). Anal. Calcd for $C_{12}H_{29}N_2O_{14}P \cdot 2H_2O$: C, 29.3; H, 6.7; N, 5.7; P, 6.3%. Found: C, 29.1; H, 6.3; N, 5.3; P, 6.8%.

The second component was the 2-phosphate **3** (18 mg, 1.5%). Anal. Calcd for $C_{12}H_{29}N_2O_{14}P \cdot 2H_2O$: C, 29.3; H, 6.7; N, 5.7; P, 6.3%. Found: C, 29.9; H, 5.9; N, 4.5; P, 6.1%.

The third component was the 4-phosphate **4** (27 mg, 2.2%). Anal. Calcd for $C_{12}H_{21}O_{14}PK_2 \cdot 2H_2O$: C, 27.0; H, 4.7; P, 5.8%. Found (for the potassium salt): C, 27.4; H, 4.5; P, 5.2%.

The final organic phosphate fraction was the 3-phosphate **5** (22 mg, 1.8%). Anal. Calcd for $C_{12}H_{29}N_2O_{14}P \cdot 2H_2O$: C, 29.3; H, 6.7; N, 5.7; P, 6.3%. Found: C, 30.8; H, 5.9; N, 4.3; P, 7.0%.

The total isolated yield of phosphate esters was 10.7%.

Some difficulty was experienced in obtaining satisfactory elemental analytical values, possibly because of the hygroscopic nature of these compounds. However, all four components were homogeneous by chromatographic analysis, using the Dionex HPLC system, and no significant impurities were apparent from inspection of either their ^1H or ^{13}C NMR spectra.

Phosphorylation also occurred under conditions similar to those described above, over a range of pH values from below 5 to above 8, as shown by chromatographic analysis. Larger amounts were observed at pH ≤ 5.5.

(b) From sodium metaphosphate "buffer", pH 2 to 5.5. The same four phosphorylated products could be similarly obtained (pH 5.5, 70°C, 5 days) in increased amounts (17% total yield) by replacing sodium orthophosphate with sodium metaphosphate (pyrophosphate and tripolyphosphate also gave greater amounts) and after passing the mixture through coupled columns of AG50-X8 (K^+ form, 25 mL) and Chelex 100 resins (K^+ form, 25 mL) prior to separation on the AG1 anion-exchange column. This step is essential in order to achieve satisfactory resolution, as this appears to be adversely effected by the presence of polyvalent cations which were present in these salts in larger amounts relative to orthophosphate.

In test samples containing metaphosphate, over a pH range of 2 to 8, more than

34% in total (based upon detector response * relative to trehalose) of trehalose monophosphates were present in the reaction mixture after 12 days at 70°C, together with an estimated (again from the detector response *) 7% of products eluted later. These later products may be diphosphorylated and/or higher phosphorylated species. Maximum phosphorylation with metaphosphate was observed between pH 2 and 4.5.

Digestion with alkaline phosphatase. — The isolated products (1 mg of each) were treated with alkaline phosphatase (10 units) in aq 0.05 M $(NH_4)_2CO_3$, pH 8.2 (1 mL) at 37°C for 3 h, and the solutions then assayed for total and inorganic phosphorus. Control samples (i.e., lacking the enzyme) were also prepared and assayed. The enzyme-digest mixtures were chromatographically analysed by Dionex HPLC as described above.

