myo-Inositol phosphorothioates, phosphatase-resistant analogues of *myo*-inositol phosphates

Synthesis of DL-*myo*-inositol 1,4-bisphosphate and DL-*myo*-inositol 1,4-bisphosphorothioate

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Syntheses of a metabolite of the second messenger *myo*-inositol 1,4,5-trisphosphate, *myo*-inositol 1,4-bisphosphate, and an analogue, the 1,4-bisphosphorothioate, are reported, by using phosphite chemistry on ($\pm$)-1,2:4,5-di-isopropylidene-*myo*-inositol. The synthesis of ($\pm$)-1,2:4,5-di-isopropylidene 3,6-bis[di-(2-cyanoethyl)]phosphite provides an intermediate that can be oxidized to either the corresponding bisphosphate or bisphosphorothioate. *myo*-Inositol phosphorothioates are proposed as novel analogues of *myo*-inositol phosphates; their resistance to phosphatase-catalysed breakdown is reported.

INTRODUCTION

D-*myo*-Inositol 1,4,5-trisphosphate [(I) in Fig. 1], derived from the hexitol *myo*-inositol [(II) in Fig. 1], is released by receptor-stimulated phosphodiesteratic hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate, and has been recently identified as an intracellular second messenger mediating calcium mobilization from non-mitochondrial ATP-dependent stores [1,2]. Additionally, other related inositol phosphates have been reported: *myo*-inositol 1,3,4-trisphosphate [3] and *myo*-inositol 1,3,4,5-tetrakisphosphate [4,5]. The latter has been proposed as a second messenger regulating Ca^{2+} entry into the cell [6]. These discoveries have refocused much biological interest on the inositol phosphates [7,8]. The isolation of such compounds from natural sources is often tedious and difficult [9], and on account of this it is important to develop synthetic approaches to the natural compounds [10–12] and analogues with novel biological properties. We have developed an approach using phosphite chemistry, and have reported syntheses of *myo*-inositol 4,5-bisphosphate [13,14] and biologically active *myo*-inositol 1,4,5-trisphosphate [15,16]. We now report the synthesis of a metabolite of the latter, *myo*-inositol 1,4-bisphosphate [17], and an analogue thereof, *myo*-inositol 1,4-bisphosphorothioate, which we introduce as an example of new analogues of *myo*-inositol phosphates and demonstrate that such compounds have the novel property of resistance to phosphatase-catalysed breakdown, an important process in second-messenger deactivation and metabolism.

MATERIALS AND METHODS

Materials

($\pm$)-1,2:4,5-Di-isopropylidene-*myo*-inositol [(III) in Scheme 1] was synthesized as described by Gigg *et al.* [18]. Di-isopropylamino-(2-cyanoethoxy)chlorophosphine was prepared as described by Sinha *et al.* [19]. *t*-Butyl hydroperoxide (3M solution in toluene) was purchased from Aldrich Chemical Co. Bovine intestinal mucosa alkaline phosphatase was purchased from Sigma

Chemical Co. as a suspension in 3.2 M-(NH_4)₂SO₄/1 mM-MgCl₂/0.1 mM-ZnCl₂, pH 7, activity 1060 units ($\mu\text{mol/min}$)/mg of protein at pH 10.4 at 37 °C with *p*-nitrophenyl phosphate as substrate.

General methods

Ion-exchange chromatography was performed on columns of DEAE-Sephadex A-25 (HCO_3^- form) with linear gradients of triethylammonium bicarbonate generated by gravity mixing. The column was attached to an LKB Ultrarac 7000 fraction collector.

Phosphate assays carried out on the fractions and to quantify the final product were performed as described by Fiske & SubbaRow [20].

³¹P-n.m.r. spectroscopy was performed on Jeol FX-60 and Bruker AM 300 n.m.r. spectrometers operating at 24.15 and 121.15 MHz respectively, with broad-band proton decoupling. ³¹P-n.m.r. chemical shifts are referenced to external 85% H₃PO₄, a positive value indicating a downfield shift from this reference; ¹³C-n.m.r. chemical shifts are reported relative to external CHCl₃.

Synthesis of ($\pm$)-*myo*-inositol 1,4-bisphosphate (VIIa) (Scheme 1)

($\pm$)-1,2:4,5-Di-isopropylidene-*myo*-inositol (III) [18] (0.325 mmol) was allowed to react with di-isopropylamino-(2-cyanoethoxy)chlorophosphine (0.715 mmol) and di-isopropylethylamine (0.715 mmol) in dichloromethane for 1 h at ambient temperature to yield the corresponding bisphosphoramidite (IV) [δ_{P} (CH₂Cl₂) 4 s, 152.24, 152.30, 152.67 and 152.71]. ³¹P-n.m.r. resonances corresponding to unequal pairs of epimers

