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SYNTHESIS AND EVALUATION OF 1-POSITION-MODIFIED INOSITOL 1,4,5-TRISPHOSPHATE ANALOGS

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Abstract: IP_3 analogs were synthesized by the modification of phosphate at the 1-position, and their affinity for the IP_3 receptor was analyzed by means of surface plasmon resonance measurements. Our results suggest that a hydrophobic and charged moiety linked to this position enhances the affinity for the IP_3 receptor.

Introduction

D-*myo*-Inositol 1,4,5-trisphosphate (IP_3) acts as a second messenger to induce the release of Ca^{2+} from Ca^{2+} stores upon binding to IP_3 receptors (IP_3R) and regulates the dynamics of intracellular Ca^{2+} concentration in many types of cells. Thus, IP_3 plays an important role in the regulation of many cellular functions, such as secretion, fertilization, muscle contraction, neuronal signaling and cell growth.[1,2] Many IP_3 analogs have been synthesized[3,4] for the study of IP_3-induced signal transduction[5,6] and the structures of IP_3R and other IP_3-binding proteins have been investigated.[7] Dye-labeled compounds have proven to be useful tools for visualization of the distribution and dynamics of cellular signaling molecules.[8,9] Such dye-labeled IP_3 analogs can be useful for the study of IP_3 chemistry and IP_3-induced signal transduction, as well as for other purposes. Here we report the synthesis in 15 steps from DL-*myo*-inositol of optically active IP_3 analogs modified at the phosphate at the 1-position with one of two dye molecules, fluorescein and malachite green. Evaluation of their binding affinity to IP_3R showed that they are highly potent ligands. It appears that a hydrophobic and charged moiety linked to phosphate at the 1-position enhances the binding of IP_3 analogs to IP_3R.

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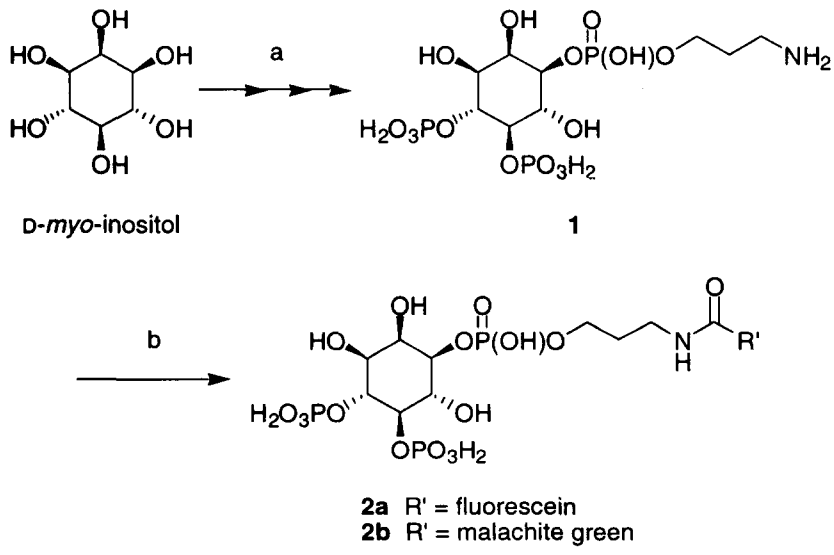
Synthesis

Since modifying phosphate at the 1-position of IP_3 has been reported to have little effect on the binding affinity to IP_3R,[10,11] we chose 1D-1-*O*-(3-aminopropyl-1-phospho)-*myo*-inositol 4,5-(bis)phosphate (**1**) as an optically active intermediate,[12] which can be coupled with many molecules at the phosphate-linked amine. We synthesized **1** according to the literature.[11,13-15] Fluorescein and malachite green were chosen because they can be excited by long-wavelength light and are appropriate for use with biological samples. The product of acidic condensation of 4-carboxybenzaldehyde (1 eq.) and dimethylaniline (2 eq.) was acylated with N-hydroxysuccinimide (NHS) in the presence of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Next oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) gave 4-carboxymalachite green-*N*-hydroxysuccinimide ester (CMG-SE) in >1.7% yield. After an addition of 5-carboxyfluorescein-*N*-hydroxysuccinimide ester (CF-SE, Research Organics, Inc.) or CMG-SE to **1** in 0.1 M $NaHCO_3$ buffer (pH 8.3), the mixture was stirred at room temperature for 2-3 hours to yield **2a** or **2b**, respectively. CMG-SE, **2a** and **2b** were purified by C_{18} reversed-phase HPLC.

The optical purity of IP_3 analogs was assessed by measurement of the specific rotation of 1D-4,5-di-*O*-allyl-3,6-di-*O*-benzyl-1-l-menthoxyacetyl-*myo*-inositol (an intermediate of **1**).[13,16] The synthetic compounds were all characterized by ^1H NMR and MS or FABMS.[16] The synthetic scheme is summarized in scheme 1.