3. Results and discussion

We have previously reported [4] that compounds containing primary and secondary alcohol groups can undergo esterification when they are dried (e.g., freeze-dried) in the presence of inorganic salts. Thus, a number of saccharides, including glucose, sucrose, amylose, cyclodextrins, alditols, and cyclitols (such as glycerol, mannitol, and inositol), produce (as shown by chromatographic and enzymic analyses and, in some instances, from spectroscopic analysis of isolated products) their corresponding phosphate esters. In addition, hydroxylated amino acids, polypeptides, and glycoproteins incorporate phosphorus when dried in the presence of phosphate buffers. The reaction occurs over a range of pH values, but it is pronounced at pH < 7. These observations were first made whilst studying the effects of buffers on a number of oligosaccharides (including α,α'-trehalose) and alditols which are used as excipients for preparing biological standards and reagents. When these conditions were applied to α,α'-trehalose (**1**) and phosphate buffer at and around neutrality, a mixture of four products was observed (Fig. 1) on anion-exchange chromatographic analysis using a Dionex BioLC system with pulsed amperometric detection. The components of the mixture were readily isolated on an anion-exchange resin, using a salt gradient (Fig. 2). Following treatment of these products with alkaline phosphatase at pH 8.2, > 90% of the organic phosphate was converted into inorganic phosphate, with the simultaneous formation of trehalose (identified chromatographically using the Dionex HPLC system). Control samples (i.e., lacking the enzyme) were unaffected. Analysis of the enzyme-digest mixtures by Dionex HPLC also showed the disappearance of those components eluted, in Fig. 1, between 12 and 16 min. Since trehalose and inorganic orthophosphate are formed upon this enzymic treatment, it follows that the products are monoorthophosphate esters of trehalose. There was some selec-

* Under the conditions used, detector response has been shown to underestimate the amounts of phosphate ester present by a factor close to 2. The actual amounts formed are therefore probably substantially greater than these values.

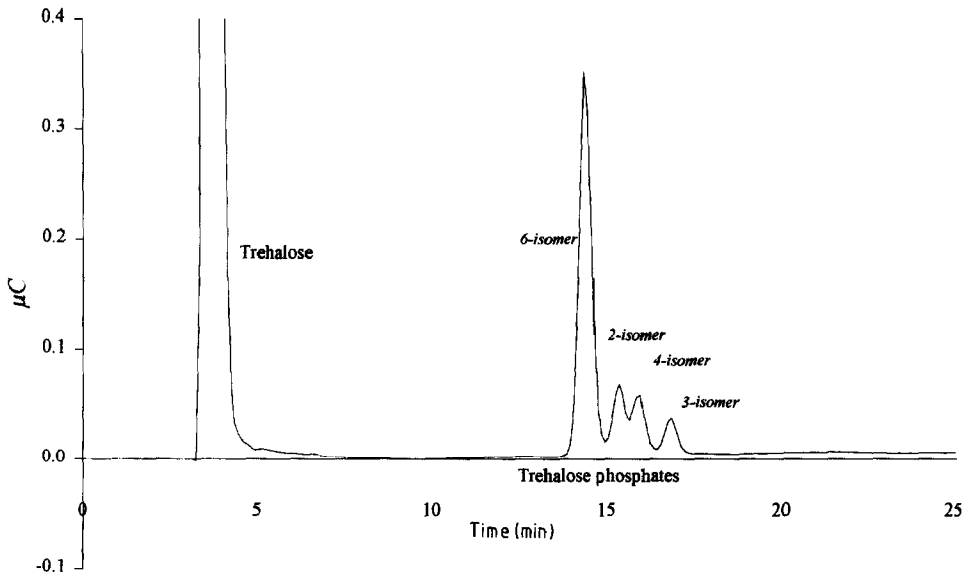


Fig. 1. Chromatogram, obtained on the Dionex BioLC system, for trehalose after drying from phosphate buffer and subsequent heating. For details, see Experimental section.

tivity observed towards enzymic hydrolysis and, as might be expected, the 6-phosphate was the most susceptible and the 2-phosphate the least susceptible. This probably reflects the ease of access of the enzyme to these sites; the 6-phosphate is at a relatively unhindered primary site, whereas the 2-phosphate group is the most sterically hindered, as it lies between the two hexopyranosyl residues. It is possible to prepare pure 2-isomer, albeit in reduced amounts, from the mixture of phosphates by complete enzymic digestion of the other three isomers followed by ion-exchange chromatography to separate the remaining 2-isomer from the released trehalose. Conversion of trehalose–orthophosphate mixtures into trehalose phosphates occurs (as shown by chromatographic analysis) over a range of pH values from below 5 to over 8, with larger amounts forming under conditions of low pH. Increased temperatures and times of heating result, as expected, in more rapid esterification.