Fig. 1. D-*myo*-Inositol 1,4,5-trisphosphate and *myo*-inositol

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Scheme 1. Synthesis of (±)-myo-inositol 1,4-bisphosphate and (±)-myo-inositol 1,4-bisphosphorothioate

R = -CH₂CH₂CN; Prⁱ = isopropyl. All products are racemic.

were observed, on account of the two chiral phosphorus centres (intensity ratios, downfield pair/upfield pair, approx 2:1). Reaction of the bisphosphoramidite with tetrazole and 2-cyanoethanol (0.715 mmol of each) gave

the bisphosphite triester (V) [δ_p (CH₂Cl₂) 2 s, 138.8 and 139.6]. Oxidation of compound (V) with *t*-butyl hydroperoxide [13–16] (1 ml, added at -78 °C, solution warmed to room temperature) gave the bisphosphate

triester (VIa) [δ_p (CH_2Cl_2) 2 s, -3.03 and -3.83]. Ammonolysis (2 h, 65°C) removed the 2-cyanoethyl groups, giving the bisphosphate possessing two isopropylidene ketals [δ_p ($^2\text{H}_2\text{O}$, pH 12) proton-coupled, d, 0.12 , $J = 7.78$ Hz, and d, 1.65 , $J = 11.69$ Hz], and treatment of the resulting ammonium salt in water with an excess of Dowex (H^+ form) ion-exchange resin for 1 h gave *myo*-inositol 1,4-bisphosphate as the free acid [δ_p ($^2\text{H}_2\text{O}$) 2 s, 0.40 and -0.60], which could be converted into the solid cyclohexylammonium salt (80% overall yield). Small amounts of impurities could be removed by ion-exchange chromatography on DEAE-Sephadex A-25 with a linear gradient of 50–700 mM-triethylammonium bicarbonate, pH 7.5, generated from 500 ml of each buffer. Fractions containing product (eluted at approx. 450 mM buffer) were combined and evaporated to give the triethylammonium salt of *myo*-inositol 1,4-bisphosphate as a colourless glass [δ_p ($^2\text{H}_2\text{O}$) 2 s, 3.83 and 4.64].

Synthesis of ($\pm$)-*myo*-inositol 1,4-bisphosphorothioate (VIIb) (Scheme 1)

The bisphosphite triester (V) (0.325 mmol) from above was oxidized to the bisphosphorothioate triester (VIb) by using sulphur (200 mg) in pyridine (2 ml) [21]. The mixture was filtered and evaporated and the residue was dissolved in methanol [δ_p (CH_3OH) 2 s, 65.85 and 67.41]. Ammonolysis (3 h, 65°C) gave a bisphosphorothioate possessing two isopropylidene ketals [δ_p ($^2\text{H}_2\text{O}$, pH 12) proton-coupled, d, 43.16 , $J = 10.96$ Hz, and d, 43.36 , $J = 10.95$ Hz], and treatment with excess Dowex (H^+ form) ion-exchange resin in water (3 h) followed by evaporation and addition of ammonia gave crude compound (VIIb) as a gum that on trituration with methanol gave the ammonium salt of ($\pm$)-*myo*-inositol 1,4-bisphosphorothioate (VIIb) (51% yield) [δ_p ($^2\text{H}_2\text{O}$, pH 12) proton-coupled, d, 44.82 , $J = 8.52$ Hz, and d, 46.34 , $J = 9.74$ Hz] contaminated with only minor amounts of impurities. Final purification could be effected on a column of DEAE-Sephadex A-25 as described for the 1,4-bisphosphate. The ^{13}C n.m.r. (phosphorus-coupled) spectrum showed δ_c ($^2\text{H}_2\text{O}$, pH 12) 73.27 ($^3J_{\text{PC}} = 2.2$ Hz), 73.52 ($^3J_{\text{PC}} = 1.71$ Hz), 74.41 ($^3J_{\text{PC}} = 4.67$), 76.39 ($^3J_{\text{PC}} = 2.42$ Hz), 77.65 ($^2J_{\text{PC}} = 6.41$ Hz) and 79.46 ($^2J_{\text{PC}} = 6.38$ Hz).

Alkaline phosphatase incubations

To *myo*-inositol 1,4-bisphosphate (50 μmol) in 100 mM-glycine/NaOH buffer, pH 10.1, containing 1 mM- MgCl_2 and 0.1 mM- ZnCl_2 (0.5 ml, approx. 60% $^2\text{H}_2\text{O}$) in a 5 mm precision n.m.r. tube was added alkaline phosphatase (60 μl , 49 units), and the ^{31}P -n.m.r. spectrum was monitored. Clear evidence for enzyme-catalysed hydrolysis of the phosphates was obtained, as indicated by the gradual disappearance of resonances due to the 1,4-bisphosphate and appearance to higher field of new resonances due to monophosphates and inorganic phosphate. When the spectrum was examined after incubation of the solution at ambient temperature overnight and addition of EDTA (21 h), only a single peak could be observed at δ 0.61, which remained sharp when broadband proton decoupling was removed and could thus be assigned to inorganic phosphate. When this experiment was repeated under similar conditions with *myo*-inositol 1,4-bisphosphorothioate (25 μmol) and alkaline phosphatase (30 μmol , 25 units) no change in the n.m.r.

spectrum could be seen when monitored over a few hours, and after overnight incubation (23 h) the spectrum showed only the two peaks assignable to the bisphosphorothioate.

