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SYNTHESIS AND CYTOTOXIC ACTIVITY OF (±) OCTADECYLPHOSPHODITHIONYL-1-MYO-INOSITOL

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Abstract: the synthesis of the phosphatidylinositol (PI) analogue (±)-octadecylphosphodithionyl-1-*myo*-inositol is described. The cytotoxic activity on K562 erythroleukaemic cells and the effect on the activity of PI specific phospholipase C (PI-PLC) from human platelets are reported.

The inositol phospholipids are precursors of second messengers in receptor-mediated intracellular Ca^{2+} mobilisation and proteins phosphorylation[1]. The key enzyme in the signal transduction system is the inositol-specific phospholipase C (PI-PLC); in fact it cleaves phosphatidyl-*myo*-inositol 4,5-bisphosphate (PIP_2) to yield the second messengers D-*myo*-inositol-1,4,5-trisphosphate (IP_3), which mediates the release of calcium ions from intracellular stores, and diacylglycerol (DG), involved in the activation of protein kinase C (Fig. 1). These are events of the mitogenic process, thus inhibition of PI-PLC is expected to produce growth inhibitory effects.

Several inhibitors of PI-PLC have been found among natural substances, even though they are not useful in therapy[2]. Ether lipids are the most promising inhibitors and are being studied in clinical trials[3].

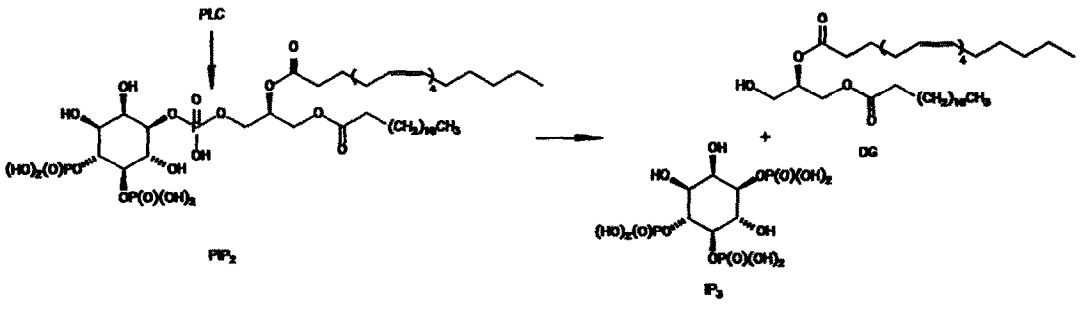
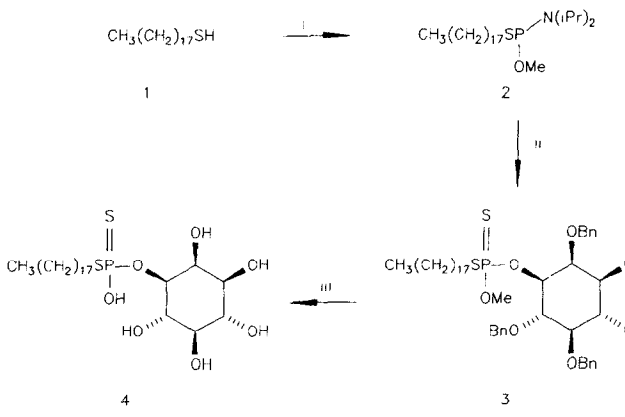


Fig. 1 - Enzymatic cleavage of phosphatidyl-*myo*-inositol-4,5-bisphosphate (PIP_2).



Scheme 1

i) n-BuLi, $(MeO)(i\text{-}Pr)_2NPCl$, THF; ii) a. (±)- 1,2,4,5,6-penta-*O*-benzyl-*myo*-inositol, $BF_4^-Py^+$, CH_3CN/H_2O 1:1; b. S_8, Py; iii) a. Me_3N, Benzene; b. EtSH, $BF_3{\cdot}Et_2O$, $CHCl_3$.

They have multiple cellular effects which could contribute to their antitumor activity. An alternative approach in this field is the use of *myo*-inositol analogues, which interfere with phosphoinositide metabolism or cause phospholipase C inhibition[4]. To date the syntheses of some PI and PIP_2 analogues as inhibitors of PLC have been accomplished, some of the synthesised compounds show only a slight degree of inhibition of PI-PLC from human platelets, while others are not effective on intact cells[5].

Along this line, we recently reported on the synthesis of some phosphothiolate analogues of phosphatidylinositol in which the diacylglycerol moiety was replaced by alkylthiols or diacylthioglycerols[6], and at the moment the synthesis of labelled compounds is in progress in order to study the interaction with PI-PLC. A previous paper described the preparation of hexadecyl-, dodecyl-, and octylthiophosphoryl-*myo*-inositol, and their use as substrates for the kinetic analysis of PI-PLC from *Bacillus cereus*. The maximal activity of the enzyme was found with the hexadecyl derivative and it was about 1% of the activity with the natural substrate[7].

As part of an ongoing programme directed toward the preparation of PI-PLC inhibitors, we synthesised the title compound and we report here the measurement of its cytotoxic activity. Compound **4** was synthesised as shown in Scheme 1.

