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SYNTHESIS OF TRITIATED 1-OCTADECYL-PHOSPHOTHIOLYL-*myo*-[1-^{3}H]-INOSITOL. A NEW INHIBITOR OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE-C[#]

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SUMMARY

The title *myo*-[1-^{3}H]-Inositol derivative was synthesized and characterized by tritium-N.M.R.. The unlabelled compound proved a potent inhibitor of the cellular key enzyme phosphatidylinositol-specific phospholipase-C (PI-PLC) involved in the production of intracellular second messengers and its tritium derivative was necessary in order to fully understand the mechanism of inhibition.

Key words: Tritium, *myo*-[1-^{3}H]-Inositol, Phosphatidylinositol-specific phospholipase-C inhibition, Tritiated inositol, NaBTx.

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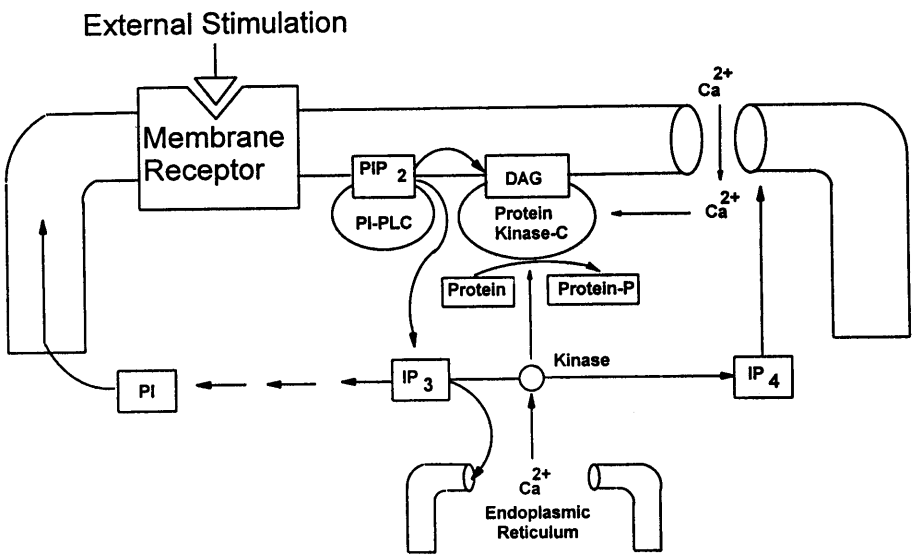
Introduction

Many cellular processes such as metabolism, excitation and cell growth are evoked by extracellular signals which give rise to a cascade of reactions. At first, external messengers such as hormones, growth factors and neurotransmitters bind to receptors on the external surface of a cell [1] .

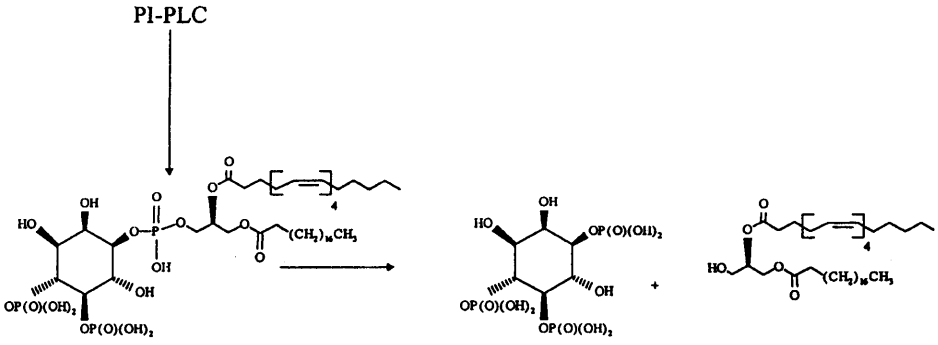
This leads to the production of active second messengers, including cAMP, cGMP, and the more recently identified diacylglycerol (DAG) and inositolphosphate (IP) [2]. Second messengers activate intracellular reactions leading to the production of the ultimate cellular effect. DAG and *myo*-inositol-1,4,5-triphosphate (IP_3) are the second messengers produced by the action of phosphatidylinositol specific phospholipase-C (PI-PLC) (Fig.1).