RESULTS

($\pm$)-*myo*-inositol 1,4-bisphosphate (VIIa) and 1,4-bisphosphorothioate (VIIb) were synthesized from ($\pm$)-1,2:4,5-di-isopropylidene-*myo*-inositol (III) by using a phosphite approach. Incubation of the 1,4-bisphosphate with alkaline phosphatase resulted in breakdown to *myo*-inositol and inorganic phosphate, as monitored by ^{31}P -n.m.r. spectroscopy. Under the same conditions the 1,4-bisphosphorothioate was not hydrolysed.

DISCUSSION

It is clear that second-messenger molecules must be rapidly degraded after they have achieved their function, in order to return the system to its basal state. This task is carried out in the case of *myo*-inositol 1,4,5-trisphosphate primarily by phosphatase action. Thus analogues of inositol phosphates wherein receptor- and enzyme-binding properties were similar, but which were resistant to such phosphatase-catalysed breakdown, might be expected to show novel biological properties and facilitate investigation of the metabolism of the 1,4,5-trisphosphate. Replacement of a phosphate group by a phosphorothioate could markedly influence the lifetime of such a second messenger and its metabolites, without greatly affecting their affinity for receptors or enzymes. Phosphorothioate analogues of nucleotides have been found to be extraordinarily useful in applications ranging from studies on the mechanism of phosphoryl- and nucleotidyl-transfer reactions to molecular biology [22,23]. A phosphorothioate analogue adenosine 3',5'-cyclic phosphorothioate has also found useful applications in the cyclic AMP second-messenger signalling system [22,23]. One important feature of these analogues is that phosphorothioate monoesters such as adenosine 5'-phosphorothioate, for example, show resistance to phosphatases [24,25]. This can lead to novel effects. For example, glycogen phosphorylase *a* is frozen in the active conformation because of phosphatase-resistance when produced by thiophosphorylation of phosphorylase *b* with adenosine 5'-*O*-(3-thiotriphosphate) [26].

D-*myo*-Inositol 1,4-bisphosphate has recently been identified as a metabolite of the second messenger D-*myo*-inositol 1,4,5-trisphosphate in stimulated cells. Its formation is thought to occur by the action of a specific 5-phosphatase located in the cellular plasma membrane and in the cytosol [27]. Such a 5-phosphomonoesterase has been isolated and purified from human platelets [28]. Subsequent phosphatase action converts this 1,4-bisphosphate into *myo*-inositol, which then re-enters the lipid cycle. The other route of metabolism appears to be phosphorylation to the 1,3,4,5-tetrakisphosphate and subsequent dephosphorylation via the 1,3,4-trisphosphate to *myo*-inositol [5]. *myo*-Inositol 1,4-bisphosphate was first synthesized some years ago, before the current interest had developed [17]. We demonstrate here a synthesis of this molecule based upon modern phosphite chemistry, primarily developed for DNA synthesis [19], and recently adapted by us to the inositol field [13–16]. Phosphitylation of compound (III) yields the bisphosphoramidite (IV), which is converted into the bisphos-

phite triester (V) {whereas a $^5J_{PP}$ spin-spin coupling was observed in the ^{31}P -n.m.r. spectrum of the vicinal 5,6-bisphosphite triester of 1,2,3,4-tetra-*O*-benzyl-*myo*-inositol [13,14], no corresponding $^7J_{PP}$ coupling was observed for compound (V)}. This key intermediate can be oxidized to the bisphosphate triester (VIa) by using *t*-butyl hydroperoxide or to the bisphosphorothioate triester (VIb) by using sulphur in pyridine. Subsequent removal of the 2-cyanoethyl groups by base-catalysed β -elimination and acid-catalysed removal of the isopropylidene groups yields either ($\pm$)-*myo*-inositol 1,4-bisphosphate (VIIa) or ($\pm$)-*myo*-inositol 1,4-bisphosphorothioate (VIIb). This use of P(III) chemistry would provide a route to the potentially valuable radioactive ^{35}S -labelled inositol phosphorothioates by oxidation with ^{35}S -labelled sulphur.

We thus undertook the synthesis of an inositol phosphorothioate, and propose the 1,4-bisphosphorothioate here as the first example of these novel non-hydrolysable analogues of inositol phosphates. We expect that inositol phosphorothioates will prove to be of significant value in future studies of the action of inositol phosphate second messengers and their metabolism.

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