Subsequently, it was shown that a major effect on the reaction was due to the nature of phosphate salt used. Increased amounts of the esters were formed upon replacing orthophosphate with pyrophosphate or tripolyphosphate, and the amounts further increased when metaphosphate was used. Metaphosphate is probably the phosphorylating agent of choice, giving, under suitable conditions, more than 34% conversion of trehalose into a mixture of its phosphate esters (as judged by HPLC — see Experimental). The reaction conditions have not yet been fully optimised for maximum phosphorylation of trehalose. However, the results demonstrate that significant amounts of phosphate esters are formed under mildly acidic conditions, and that these conditions should therefore be applicable for

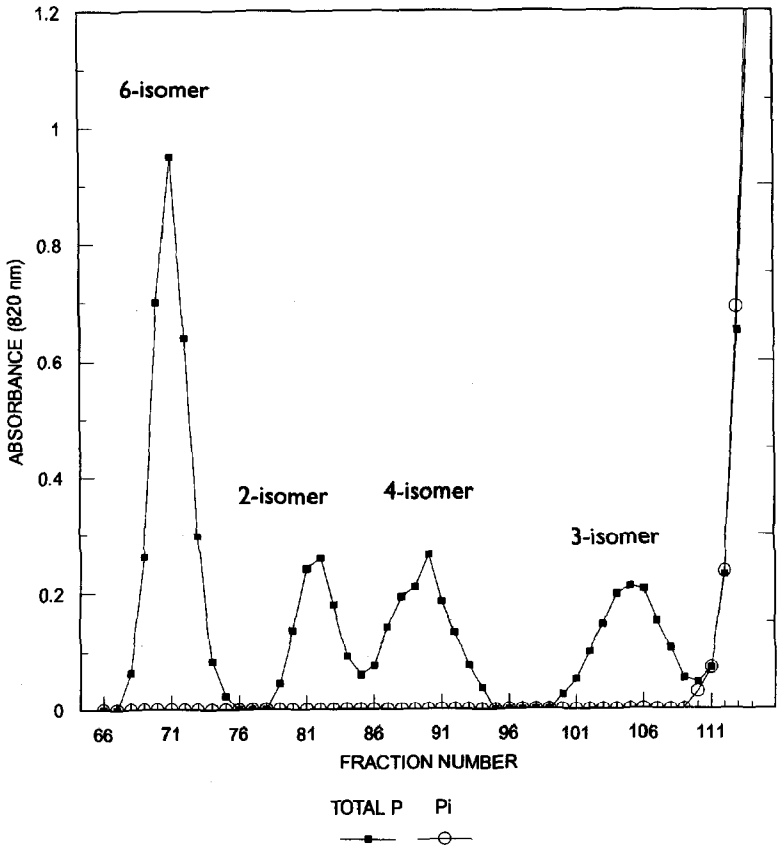
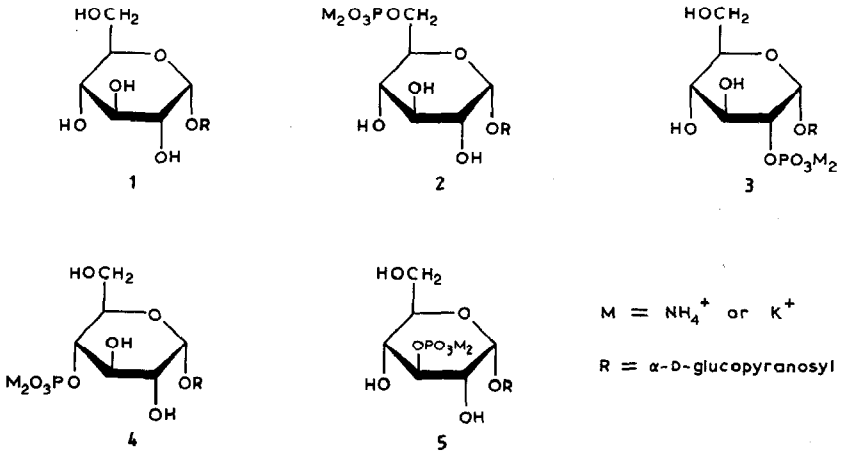


Fig. 2. Phosphorus profile obtained after fractionation of the trehalose phosphate mixture on an AG1 ion-exchange column. For details, see Experimental section.



phosphorylation of compounds which are more sensitive than trehalose to acidic conditions.