Scheme 1



(a) 14 steps[11, 13-15] (b) R'-*N*-hydroxysuccinimide ester, DMF, 0.1 M $NaHCO_3$, under Ar, dark, r.t., 2-3 hours

Binding assay

Expression and purification of human IP_3 receptor type 1

The IP_3-binding domain (IBD, amino acid residues 1-885)[17] of human IP_3 receptor type 1[18] was subcloned into pGEX2T (Amersham Pharmacia) for bacterial expression. The N-terminus of IBD was fused to glutathione S-transferase with a linker sequence, Leu-Val-Pro-Arg-Gly-Ser, while the C-terminus of IBD was His-tagged with a linker sequence, Leu-Val-Pro-Arg-Gly-Ser. After expression in *E. coli*, BL-21 (DE3), the recombinant protein was purified using an Ni-NTA-immobilized resin and HiTrap NHS-activated affinity column (both from Amersham Pharmacia) conjugated with **1**. The purity of the protein was estimated to be 22% from SDS-PAGE.

Measurements of surface plasmon resonance

Measurements of surface plasmon resonance (SPR) were carried out using a BIAcore 2000 (Amersham Pharmacia). After activation with NHS and EDC, the surface of a CM5 sensor chip was coupled with 10 mM **1** in 100 mM borate buffer (pH 8.5), followed by inactivation with ethanolamine. In order to evaluate the dissociation constants of IP_3, **1**, **2a** and **2b**, previously purified IBD was dissolved in the running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA and 0.005% Tween 20 at pH 7.4) with the respective compounds. The chip surface was regenerated with 100 mM NaOH after sample injection.

Results and Discussion

Injections of various concentrations of IBD or bovine serum albumin (BSA, Fig. 1A) confirmed that the **1**-immobilized sensor chip was suitable for our assays, because IBD bound to the chip in a concentration-dependent manner, while no non-specific binding was found with BSA (or glutathione S-transferase, not shown). In order to measure the affinity of IP_3, **1**, **2a** and **2b** for IBD, the following competition assays were conducted. Aliquots of IBD (52.8 nM) were mixed with various concentrations of one of the compounds. When injected onto the chip, the compounds competed with **1** immobilized on the chip surface for binding to IBD (Fig. 1B). From these assays, the dissociation constants (K_d) of the compounds were calculated by fitting the obtained SPR to an equation.[19] The K_d of **2b** could not be calculated accurately, because it was much lower than half the concentration of IBD. Therefore we reduced the IBD concentration to 1.76 nM and conducted the competition assay of **2b** again (Fig. 1C). We carried out the binding assays under light and dark conditions, and found that the binding was not affected by light. In order to assess non-specific binding of **2b** to IP_3R, carboxymalachite green (CMG, hydrophobic moiety of **2b**) was injected in the competition assay (Fig.

1B,1C). The result showed that non-specific binding was not observed and thus, **2b** was specifically bound to IP_3R.