Fig.1



Phosphatidyl-inositol specific phospholipase-C (PI-PLC) [E.C. 3.1.4.10] [3] is an intracellular enzyme which catalyses the cleavage of cell-membrane phosphatidylinositol lipids. In mammalian cells PI-PLC occurs as a family of about fifteen isoenzymes of rather different primary structure and size ranging between 60 and 150 KDa. PI-PLC can be found either as a membrane-associated or as a cytosolic enzyme. Its activation leads to the hydrolysis of phosphatidylinositol-4,5-diphosphate and thus to DAG and IP_3 (Fig.2).

Fig.2



The formation of DAG is involved in the activation of protein-kinase-C, IP_3 mediates the release of calcium ions from intracellular stores. These events eventually lead to mitogenesis. Therefore inhibitors of PI-PLC are sought for, for example, as potential inhibitors of cell growth in cancer theraphy. DAG is also a substrate for phospholipase-A_2, which thus produces a number of biologically important molecules named eicosanoids, which include leukotrienes, prostaglandins and thromboxanes, involved in the activation of many cellular events of both physiological and pathological nature. For example, specific inhibitors of PI-PLC have enormous therapeutic potential especially as anti-inflammatory agents. It is therefore apparent the importance of a study of the PI-PLC regulation, through the action of drugs.

To date, several non-specific PI-PLC inhibitors are known and only lately the synthesis of some PI and PIP_2 analogues, as inhibitors of PI-PLC, has been accomplished **[4]**.

Along with this line, some of us recently synthesized **[5]** a few thiophosphate analogues (C-S-P bond) of phosphatidylinositol as inhibitors of PI-PLC. Among these, one of the most active proved to be the unlabelled title compound.

Concerning the mechanism of inhibition, it has been hypothesized that these compounds lower the activity of PI-PLC by competition with the substrate, being substrate analogues, rather than being

true inhibitors. This was shown to occur for the analogue hexadecyl derivative with respect to *Bacillus Cereus* PI-PLC [6].

In our case the usual procedure involving colorimetric methods [5] was not applicable, since we had the opportunity to use PI-PLC contained in a lysate from human platelets. Therefore it was necessary to prepare the tritiated title compound. Its use in the inhibition tests could permit a deeper investigation into the mechanism of inhibition, verifying whether the inhibitor undergoes enzymatic hydrolysis.

Results and Discussion

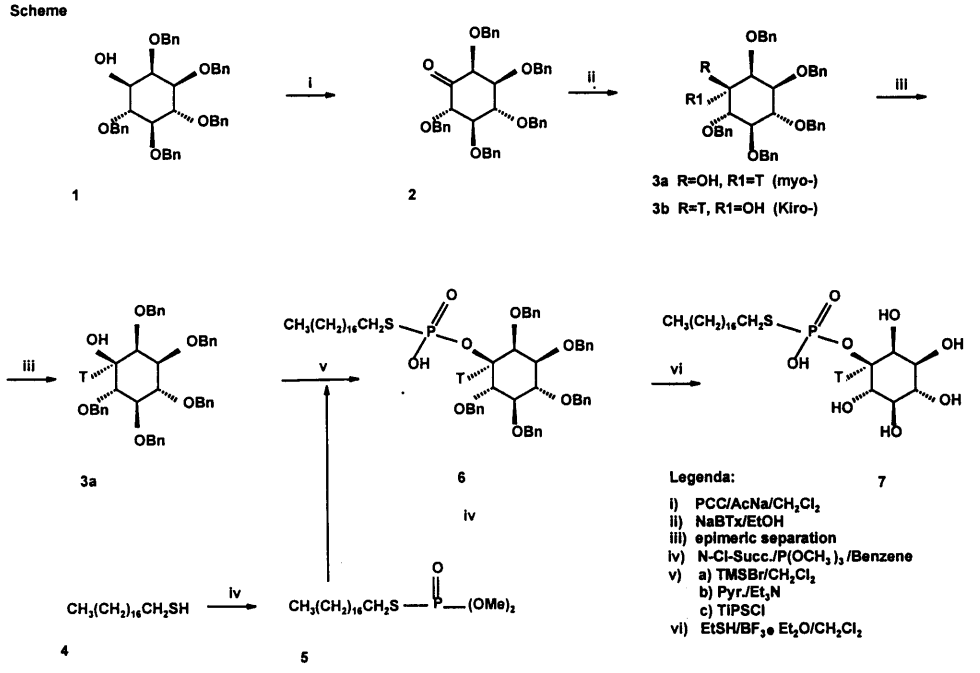
To prepare the labelled title compound, we decided first to label the inositol moiety and then to introduce the octadecylphosphothiolyl group by the well established synthetic route used for the unlabelled compound [5].

