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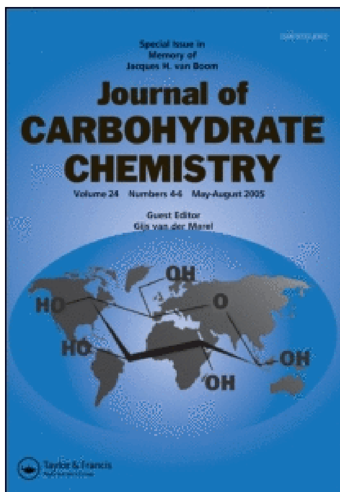

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INOSITOL MONOPHOSPHATES PREPARED IN A SINGLE STEP FROM *MYO*-INOSITOL AND INORGANIC PHOSPHATE

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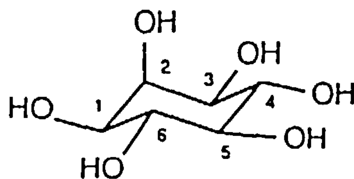
ABSTRACT

A mixture of *myo*-inositol and inorganic metaphosphate (prepared by freeze drying an aqueous solution at pH 4) produced, upon warming, in moderate yield every inositol monophosphate ester, thereby providing in a single step a complete library of these compounds. The individual inositol monophosphates, both *meso*-compounds and racemic mixtures of the two pairs of enantiomers, were isolated by ion-exchange chromatography which was facilitated by the addition of borate to the mobile phase. The phosphates were characterised using phosphorus analysis in conjunction with alkaline phosphatase, mass spectrometry and ^{1}H NMR spectroscopy.

INTRODUCTION

Inositol phosphates, derivatives of *myo*-inositol **1**, are intracellular second messengers produced in response to extracellular stimulation of cell surface receptors by hormones, neurotransmitters, growth factors etc.. The initial response to stimulation is the production



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of D-1,4,5- inositol triphosphate from hydrolysis, by phospholipase C, of phosphatidyl 4,5-bisphosphate and this leads to activation of the cell by mobilisation of stored Ca^{2+}; a number of other inositol phosphates, including monophosphates, are elaborated subsequently. [1-4] Inositol phosphates are also components of GPI anchors through which some glycoproteins embed into cell membranes. [5] Consequently, there is considerable interest in the preparation of inositol phosphates and their analogues, synthetic strategies usually comprising several steps to selectively expose hydroxyls in order that they may be esterified with a phosphorylating agent. [4,6,7]

Recently it has been shown that compounds containing primary and/or secondary alcohol groups undergo direct phosphorylation after they are dried from solutions containing inorganic phosphate salts. [8-12] This reaction is faster and more extensive upon warming, and at acid pH (optimal around pH 4). Since a mixture of all isomers results, albeit only in moderate yield, the methodology enables the direct preparation, in a single step from readily available materials, a library of phosphate esters some of which would be difficult to prepare by other means. The isomeric phosphates can usually be resolved by ion-exchange chromatography and a number of pure oligosaccharide phosphates have been isolated and characterised by these means. [9-12] The results obtained by exposing *myo*-inositol **1**, to these mild conditions are now described.

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RESULTS AND DISCUSSION

With metaphosphate at pH 4, *myo*-inositol **1**, produced a mixture of three major products in the ratio 2:6:9 possessing retention times expected from monophosphate esters using high pH anion exchange chromatography (HPAEC, Fig.1a). Although six monophosphates can be derived from *myo*-inositol, because of molecular symmetry, four, the 1and 3 and the 4 and 6 isomers (see **1**), exist as pairs of enantiomers. The enantiomers are named (according to the stereospecific numbering system described by the International Union of Biochemistry: *Eur. J. Biochem.*, **180**, 485 (1989)) D-*myo*-inositol 1-phosphate, D-*myo*-inositol 3-phosphate, D-*myo*-inositol 4-phosphate and D-*myo*-inositol 6-phosphate. However, because a non-chiral synthesis and isolation procedure are described in this report, for simplicity the racemic mixture of the 1 and 3 isomers is denoted throughout as *rac*-inositol 1-phosphate and similarly the mixture of the 4 and 6