The four components were further identified by NMR spectroscopy, primarily ^{1}H NMR, and these spectra are shown in Fig. 3 with the chemical shifts and coupling constants, including those for trehalose, tabulated in Table 1. The identities of the products were confirmed from their ^{1}H-decoupled ^{13}C spectra, all of which contained 12 resonances (Table 2) in keeping with their being disaccharide derivatives.

As expected from monosubstituted trehalose derivatives, the ^{1}H NMR spectra were a composite of two sets of signals, one from each moiety, with signals attributable to the nonphosphorylated residue being, in the main, similar to those of trehalose itself. The first eluted fraction (Fig. 2) was identified as the 6-phosphate **2** * because of the two-proton multiplet observed at δ 3.91–3.99 attributable to the two methylene hydrogens (H-6a and H-6b). The neighbouring H-5 was observed as a complex multiplet at δ 3.84. The pertubations of these resonances (+0.2 to +0.3 ppm for H-6's, +0.04 ppm for H-5) from the corresponding resonances for trehalose are as expected [5] from the introduction of a phosphate group at the 6-position of a hexopyranoside. The chemical shifts and coupling constants of the remaining signals are also as expected from this structure.

In the proton-decoupled ^{13}C NMR spectrum **, signals at 60.8 and 64.0 ppm were present. The former is at a shift value expected for an unsubstituted hydroxymethyl carbon and the latter is in keeping with C-6 of a hexopyranosyl 6-phosphate residue, i.e., with trehalose 6-phosphate (**2**). The observed shift (+2.5 ppm from C-6 of trehalose) is similar to those reported [5] for related primary phosphate esters of lactose. The spectra obtained from this component were identical to those for an authentic sample of trehalose 6-phosphate obtained from the Sigma Chemical Company.

The second component eluted from the ion-exchange column was identified as the 2-phosphate **3** for the following reasons. In its proton spectrum, one of the anomeric proton signals was observed at a chemical shift of δ 5.28, with H-2 at δ 3.97 and H-3 at δ 3.92. These shift values are, respectively, +0.11, +0.35, and +0.09 ppm from the resonance positions of H-1, H-2, and H-3 of trehalose, and accord with the presence of a phosphate group at the 2-position. Of further note in this spectrum are the observed chemical shifts for two protons (H-4′ and H-5′) in the nonphosphorylated residue. These resonated at values −0.16 and +0.28 ppm, respectively, from those of the parent disaccharide. Inspection of molecular models suggests that the 2-phosphate group can be in close proximity to these hydrogens, thereby influencing their environment and hence their chemical shifts. The ^{13}C NMR spectrum possessed two anomeric carbon signals at 93.7 and 92.4 ppm, the former being close to that observed for C-1 of trehalose itself whilst the latter, shifted by −1.6 ppm, again accords [5] with the presence of an adjacent phosphate

* *Unprimed* numbers refer to the phosphorylated moiety, and *primed* numbers to the nonphosphorylated moiety.