It is possible to find in literature some syntheses of tritium labelled *myo*-inositols [7-10]. All these syntheses have as final goal the preparation of the corresponding 1,4,5,-triphosphate inositol derivatives and some of them have a fluorine [9,10] or an azido [8] group on the inositol framework. Generally, it is mandatory the protection of the hydroxyl groups for the introduction of the tritium. Taking in account the peculiarities of the phosphothiolyl bond, characterizing the title compound (7), we chose, on the basis of our previous synthetic experimental data [5], the pentabenzyl as the better hydroxyl group protection during the radiosynthetic route (see Scheme),.

In all cases, the introduction of tritium is accomplished by reduction of the corresponding inosose derivatives (see Scheme, compd. 2) with NaBTx, obtaining a mixture of labelled epimeric alcohols. Irrespective of the protection groups, the labelled *myo*-inositols were obtained in epimeric excess with respect to the *scyllo* (*kiro* in our case)-inositols (generally 3:1) and recovered in pure form by silica-gel column chromatography.

Our radiosynthesis (Scheme) started with the reduction by NaBTx (620 mCi, 42.9 Ci/mmol), in absolute ethanol for one hour, of the 2,3,4,5,6-penta-O-benzyl-inosose (2), prepared according to

Scheme



Corey [**11**] from the corresponding 1,2,4,5,6-penta-O-benzyl-*myo*-inositol (1). This pentabenzyl derivative was prepared following the Gigg's method [**12**].

To achieve complete reduction of fully protected inosose (2), without detriment to the transfer of tritide (i.e. the best radiochemical yield) we decided to use a slight excess of NaBTx.

The epimeric alcohols so obtained (3) were diluted with cold 1,2,4,5,6-penta-O-benzyl-*myo*-inositol (1) (10 mg) and purified on a silica gel column (hexane/AcOEt). The protected tritiated *myo*-inositol (3a) was so recovered (13 mg; 52.87 mCi) as a white solid and diluted with 77 mg of cold compound (1) (final specific activity after the dilution: 364 mCi/mmol). This compound (3a), in anhydrous pyridine, was coupled with pre-formed dimethyloctadecylphosphothiolate (5) [**5**], in the presence of anhydrous triethylamine and triisopropylbenzenesulphonylchloride (TiPSCl). The mixture so obtained was stirred at room temperature overnight and then was quenched with water and methanol.

The solvents were evaporated under high vacuum and the recovered red oil was purified by column chromatography on deactivated (15% water w/w) acidic silica gel with a step elution from net

$CHCl_3$ to 95:5 $CHCl_3/CH_3OH$ to yield (80%) of octadecylphosphothiolyl-2,3,4,5,6-penta-O-benzyl-*myo*-[1-^{3}H]-inositol (6) (113 mg; 40.3 mCi; 350.4 mCi/mmol).

