SYNTHESIS OF MIXTURES OF [$^2H_6$]myo-INOSITOL MONOPHOSPHATES SUITABLE AS INTERNAL STANDARDS FOR QUANTITATIVE GC/MS AND A SIMPLE SYNTHESIS OF DL-myo-INOSITOL-1-PHOSPHATE

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SUMMARY

A mixture of DL-[$^2H_6$]myo-inositol-1- and -4-phosphate and (meso) myo-inositol-2- and -5-phosphate is readily prepared on a 100 milligram scale from the mixed isomers of di-O-cyclohexylidene-[$^2H_6$]myo-inositol by phosphorylation with tetrabenzylpyrophosphate. The deblocked [$^2H_6$]myo-inositol monophosphate isomers are obtained where the relative amounts of racemic myo-inositol-1-phosphate > -4-phosphate > -2-phosphate > -5-phosphate. In addition a simple synthesis of DL-myo-inositol-1-phosphate was developed which was found to be unsatisfactory for the preparation of deuterium-labeled product.

Keywords: myo-inositol, inositol, deuterium, inositol monophosphate, inositol-1-phosphate, GC/MS.

INTRODUCTION

myo-Inositol-1- and -3-phosphate (which are enantiomers; see Figure 1)** and myo-inositol-4-phosphate are metabolic products of the second messenger signalling system that utilizes the phosphoinositide lipids (for a recent review see reference 1). myo-Inositol-2- and -5-phosphates have also been identified in brain tissue although their source is unknown

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**Unless otherwise indicated, numbering of the myo-inositol monophosphates assumes the D-enantiomer. Only the 1(3)- and 4(6)-phosphates occur in enantiomeric form; the 2- and 5-phosphates are meso molecules.

0362–4803/90/020181–05$05.00

Received July 13, 1989
Revised September 5, 1989

(2,3). Measurement of changes in the levels of inositol monophosphates following stimulation gives evidence for the involvement of this signalling pathway in a stimulus-response event (3). The most sensitive quantitative assay for the inositol monophosphates at this time is GC/MS

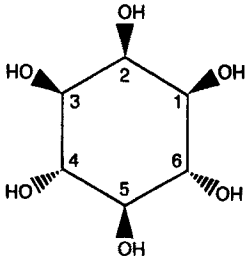

Figure 1. myo-Inositol, numbered in the D-series of enantiomers.

of their per(trimethylsilyl) derivative, an analysis which is best performed using an internal standard (4). We have described the preparation and use of L-[$^2H_6$]myo-inositol-1-phosphate obtained enzymatically from D-[$^2H_7$]glucose-6-phosphate by the use of L-myo-inositol-1-phosphate synthase (5,6). The enzymatic procedure is a difficult one for many laboratories, one that is complicated by uncertain yields of the enzyme from biological sources (unpublished results). In this paper we describe a three-step synthesis of mixed inositol monophosphates from [$^2H_6$]myo-inositol. 1,2,3,4,5,6-[$^2H_6$] myo-Inositol is first converted to the mixed di-O-cyclo-hexylidene isomers and then phosphorylated with tetrabenzylpyrophosphate. Deblocking gives a useful mixture of deuterium-labelled inositol monophosphate isomers. We also describe a simple synthesis of DL-myo-inositol-1-phosphate, largely free of other inositol monophosphates, but one which could not be successfully applied to obtain DL-[$^2H_6$]myo-inositol-1-phosphate in satisfactory yield.

EXPERIMENTAL

Mixed isomers of di-O-cyclohexylidene-[$^2H_6$]myo-inositol. A racemic mixture of inositol 1,2:4,5-, 1,2:3,4- and 1,2:5,6-di-O-cyclohexylidene ketals was prepared according to the procedure of Garegg et al (7). Briefly, 100 mg (0.54 mmole) of either myo-inositol or 1,2,3,4,5,6-[$^2H_6$]myo-inositol (MSD Isotopes, Montreal, Canada), 2.5 ml dry N,N-dimethyl formamide (DMF), 800 μl (~ 6.4 mmole) of 1-ethoxycyclohexene (prepared from cyclohexanone and triethyl orthoformate with careful fractional distillation of the product [8])

and 5 mg of p-toluene sulfonic acid was heated at 100°C for 2 h, cooled and volatile materials removed at 50°C (20mm Hg) on a rotary evaporator. The residual inositol bisketal mixture was a viscous yellow oil that did not crystallize; it was further dried under high vacuum. Yield: 165 mg (87.5%) when starting with d_0-inositol; 137 mg (73%) with d_6-inositol. The mixtures were used without further purification for the next step.