** All carbon spectra were determined at a pD of 3.6, previously [5] suggested as being optimum.

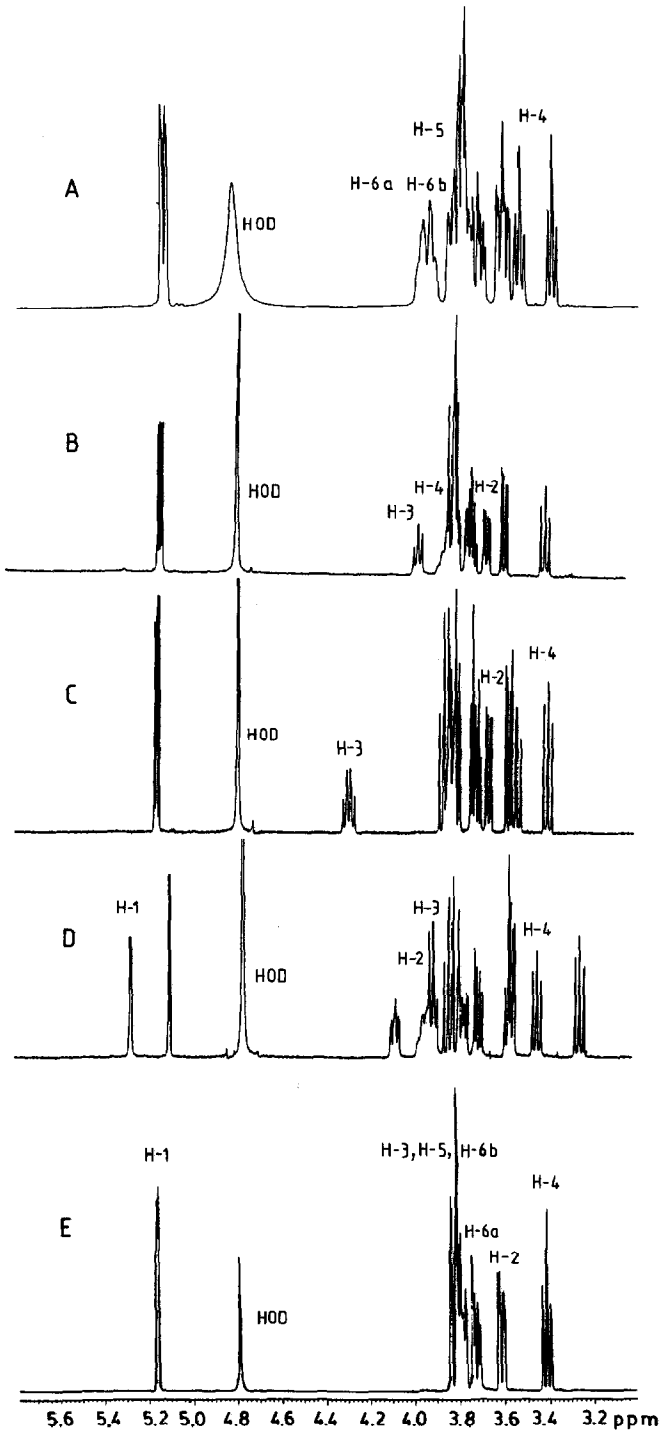


Fig. 3. ^{1}H NMR spectra of trehalose and isomeric trehalose phosphates: **A**, the 6-isomer; **B**, the 4-isomer; **C**, the 3-isomer; **D**, the 2-isomer; **E**, trehalose. The approximate resonance positions of protons in the phosphorylated moiety are indicated.

Table 1
^1H chemical shifts (ppm from Me_4Si) and $^1H-^1H$ coupling constants (Hz) obtained for the four isomeric mono-orthophosphates (NH_4^+ salts) of α,α'-trehalose in D_2O at 500 MHz. Included, for comparison, are the values obtained for α,α'-trehalose.

H	Trehalose	2-P	3-P	4-P	6-P
1	5.17	5.28	5.14	5.11	5.13
2	3.62	3.97	3.64	3.64	3.62
3	3.83	3.92	4.27 [a]	3.95	3.80
4	3.42	3.47	3.52	3.81	3.54
5	3.80	3.78	3.81	X	3.84
6a	3.74	X	X	X	3.91
6b	3.84	X	X	X	3.99
1′	5.17	5.11	5.13	5.13	5.15
2′	3.62	3.56	3.55	3.56	3.59
3′	3.83	3.84	3.84	3.81	3.80
4′	3.42	3.26	3.37	3.38	3.39
5′	3.80	4.08	X	3.88	X
6a′	3.74	3.58	X	X	3.70
6b′	3.84	X	X	X	X
X		3.57–3.84	3.54–3.86	3.73–3.88	3.68–3.81
$J_{1,2}$	3.6	3.4	3.7	3.9	4.0
$J_{1',2'}$	3.6	3.7	3.7	3.7	3.0
$J_{2,3}$	9.7		9.7	9.0	9.9
$J_{2',3'}$	9.7	9.9	9.7	9.9	9.9
$J_{3,4}$	9.6	9.3	9.85	9.0	9.4
$J_{3',4'}$	9.6	9.6	9.6	9.2	9.4
$J_{4,5}$	9.6	9.4	9.85		9.4
$J_{4',5'}$	9.6	9.6	9.6	9.3	9.4