isomers as *rac*-inositol 4-phosphate. These racemates, together with the 2- and 5-*meso*-isomers would therefore be expected to appear as four components (on non-chiral analysis) rather than three as in Fig.1a. Despite extensive modifications to the normally used NaOH/NaOAc mobile phase, further NaOH/NaOAc mobile phase, further resolution of the mixture was not achieved. However, addition of sodium tetraborate (0.125% w/v) to the eluant increased retention times and resolved the last-eluted peak in Fig.1a into two components, that is into four product peaks denoted as **A, B, C** and **D** (Fig. 1b), with **D** appearing as a broad peak eluting over several minutes. Borate can complex with polyols [13] and addition of borate to chromatographic mobile phases has been used to assist the separation of saccharides and their phosphates [14,15,16] but has not been used widely in HPAEC.

On a preparative scale, preliminary separation of the products from inositol and P_i was achieved by fractionation of the mixture on AG1 anion exchange resin (acetate form),

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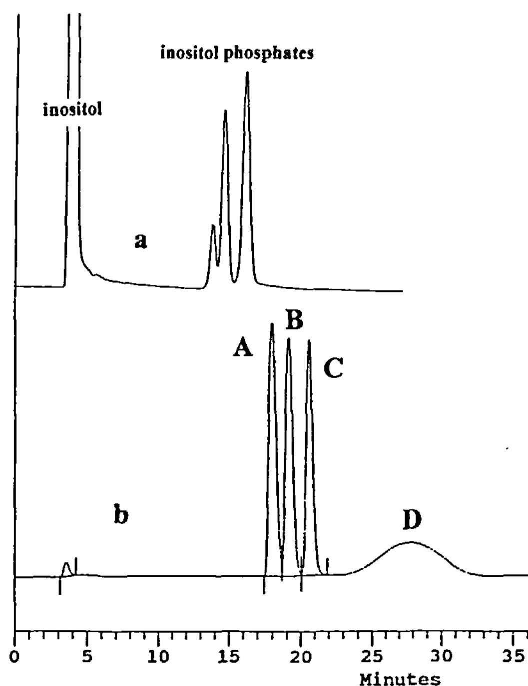

Figure 1. HPAEC chromatogram of the mixture obtained from *myo*-inositol and metaphosphate (a) using mobile phase (i), (b) using mobile phase (ii).

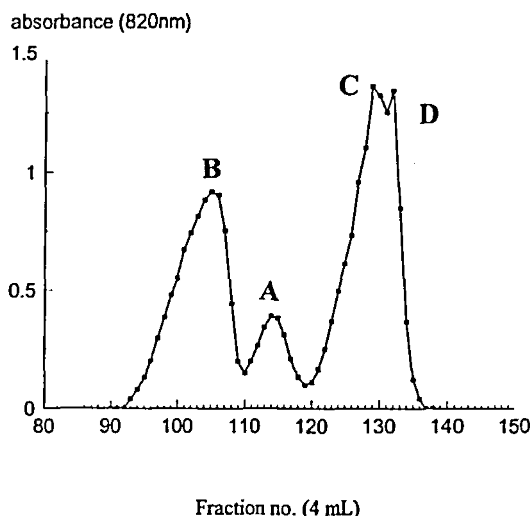

Figure 2. Phosphorus profile from fractionation on AG1 (acetate form) of the mixture obtained from *myo*-inositol and metaphosphate. Products **A, B, C**, **D** are indicated.

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as previously used for separating oligosaccharide monophosphates.[10,11] This resulted in good separation, in order of elution, of **B** and **A**, and a partial separation of **C** from **D**. The latter two could be obtained in up to 80% purity (estimated by HPAEC and NMR) by selecting the leading and trailing edges of the last eluted peak in Fig. 2. All of the products were separated from, and eluted before P_i. Pure samples of **C** and **D** were obtained by further fractionation on HPAEC using borate-containing mobile phase (ii). Subsequent treatment with ion exchange resins and gel-chromatography followed by lyophilisation afforded **A, B, C** and **D** in the approximate ratio of 3:1:1.5:3 as white amorphous solids. Their structures were ascertained using a combination of elemental, enzymatic, mass spectrometric and ^{1}H NMR analysis. The organic phosphorus-containing fraction obtained after chromatography on AG1 resin contained [17] 9.8% w/w phosphorus indicating the presence of one phosphorus per molecule. Upon treatment with alkaline phosphatase, more than 90% of this phosphorus was converted into P_i with the simultaneous formation of *myo*-inositol (identified by HPAEC) confirming the products as orthophosphate esters of *myo*- inositol.

