Bioorganic & Medicinal Chemistry Letters, Vol. 1, No. 1, pp. 17-20, 1991
Printed in Great Britain

0960-894X/91 $3.00 + .00

Chemical and Enzymic Syntheses of D- and L-*myo*-Inositol 1-Phosphorothioate, Substrates for Inositol Monophosphatase: D-Glucose 6-Phosphorothioate is not a Substrate for Inositol Synthase

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(*Received* 8 *November* 1990)

Abstract: D- and L-*myo*-inositol 1-phosphorothioate have been synthesized from 2,3,4,5,6-*O*-pentabenzyl-*myo*-inositol. Both enantiomers are slow substrates for inositol monophosphatase and weak competitive inhibitors, therefore, ideal probes for the phosphatase reaction. D-glucose 6-phosphorothioate did not serve as a substrate for the enzyme inositol synthase in an alternative synthesis of L-*myo*-inositol 1-phosphorothioate.

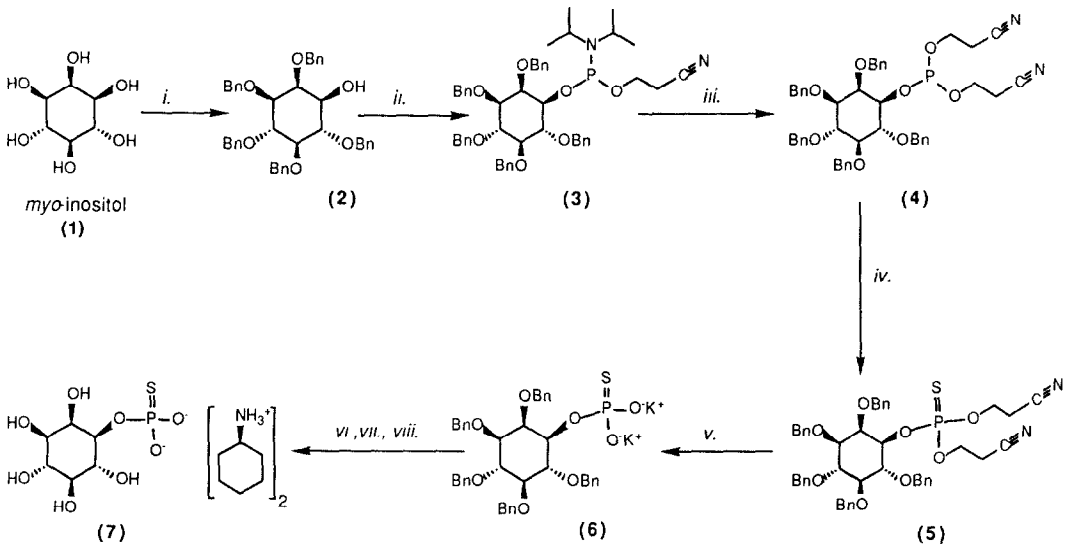
Aspects of the metabolic pathways associated with the release of calcium from intracellular stores during signalling processes have been defined recently.[1] An important and key step involves the hydrolysis of inositol 1-phosphate by the lithium-sensitive phosphoesterase, inositol monophosphatase, to give free inositol.[2] Free inositol is recycled to provide the precursor, phosphatidylinositol 4,5-bisphosphate, for the two secondary messengers, diacylglycerol and inositol 1,4,5-trisphosphate.

The monophosphatase is capable of hydrolysing both enantiomers of *myo*-inositol 1-phosphate and *myo*-inositol 4-phosphate although the kinetic parameters, V_{max} and K_m for each substrate are different. The modes of inhibition by lithium cation are similar for the 1- and 4-phosphates and both show apparent uncompetitive inhibition, but the K_i value for inositol 4-phosphate (0.11 mM) is 7-fold lower than that for the 1-phosphate.[3,cf.4]

Recently we proposed that the enzyme operated *via* a substituted enzyme mechanism in which the phosphate group of the substrate was first transferred to an enzyme bound nucleophile, and in a subsequent step to water.[5] Based on the observation of the burst-phase release of [^{14}C]-inositol from [^{14}C]-inositol 1-phosphate at high [Li^+], together with the established uncompetitive mode of lithium cation inhibition, we further proposed that Li^+ might act by retarding the rate of conversion of the phosphoryl enzyme to free enzyme relative to the rate of phosphorylated enzyme formation.

In order to further probe the mechanism of the phosphatase, we sought substrates which might display more pronounced differences in the rates of formation and break-down of the putative phosphorylated enzyme intermediate. Here we report on the synthesis of both enantiomers of *myo*-inositol 1-phosphorothioate from inositol and demonstrate that both enantiomers are substrates for inositol monophosphatase. In an alternative synthesis of the L-enantiomer, D-glucose 6-phosphorothioate did not serve as a substrate for inositol synthase.