[a] J_{POCH} inferred as ca.9 Hz.

Table 2
^{13}C Chemical shifts (ppm from Me_4Si) obtained for the four isomeric monoorthophosphates (K^+ salts) of α,α'-trehalose in D_2O at pD 3.6 at 125 MHz. Assignments have been made on *best fit* and are therefore tentative. Included, for comparison, are the values obtained for α,α'-trehalose.

Carbon	Trehalose [10]	2-P	3-P	4-P	6-P
1	94.0	92.4	93.5	93.5	92.6
1′	94.0	93.7	93.7	93.8	92.6
2	72.0	74.6 *	70.7 *	71.1	71.2
2′	72.0	71.4	71.4	71.4	71.2
3	73.5	71.8 *	78.0 *	71.5 *	72.7
3′	73.5	73.0	72.8	72.9	72.6
4	70.6	69.6	69.4 *	74.0 *	69.4
4′	70.6	70.2	70.0	70.0	69.9
5	73.0	72.0	72.2	72.5	71.5 *
5′	73.0	72.4	72.4	72.3	72.4
6	61.5	60.8	60.7	60.9	64.0
6′	61.5	60.8	60.8	60.7	60.8

group. The next lowest field signal (74.6 ppm) may be assigned to C-2, shifted by +2.6 ppm from the resonance position of C-2 of trehalose as would be expected [5] from *O*-phosphorylation.

The third component was identified, from its ^{1}H NMR spectrum, as the 4-phosphate **4** by virtue of the multiplet observed for H-4 at δ 3.81, a shift of +0.41 ppm from the value for H-4 in trehalose. A significant perturbation (+0.12 ppm) was also observed for H-3 (a triplet at δ 3.95) in keeping with its being adjacent to an *O*-phosphorylated carbon. The carbon spectrum had, amongst its 12 resonances, a signal at 74.0 ppm, which can be ascribed to C-4, shifted by +3.4 ppm from that of C-4 of trehalose.

The final component eluted from the ion-exchange column was identified as the 3-phosphate **5** on the basis that H-3 was observed as a quartet at δ 4.27 (+0.44 ppm relative to H-3 of trehalose) with H-4 experiencing a shift of +0.1 ppm. In the ^{13}C NMR spectrum, one carbon resonated at 78.0 ppm and this can be assigned to C-3 — a pertubation of +4.5 ppm from C-3 of trehalose. These data accord with structure **5**. For all of these isomers, the anomeric protons were observed as a pair of doublets (J 3.4 to 4.0 Hz, Table 1) at low field and, with the exception of the 2-phosphate, not significantly different from the resonance position of the anomeric protons of trehalose itself. The chemical shifts and proton–proton coupling constants are as would be expected from the proposed structures. $^{3}J_{POCH}$ coupling is almost certainly present in the spectra of all the isomers, but was only readily observable in the case of the 3-phosphate for which H-3 appeared as a 1:4:4:1 quartet indicating a $^{31}P-^{1}H$ coupling of ca. 9 Hz similar to that previously reported [5] in secondary phosphate esters of lactose.

$^{2}J_{POC}$ and $^{3}J_{POCC}$ couplings should be present in the carbon spectra; in keeping with this, were the doublets observed in the spectra of the secondary phosphates, and the doublet and narrow triplet in that of the 6-isomer (coupled signals marked * in Table 2).