Table 1. ^{1}H NMR Spectral Data[a] for *myo*-inositol and **A, B,C, D** in D_2O.

	Inositol	A (*meso*-2)	B (*rac*-1)	C (*meso*-5)	D (*rac*-4)
H-1	3.42q[b]	3.41d	**3.83nt**	3.48t	3.45q
H-2	3.95nt	**4.41d**	4.15tt	3.93nt	3.95nt
H-3	3.42q	3.41d	3.42t	3.48t	3.59t
H-4	3.51t	3.60t	3.54t	3.64t	**4.02q**
H-5	3.17t	3.16t	3.22t	**3.70q**	3.32
H-6	3.51t	3.60t	3.64t	3.64t	3.59t
$J_{1,2}$	2.9	2.9	2.9	2.9	2.9
$J_{2,3}$	2.9	2.9	2.9	2.9	2.9
$J_{3,4}$	10.1	10.1	10.1	9.3	9.3
$J_{4,5}$	10.1	10.1	10.1	9.3	9.3
$J_{5,6}$	9.3	9.3	9.3	9.8	9.3
$J_{6,1}$	10.3	10.3	10.3	9.3	9.8
$^{3}J_{PH}$		~8	~8	~8	~8

a. K^{+}salts. Chemical shifts) in ppm relative to internal acetone with those in bold from protons geminal to phosphate. Coupling constants (Hz).
b. multiplicity, q = quartet; nt = narrow triplet; t = triplet; d = doublet:

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Negative ion FAB mass spectra of **A, B, C** and **D** contained molecular ions at *m/z* 259 +/- 0.3 (calculated 259.0 for the deprotonated species, $C_6H_{12}O_9P$) in agreement with them being inositol monophosphates.

The positions of substitution by phosphate were determined from inspection of the ^{1}H NMR spectra of **A, B, C** and **D,** the chemical shifts and coupling constants of which are recorded in Table 1 together with those for *myo*-inositol. The spectra of *meso* isomers contain only four resonances, because of their molecular symmetry, and *rac* isomers contain six resonances and so these are readily distinguished. Within each spectrum one resonance, relative to those of *myo*-inositol, was shifted downfield by about 0.5 ppm, and could be attributed to a proton geminal to a phosphate. [12-14,18,19] Smaller downfield shifts were also observed for protons adjacent to the phosphorylated site. These observations together with the multiplicity of the signals, the magnitude of the coupling constants, and

by comparison with published spectral data, [20] identified **A, B, C and D** as, respectively, *meso*-inositol 2-phosphate, *rac*-inositol 1-phosphate, *meso*-inositol 5-phosphate and *rac*-inositol 4-phosphate. It is therefore the *rac* 4-phosphate which produces the broad peak on HPAEC in the presence of borate. By analogy with the borate complex of *myo*-inositol [21] the *rac* 4-phosphate probably forms a tridentate complex through its 1, 3 and 5 hydroxyls in the conformation where these are all axial (with the phosphate group trans-axial) as in **2**. An equatorial phosphate, as would occur with the 2-phosphate in this conformation, would appear to significantly affect the ability to complex in this manner, since this isomer remained chromatographically well resolved. Roughly equal amounts of each of the six isomeric inositol monophosphates were obtained with the 2- and 5-phosphates obtained as pure isomers. The methodology thus provides a route for obtaining them from a single reaction. It should, however, be possible to resolve the two pairs of enantiomers by derivatisation with chiral agents as has been used previously. [22] An

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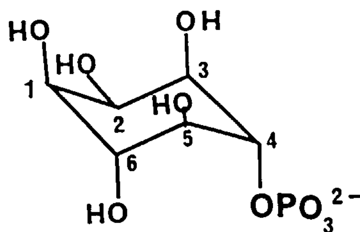

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alternative method to achieving chiral purity is by direct phosphorylation of optically pure inositol analogues such as quebrachitol. Such an approach using chemical syntheses (but requiring several steps) has been used successfully [23,24] and this is currently under investigation. Finally, the process described here allows the recovery of unreacted starting materials and these can be re-recycled to increase overall yields. Furthermore because of the low selectivity of this reaction, the method provides a combinatorial single step process to obtain libraries of esters for possible biological evaluation.