Because of the structural limitations of (6), the Fujita's method **[13]** was found to be the best one to cleave the benzylic protection groups in the presence of a phosphothiolyl moiety, with respect to other known benzylic deprotection methods such as catalytic hydrogenolysis or reductive cleavage with sodium in ethanol or in liquid ammonia.

Following the Fujita's method **[13]**, to a solution of the protected tritiated compound (6), in anhydrous CH_2Cl_2, ethylmercaptan and borontrifluoride etherate complex were added. The mixture was stirred at room temperature until disappearance of the substrate (6), checked by TLC. Water was added and the mixture was extracted with diethylether. The aqueous phase was lyophilized to yield the 1-octadecylphosphothiolyl-*myo*-[1-^{3}H]-inositol (7) (61 mg; 39.3 mCi; 341.7 mCi/mmol). Total radiochemical yield, referred to the starting compound (3a) was about 50%.

The title compound (7) was fully characterized by ^{1}H-NMR and also by tritium-NMR analysis, to check the position of the tritium.

In Fig.3 we report a comparison between the proton and the tritium-NMR spectra in the range of interest.

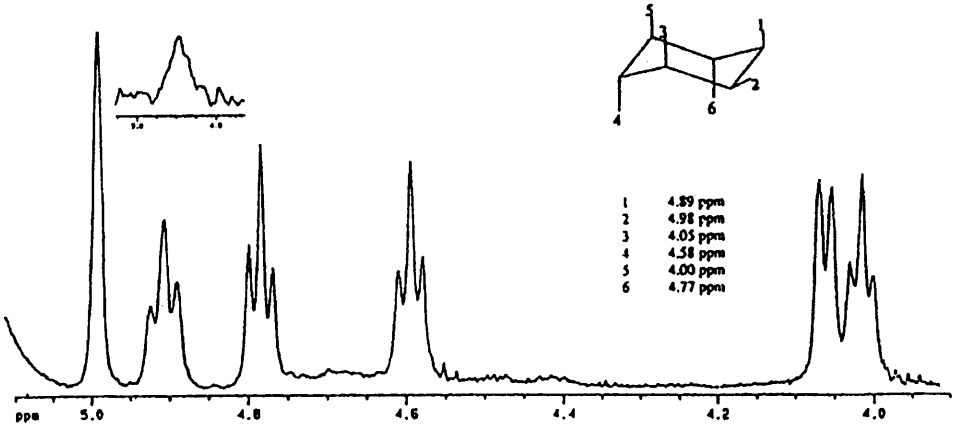


Fig.3 ^{1}H-NMR 600 MHz spectrum of the title compound. The insert shows the only signal in the ^{3}H-NMR spectrum

Preliminary tests, carried out *in-vitro* on PI-PLC from human platelets, show that 1-octadecylphosphothiolyl-*myo*-[1-^{3}H]-inositol (7) is not hydrolyzed by the enzyme, suggesting it to act as a competitive reversible inhibitor of this enzyme, acting as a resistant substrate analogue. Further studies are in progress.

Experimental part

Materials.

- Sodium borotritide was obtained from Amersham with a specific activity of 42.9 Ci/mmol.
- The 2,3,4,5,6-penta-O-benzyl-inosose (2), the cold 1,2,4,5,6-penta-O-benzyl-*myo*-inositol (1) and the dimethyloctadecylphosphothiolate (5) were provided by Mediolanum Farmaceutici S.p.A..
- All the chemicals, anhydrous solvents or chromatographic solvents were of the highest chemical purity or HPLC- grade and furnished by Fluka or Aldrich. Water was Milli-Q®-grade.
- Thin Layer Chromatography (TLC) plates (Silica gel 60 F254) and Silica gel 60 (70 -230 mesh) for column chromatography were purchased from Merck.
- Radioactive samples were counted using a Packard Tri-Carb 2200 liquid scintillation counter.
- In the synthetic description, the correct IUPAC numbering for the hexitol rings is used, in which numbering priorities change during the synthesis [**14**].

Preparation methods

2,3,4,5,6-penta-O-benzyl-*myo*-[1-^{3}H]-inositol (3a).