Racemic 2,3,4,5,6-*O*-pentabenzyl-*myo*-inositol (2) was prepared from *myo*-inositol (1) in six steps using literature procedures.[6] The alcohol (2) was then treated[7] as outlined in Scheme 1 to give the pentabenzyl-*myo*-inositol 1-phosphorothioate triester (5) in 75% overall yield from the protected inositol. The phosphorothioate triester was converted to the dipotassium salt (6) quantitatively *via* base catalysed β-elimination and the protecting groups were removed with sodium and ammonia in tetrahydrofuran to give the disodium salt of *myo*-inositol 1-phosphorothioate.[8] After purification on Amberlite IR118H the compound was converted to the crystalline *bis*-cyclohexylammonium salt (7), in 70% overall yield from the protected inositol (2).



i. As ref. 6, ii. $iPr_2NClPOCH_2CH_2CN$, iPr_2NEt, CH_2Cl_2; iii. $HOCH_2CH_2CN$, 1H-Tetrazole, MeCN; iv S_8, Pyridine; v MeOK, MeOH; vi Na, NH_3; vii. Amberlite IR 118 [H^+], H_2O; viii $C_6H_{13}N$

Scheme 1

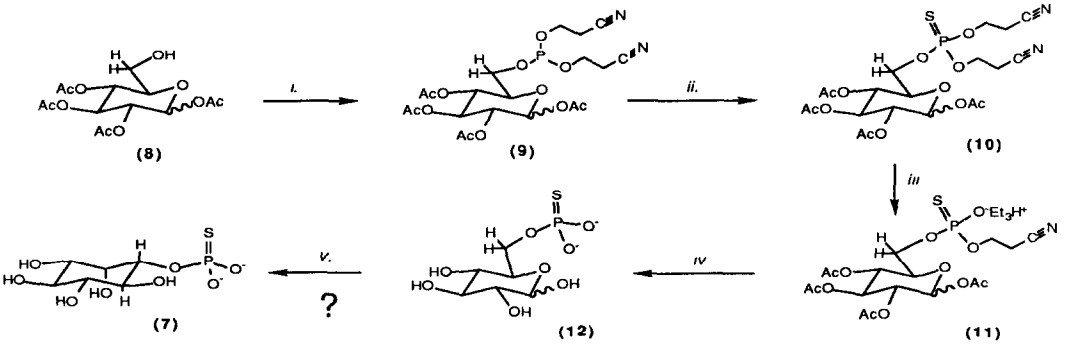
To synthesise the enantiomers of the phosphorothioate (7), the racemic 2,3,4,5,6-*O*-pentabenzyl-*myo*-inositol (2) was resolved.[6] Treatment of each separate enantiomer in the manner

outlined above gave the *bis*-cyclohexylammonium salt of the chiral inositol 1-phosphorothioates in 57% overall yield from the resolved alcohols.

In order to test the phosphorothioates as inhibitors for inositol monophosphatase, the compounds were added at various concentrations to standard activity assay incubations[2] containing inositol 1-phosphate (1 mM). Analysis of the kinetic data indicated that each of the enantiomers acted as a weak competitive inhibitor, K_i = 1.0 and 1.0 mM for the D- and L-antipodes, respectively.

In order to test the phosphorothioates as substrates for the enzyme, the phosphorothioates, together with Mg^{2+} ions (2 mM) and ammonium bicarbonate buffer (20 mM), were incubated at 37 ^{o}C and at pH 7.8, with pure inositol monophosphatase. After 18 hours, the protein in each incubation was denatured with ethanol and was then removed by filtration. Examination of the lyophilised filtrate by ^{1}H-NMR spectroscopy indicated that each of the phosphorothioates were converted to inositol, but more slowly than inositol 1-phosphate. The phosphorothioate ester hydrolyses were inhibited by lithium cation and control experiments which contained the phosphorothioates but, no enzyme, showed no reaction or decomposition. Thus, both of the phosphorothioates were substrates and would be suitable as probes for a kinetic and mechanistic study of the phosphatase.

In order to facilitate the introduction of labels into the inositol and phosphorothioate moieties of the new substrates and, specifically to allow the synthesis of the ^{14}C-compound and compounds containing a chiral phosphorothioate group,[9] a new synthesis using inositol synthase[10] and D-glucose phosphorothioate was examined, Scheme 2.



i. $^{i}Pr_2NP(OCH_2CH_2CN)_2$, 1H-Tetrazole, MeCN; *ii* S_8, Pyridine;
iii 3:1 Pyridine:Et_3N, *iv.* MeOK, MeOH; *v.* Inositol Synthase, NAD

Scheme 2

Accordingly, 1,2,3,4-tetraacetyl-D-glucose (8) was prepared by the method of Stacey[11] and was converted to D-glucose 6-phosphorothioate dipotassium salt (12). Incubation of compound (12)

with partially purified inositol synthase from bovine testes at pH 7.7 and at 37°C in the presence of a catalytic amount of NAD, surprisingly, did not give inositol 1-phosphorothioate.[12] Under similar conditions D-glucose 6-phosphate was rapidly converted to L-*myo*-inositol 1-phosphate. This is a curious result and suggests that the phosphate moiety serves a more important role in the synthase reaction than we had expected. Just how the phosphate moiety interacts with the synthase and why replacing just one phosphorus ligand by sulphur completely prevents reaction are not known. Nevertheless, the finding excludes the possibility of using the synthase in the synthesis of inositol 1-phosphorothioate as outlined in Scheme 2.[13]

Acknowlegements

We thank the SERC and Merck, Sharp and Dohme for a CASE studentship to G.R.B.

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13. All spectral and analytical data for the compounds and their intermediates were consistent with their expected structures; full details will be reported elsewhere.