During the course of this and related studies involving other hydroxylated organic compounds, we have observed that the rate and the extent of their phosphorylation in dried mixtures are dependent upon a number of factors including the pH and water content of a particular preparation. Esters are formed, albeit slowly, following drying from phosphate buffer with pH > 8; however, their formation is very much greater under acidic conditions (especially $\leq$ 5.5). There is also a degree of selectivity, and the product profile obtained from phosphorylation of trehalose reflects the increased reactivity (by a factor of ca. 3) of the primary relative to the secondary hydroxyl groups (which are of similar reactivity). This can be explained by the greater accessibility and nucleophilic character of the primary hydroxyl groups. For corresponding (i.e., similarly heat-treated) aqueous solutions, little or no (at least 2 orders of magnitude less) phosphorylation is detected. The influence of water may be rationalised in terms of a mass action effect; since water is produced upon ester formation, low water activity will result in driving the esterification reaction. However, from our (limited) observations, some residual water in dried preparations appears to be necessary for the reaction to proceed at an appreciable rate. This is similar to the formation of Schiff's bases in "dry"

mixtures of reducing sugars and proteins, for which it is reported [6] that the presence of a small amount of residual water in the mixture is a requirement of the reaction despite water being a product of this condensation. It was suggested that the effect of residual water was, at least in part, physical, perhaps providing a degree of mobility within the solid "dry" matrix, thereby facilitating the progress of the reaction. In the present case of esterification, there may also be a chemical (mechanistic) function for water. Thus, it has been proposed [7] that the *hydrolysis* of phospho-monoesters, at and around pH 4, proceeds via an intermediate which involves the participation of a molecule of water that is subsequently eliminated. Hydrolyses under these conditions also proceed [7,8] with retention of configuration at chiral centres because it is O–P bond fission which takes place. Esterification as described here may simply be the reverse of this process, with O–P bond formation taking place as a result of nucleophilic attack by a hydroxyl oxygen on the electrophilic centre (phosphorus) of inorganic phosphate (orthophosphate or related [equilibrium] species). In keeping with this proposition are our observations that: (*a*) phosphorylation occurs with retention of configuration at chiral centres; (*b*) products other than orthophosphate esters are not detected; (*c*) inorganic phosphate salts other than orthophosphate, viz., pyrophosphate, tripolyphosphate, and metaphosphate, react in a similar manner to orthophosphate and, furthermore, these *other salts* are more reactive; this can be ascribed to the increased electrophilic character of their phosphorus atoms; (*d*) the rate of reaction appears to be reduced when less than a stoichiometric amount of water is present; (*e*) esterification takes place over a range of pH values, but is especially pronounced over the range of 2–5.5.

In conclusion, the methodology described here for the preparation of phosphate esters is simple in that it uses readily available, inexpensive reagents and does not require multistep syntheses. The products are readily isolated and the method should therefore be of general applicability to the direct syntheses of a variety of phosphate esters of carbohydrates and other hydroxyl-containing compounds. Furthermore, because of their greater reactivity, it is preferable to use pyro-, tripoly-, or meta-phosphate salts rather than orthophosphate. In preliminary experiments with several polyols, yields of esters (HPLC data) have been increased to more than 35% when orthophosphate is replaced by metaphosphate and the pH is lowered to below 4. The rate at which esterification occurs also increases under these conditions. The methodology for carrying out esterification by the procedures described here is included in a patent application [9].

The relative ease of formation of esters in dried mixtures also provides a simple mechanism whereby biochemically important phosphate esters could have arisen in Nature since all that is needed, in addition to a hydroxylated organic compound, are solutions containing inorganic phosphate and a drying environment. Preliminary studies (unpublished) have shown this to be the case for glucose, ribose, "2-deoxyribose", and adenosine, all of which gave products that exhibited properties in ion-exchange chromatography similar to those of their orthophosphate esters.

Finally, because phosphate buffers and drying are widely used laboratory

reagents and techniques, careful consideration should be given to the possible formation of phosphate esters when hydroxylated organic compounds and phosphate-buffered solutions are dried.

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