EXPERIMENTAL

General methods. *Myo*-inositol and metaphosphoric acid (described as $(HPO_3)_n$, stabiliser 57-63% $NaPO_3$) was obtained from Aldrich Chemical Co. Gillingham, Dorset, U.K. Analytical HPAEC was performed using a Dionex BioLC system with pulsed amperometric detection on a Carbopac PA 100 (4 x 250 mm) column with the following mobile phases- (i) 50 mM sodium hydroxide, 200-750 mM sodium acetate over 40 minutes (ii) as (i) plus 0.125% w/v sodium tetraborate. Preparative HPAEC was performed on a PA1 column (9 x 250 mm) Dionex, UK, using eluent (ii) and a flow rate of 3 mL/min. Fractions (1 mL) after passage through an ion-membrane suppresser (Dionex) were collected. ^1H NMR spectra were determined under ambient conditions on solutions of potassium salts in D_2O at 500 MHz on a Varian Unity Spectrometer. Fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS 80 RFA Mass Spectrometer equipped with an Ion-Tech saddle field atom gun and standard Kratos FAB source (Kratos Analytical, Manchester, England). Xenon atoms were used as the bombarding particles and the liquid matrix was a 1:1 mixture of glycerol and a eutectic mixture of dithiothreitol and dithioerythritol (5:1). Approximately 3μg of sample dissolved in 1 μL 30% acetic acid was mixed with an equal volume of matrix on the stainless steel target. The instrument was operated at 4kV accelerating voltage and the magnet was scanned at 10 seconds per decade over a mass range of 1500 to 100. Masses were assigned, in the positive mode, by comparison with caesium iodide clusters. Approximately 10 scans were acquired and averaged using the raw data program supplied with the DS90 data system. For other details, see reference 10.

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***meso*-Inositol 2-Phosphate, *rac*-Inositol 1-Phosphate, *meso*-Inositol 5-Phosphate, and *rac*-Inositol 4-Phosphate.** *myo*-Inositol (1.0 g) was dissolved in aqueous sodium metaphosphate (0.1M, pH 4.0, 100 mL, prepared by titrating metaphosphoric acid with sodium hydroxide) and the solution freeze-dried. The container was then sealed and heated at 66 °C for 6 days. The resulting solid was then dissolved in water (100 mL), loaded onto a column (2 x 100 cm) of AG1-X8 resin (acetate form) and the column was eluted using a linear gradient of ammonium acetate (0.3-0.6M) and 4 mL fractions were collected. The phosphorus containing fractions (Fig. 2), which eluted

before P_i, were assayed by HPAEC using mobile phase (ii) and appropriate fractions were combined and freeze-dried several times to constant weight. **B** and **A** were obtained homogeneous by HPAEC analysis and **C** and **D** were partially separated by these means. The total amount of organic phosphorus was 12 mg representing a yield of 120 mg (12%) of inositol monophosphates. Fractions were further purified to obtain analytical samples by chromatography on a PA-1 (9 x 250 mm) column using mobile phase (ii) and fractions (1 mL) collected after the eluant had passed through a membrane suppresser (Dionex) in order to remove sodium ions. The fractions were inspected by analytical HPAEC using mobile phase (ii), combined as appropriate, desalted on a polyacrylamide Biogel P2 (BioRad) column (42 x 1.5 cm) and converted to their potassium salts by passage through a column (12 x 1 cm) containing AG50 and Chelex 100, (K^+ forms, BioRad) as described. [10] After filtration and freeze drying, analytical samples of **A, B, C** and **D** which are, respectively, *meso*-2, *rac*-1, *meso*-5 and *rac*-4 phosphate esters of inositol were obtained as white amorphous solids in the ratio of 3:1:1.5:3.

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Digestion with Alkaline Phosphatase. Each product (0.5 mg) was treated with alkaline phosphatase (5 units) in aqueous 0.05M $(NH_4)_2CO_3$, 0.01% NaN_3, pH 8.2 (1 mL) at 37 °C and the solutions assayed for total and inorganic phosphorus. The reaction mixtures were also inspected by HPAEC. Control samples, lacking enzyme were similarly prepared and assayed.

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