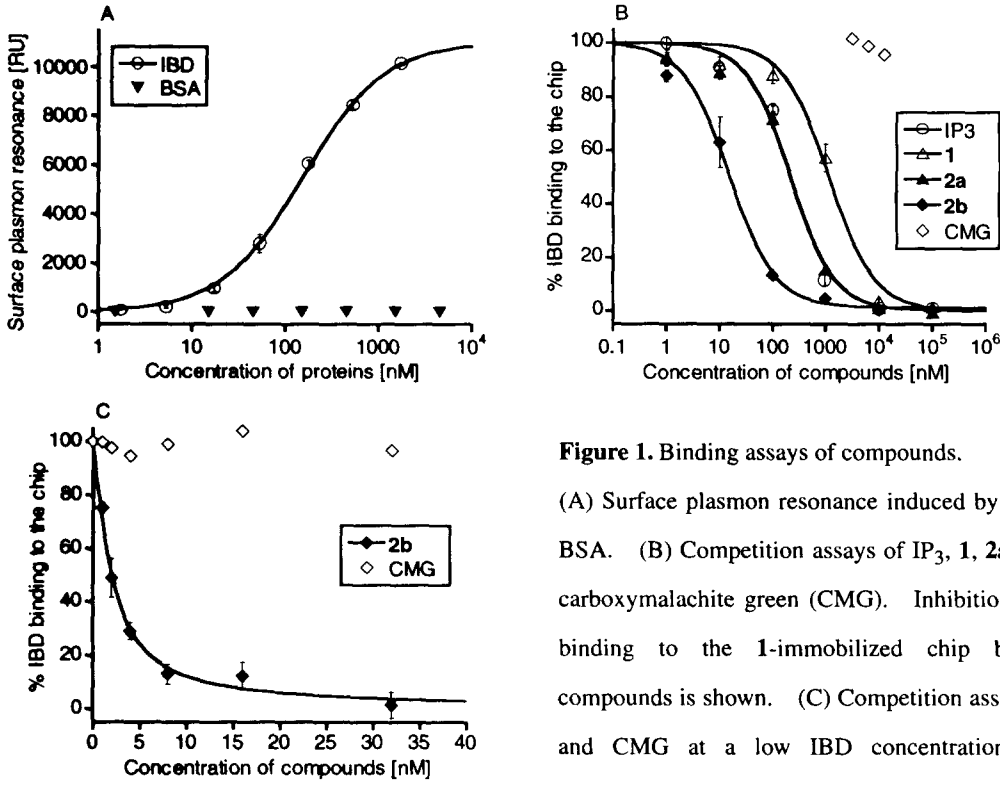


Figure 1. Binding assays of compounds.

(A) Surface plasmon resonance induced by IBD and BSA. (B) Competition assays of IP_3, **1**, **2a**, **2b** and carboxymalachite green (CMG). Inhibition of IBD binding to the **1**-immobilized chip by these compounds is shown. (C) Competition assays of **2b** and CMG at a low IBD concentration. IBD concentration was 52.8 nM (B) or 1.76 nM (C).

Table 1. Dissociation constants of compounds

Compounds	K_d [nM]
IP_3	195
1	1116
2a	188
2b	1.17

It should be noted that although **1** was about 6-fold less potent than IP_3,[11] **2a** was equipotent to IP_3 and **2b** was 167-fold more potent than IP_3 (Table 1). Among many IP_3 analogs so far reported, **2b** possesses the highest affinity for IP_3R. Only adenophostins, metabolites isolated from the culture broth of *Penicillium breviocompactum*, are comparably potent agonists of the IP_3R.[20] Our results show that linking dye molecules to the phosphate at the 1-position of **1** enhances the binding affinity of the IP_3 analogs to IBD. Since the dye molecules carry a hydrophobic moiety, we speculate that it might interact with a hydrophobic region near the IP_3 binding site to stabilize the binding of the IP_3 analogs to IBD. Since **2b** has a higher affinity than **2a**, there could also be an electrostatic interaction, in favor of cationic ligands, between the IP_3 analogs and IBD.

In conclusion, the novel IP_3 analog, **2b**, was found to be a highly potent ligand of IP_3R, and could be an important lead compound for the design of high-affinity IP_3 analogs and for the study of IP_3-binding molecules.

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16. Spectral data of selected compounds[21] : 1D-4,5-di-*O*-allyl-3,6-di-*O*-benzyl-1-l-menthoxyacetyl-*myo*-inositol $[\alpha]_D^{25}$ = - 52.5 (c 2.2, $CHCl_3$) : CMG-SE ^1H NMR ($CDCl_3$) 2.96 (s, 4H), 3.37 (s, 12H), 6.96 (d, *J* = 8.79 Hz, 4H), 7.37 (d, *J* = 8.79 Hz, 4H), 7.49 (d, *J* = 8.22 Hz, 2H), 8.29 (d, *J* = 8.22 Hz, 2H) ; FABMS 470 $(M)^+$: **2a** ^1H NMR (D_2O) 1.88 (m, 2H), 3.46 (m, 2H), 3.58 (dd, *J* = 2.37, 9.78 Hz, 1H), 3.77 (t, *J* = 9.15 Hz, 1H), 3.87-4.00 (m, 4H), 4.14 (t, *J* = 2.19 Hz, 1H), 4.22 (dd, *J* = 8.79, 9.69 Hz, 1H), 6.96 (dd, *J* = 2.19, 9.24 Hz, 2H), 7.08 (d, *J* = 2.19 Hz, 2H), 7.29 (d, *J* = 9.15 Hz, 2H), 7.38 (d, *J* = 7.71 Hz, 1H), 8.07 (dd, *J* = 1.65, 7.95 Hz, 1H), 8.50 (d, *J* = 1.11 Hz, 1H) ; FABMS 836 $(MH)^+$: **2b** ^1H NMR (D_2O) 1.80 (m, 2H), 3.14 (s, 12H), 3.35 (m, 2H), 3.47 (dd, *J* = 2.58, 9.80 Hz, 1H), 3.69 (t, *J* = 9.54 Hz, 1H), 3.82-3.97 (m, 4H), 4.07 (t, *J* = 2.91 Hz, 1H), 4.21 (dd, *J* = 6.21, 9.54 Hz, 1H), 7.20 (d, *J* = 8.25 Hz, 2H), 7.32 (d, *J* = 8.79 Hz, 4H), 7.46 (d, *J* = 8.97 Hz, 4H), 7.59 (d, *J* = 8.43 Hz, 2H) ; FABMS 832 $(M)^+$
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