Octadecanethiol **1** was reacted with n-BuLi in THF at 0°C for 30 min to generate the anion and then with N,N-diisopropylmethylphosphonamidic chloride at r.t. for 30 min to give **2**. The phosphithiolate **2** was converted to **3** directly (without purification) treating with (±)-1,2,4,5,6-penta-*O*-benzyl-*myo*-inositol[8] and pyridinium tetrafluoroborate[9] in acetonitrile/THF 1:1 for 5 h, followed by an excess of S_8 and pyridine. The resulting phosphodithionate **3** was deprotected using sequentially trimethylamine in benzene for 24 h (to demethylate phosphorodithionate function) and, after purification by column chromatography on silica gel eluted with $CHCl_3$/MeOH 95:5 (yield 25% calculated with respect to pentabenzylinositol), debenzylated using BF_3-etherate in ethyl mercaptane[10] for 1 h. The dithionate **4** was purified by column chromatography on silica gel eluted with $CHCl_3/MeOH/H_2O$ 65:35:5 (yield 58%)[11].

Compound **4** is a mixture of four stereomers being chiral at C(1) of inositol and at phosphorus. The presence of the 50% *R* and *S* isomers at phosphorus was shown by the occurrence of two equal-intensity

Table I

	Blank	10^{-6} M	$4*10^{-6}$ M	10^{-5} M	$4*10^{-5}$M	10^{-4} M	$4*10^{-4}$ M	10^{-3} M
1 h	14.7	14.0	13.3	13.3	15.5	18.4	48.2	48.9
2 h	16.5	18.2	16.8	16.6	17.0	22.7	45.9	52.52
24 h	26.9	27.2	22.5	23.3	24.4	40.0	71.1	96.2

Cytotoxic activity of (±)-octadecylphosphodithionyl-1-*myo*-inositol (4) on K562 erytroleukaemia cell line. Results are expressed as percentage of dead cells.

^{31}P NMR resonances at 71.48 and 67.50 ppm (external reference H_3PO_4), but the product gives only one spot by TLC.

It is known that, PI-PLCs (*B. cereus* and *B. thuringiensis*) are essentially stereospecific for D-*myo*-inositol stereomers, while there is no appreciable binding of the L enantiomer to the active site[12]. Furthermore, R_P stereomers of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoinositol are the preferred substrate for both bacterial and mammalian PI-PLCs[13]. Nontheless, due to the difficulty in resolving the mixture, compound **4** was tested as such.

The cytotoxic activity was measured by the following assay: cells (K562 erytroleukaemia cell line[14]) were washed and suspended to the final concentration of 10^6/mL in a medium containing 5% (v/v) foetal calf serum (FCS). (±) Octadecylphosphodithionyl-1-*myo*-inositol (**4**) was diluted from a 2 mM stock solution containing 5% (v/v) FCS and 0.2% (v/v) dimethylsulphoxide (DMSO); when necessary, to enhance solubility, solutions were treated with short sonication pulses. 0.2% DMSO was added to controls. Compound **4** was added to a final concentration ranging from 10^{-6} to 10^{-3} M, and the test was carried out in a humidified atmosphere with 5% (v/v) CO_2 at 37 °C. Cell death was visualised by adding 50 μM propidium iodide (Sigma, USA) to 100 μL aliquots of unfixed cells. The 560-580 nm fluorescence of the dead cells, excited at 490-495 nm, was monitored on a FACS-analizer flow cytofluorometer (Becton Dickinson, CA, USA) using a Consort 30 programme. About 10.000 events were collected for each point, at 1, 2, 24 hours. The results are shown in Table I.

To investigate if the citotoxicity is due to PI-PLC inhibition, we tested compound **4** on PI-PLC from human platelets.

The inhibition assay mixture (0.3 mL total volume) contained: Tris-Maleate (pH 6.5) 50 mM; $CaCl_2$ 1 mM; L-3-phosphatidyl(2-^{3}H)inositol (10-15 Ci/mmol) 100 nCi; L-3-phosphatidylinositol from bovine liver 0.3 mM; the concentrations of the inhibitors were comprised between 10^{-4} M and 10^{-5} M. Before starting the reaction, substrate or substrate and inhibitor in the buffer were sonicated for 5 min at r.t. in an ultrasonic Uniset-AC AGE. The reaction was then started by the addition of 30 μL of human platelet lysate, containing an amount of PI-PLC which proved able to hydrolyze 0.885 nmoles of the above substrate in one minute. After 20 min at 37 °C the reaction was stopped by addition of 1 mL of a mixture of $CHCl_3$/MeOH/H_2O (100:100:0.6) and 0.3 mL HCl 1 N. A portion of the aqueous phase (0.2 mL) was taken and counted in a liquid scintillation analyzer Tricarb 2260XL (Packard).

Compound **4** gave 50% inhibition at 10^{-4} M, while at the concentration $5*10^{-5}$ M the inhibition was only of 15%.

It is not proved which of the isomers are responsible for the observed effects. However these results suggest a certain relationship between cytotoxicity and enzymatic inhibition, in fact compound **4** causes 40% of cellular death *in vivo* at the same concentration giving 50% PI-PLC inhibition *in vitro*. Since the analogue hexadecyl derivative proved to be a poor substrate for *Bacillus cereus* PI-PLC[7], it is very likely that also compound **4** lowers the activity of human platelets PI-PLC by competition, being a poor substrate for the enzyme rather than being a true inhibitor[13]. Nevertheless we cannot exclude that the cytotoxicity may be due to other activities of the compound.

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