To a solution of 2,3,4,5,6-penta-O-benzyl-inosose (2) (6 mg; 9.5 μmol) in 0.5 mL of absolute EtOH was added NaBTx (620 mCi; 42.9 Ci/mmol) dissolved in the same solvent (2 x 0.5 mL). The mixture was stirred, under N_2 and at R.T., for one hour and checked by TLC (8:2 hexane/AcOEt; r.f.=0.2 for the inosose) until the starting compound was completely reduced. Then water was added (1.5 mL) and the mixture extracted with $CHCl_3$ (3 x 1.5 mL).

To the overall organic solution (137 mCi, with likely water traces), cold 1,2,4,5,6-penta-O-benzyl-*myo*-inositol (1) (10 mg, 0.016 mmol) were added and the solution dried over anhydrous Na_2SO_4. After the desiccant filtration, the solution was evaporated to dryness *in-vacuo* and the solid so obtained was purified by column chromatography (8:2 hexane/AcOEt).

We recovered 13 mg of pure 2,3,4,5,6-penta-O-benzyl-*myo*-[1-^{3}H]-inositol (52.87 mCi) (3a) as a white solid to which further 77 mg of cold (1) were added (overall 90 mg, 364 mCi/mmol).

1-octadecylphosphothiolyl-2,3,4,5,6-penta-O-benzyl-*myo*-[1-^{3}H]-inositol (6).

Trimethylsilylbromide (64 μL; 0.49 mmol) was added, under N_2, to a solution of dimethyl-octadecylphosphothiolate (64 mg; 0.162 mmol) in anhydrous CH_2Cl_2 (0.43 mL) and the mixture was stirred until the phosphothiolyl disappeared (about one hour; TLC-check, $CHCl_3$). The solvent was evaporated to dryness *in-vacuo* and the previously prepared solution of 2,3,4,5,6-penta-O-benzyl-*myo*-[1-^{3}H]-inositol (3a) in anhydrous pyridine (3 x 0.5 mL), anhydrous triethylamine (0.11 mL) and triisopropylbenzenesulfonylchloride (161 mg; 0.53 mmol) was added to the crude red oil. The mixture so obtained was left at R.T. , under N_2 , for 24 hours. Water (0.5 mL) and MeOH (1.0 mL) were added and the mixture was stirred for 30 min. After evaporation to dryness the residue, a dark red oil, was dissolved in $CHCl_3$ (5 mL) and purified by column chromatography (silica gel washed with 1N HCl, dried and deactivated with 15% w/w of water) eluting with $CHCl_3$ and after the pyridine elution, with 95:5 $CHCl_3/CH_3OH$. By TLC-check of the eluted fractions (5 mL), we recovered, after evaporation, pure 1-octadecylphosphothiolyl-2,3,4,5,6-penta-O-benzyl-*myo*-[1-^{3}H]-inositol (6) (113 mg; 0.115 mmol; 40.3 mCi; 350.4 mCi/mmol. Chemical yield: 80%).

1-octadecylphosphothiolyl-*myo*-[1-^{3}H]-inositol (7).

To a solution of the obtained 1-octadecylphosphothiolyl-2,3,4,5,6-penta-O-benzyl-*myo*-[1-^{3}H]-inositol (6) in anhydrous CH_2Cl_2 (2 mL) , under N_2 , ethylmercaptane (2 mL) and borontrifluoride

etherate complex (0.45 mL) were added and the mixture was stirred at R.T. until the starting protected inositol (6) disappeared (TLC-check; 65:35:5 $CHCl_3/CH_3OH/H_2O$). Then water (2 mL) was added and the mixture was extracted with Et_2O (3 x 2 mL). The aqueous phase was lyophilized to give a white foamy solid of 1-octadecylphosphothiolyl-*myo*-[1-^{3}H]-inositol (7) (61 mg; 0.115 mmol; 39.3 mCi; 341.7 mCi/mmol. Chemical yield: ~100%).

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