Mixed myo-inositol monophosphates prepared using tetrabenzyl pyrophosphate. The mixture of di-O-cyclohexylidene isomers, prepared as above, was dissolved in 1 ml of dry DMF, 1.5 molar equivalents of NaH was added and the resulting slurry was cooled to 0°C. Phosphorylation of the bisketal mixture was carried out using 2 molar equivalents of solid tetrabenzyl-pyrophosphate (9) prepared by the method of Khorana and Todd (10). The reaction mixture was stirred at 0°C for 3-4 h, then at 4°C for 48 h, after which 10 ml water was added. The di-O-cyclohexylidene-inositol dibenzyl phosphates were extracted into methylene chloride (3 x 8 ml), taken to dryness in vacuo and the residue was taken up in 40 ml of 20% aqueous ethanol. Hydrogenolysis of the benzyl groups was carried out with 50 mg of 5% Pd on carbon, at 75 p.s.i. of H_2 and at room temperature for 18 h. The catalyst was then removed by centrifugation, the supernatant made 0.1 M with HCl and stirred at room temperature for 30 min to remove the cyclohexylidene groups. The resulting product was evaporated to dryness, dissolved in 25 ml of water and washed with methylene chloride (3 x 10 ml) and the organic phases discarded. Yield, (based on starting with 100 mg of inositol) was 22 mg (21%) using d_6-inositol and 34 mg (27%) using d_0-inositol. The relative amounts of the isomers formed in the deuterium-labeled case, based on GC/MS total ion current, were: myo-inositol-1(3)-P (58%), -4(6)-P (24%), -2-P (12%) and -5-P (6%) The results with unlabelled myo-inositol were similar, i.e., all of the isomers of inositol monophosphate were formed and in similar yields.

A SIMPLE SYNTHESIS OF DL-myo-INOSITOL-1-PHOSPHATE.

Racemic 1,2: 4,5-di-O-cyclohexylidene-myo-inositol (500 mg, 1.48 mmole, reference 7) was dissolved in 4 ml of dry pyridine and diphenyl chlorophosphate (307 ul, 1.48 mmole), in 4 ml of dry pyridine, was added over a 10 min period with stirring at room temperature. The reaction mixture was then stirred at room temperature for 18 h, diluted with ice cold water and the product extracted into methylene chloride. After removing the solvent the product was crystallized from acetone-ethanol (yield 62%). The product contained < 3% of the 6(4)-diphenyl phosphate.

Deblocking of the inositol-1,2:4,5-di-O-cyclohexylidene-3-diphenyl phosphate was carried out by hydrogenation (70 p.s.i. H_2, 18 h, room temperature) with PtO_2 in 9% ethanol containing 0.01 M HCl. The yield of DL-myo-inositol-1-phosphate was 95% containing < 3% of DL-myo-inositol-4-phosphate.

DISCUSSION

Several methods for the synthesis of racemic myo-inositol-1-phosphate have been described previously (11 and references therein). While some of these methods are potentially suitable for the synthesis of deuterium-labelled material, the preparation and separation of individual di-O-cyclohexylidene myo-inositols on a small scale is subject to low yields through product losses, an important consideration when using expensive isotopically labelled inositol. Furthermore, for use as a GC/MS internal standard it is useful to have the mixture of inositol monophosphates since all of the isomers are found in biological samples. Since tissue abundances of the four inositol monophosphates are similar to the ratio of their amounts in the synthetic mixture, it is convenient to have them all present in the internal standard. Also, since the absolute amounts of the internal standards are unimportant as long as the standard curve and the samples are prepared with the same concentrations of the deuterated species, the presence of all of the isomers where only one species is measured is of no consequence.

The formation of myo-inositol-1-, -4- and -5-phosphate is readily understandable; the three di-O-cyclohexylidene-myo-inositols have these positions available for substitution. The formation of myo-inositol-2-phosphate is surprising; all three di-O-cyclohexylidene myo-inositols have been characterized as having the 1(3)- and 2-positions blocked with the cyclohexylidene moiety (12), the 1, 2 and 3 positions bearing the only cis-hydroxyls of myo-inositol and thus forming the most stable ketals. While phosphate migration can occur by intramolecular transesterification between positions 1, 2, and 3, this is known to occur only slowly, even at low pH (13). Whether the formation of the 2-isomer occurs as a special circumstance accompanying the acid-catalyzed removal of the cyclohexylidene groups is not known; in the case of the simple synthesis of myo-inositol-1-phosphate where no 2-phosphate was obtained, the fully blocked substance is crystallized, perhaps with the loss of the 2-isomer.

It is possible that small amounts of myo-inositol bisphosphates are formed in this procedure, however they would not be detected due to the difficulty in gas chromatographing these substances (4).

In early attempts to obtain DL-[$^2H_6$]myo-inositol-1-phosphate we found that we could readily prepare the unlabelled molecule by phosphorylation of racemic 1,2:4,5-di-O-cyclohexylidene-myo-inositol with diphenyl chlorophosphate followed by deblocking, however, for unknown reasons, repeated attempts to perform this reaction on [$^2H_6$]myo-inositol resulted in yields of 10% or less.

ACKNOWLEDGEMENTS

This work was supported by N.I.H. grants NS-05159, AM-20579 and RR-00954.

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