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SYNTHESIS AND BINDING PROPERTIES OF *MYO*-INOSITOL 4,5,6-TRIS(PHOSPHATE) AN ANALOGUE OF *MYO*-INOSITOL 1,4,5-TRIS(PHOSPHATE). CORRELATION WITH THE IONIZATION STATE OF THE MOLECULES

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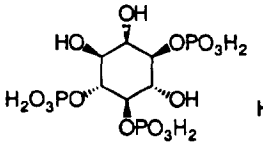
Abstract: *Myo*-inositol 4,5,6-tris(phosphate) is synthetized. The binding activity for this analogue with the CNS Ins(1,4,5)P_3 receptors is explained by taking into account steric modification and, the ionization state of the entire molecule, determined by potentiometric analyses, and the ionization state of each phosphate group determined by ^{31}P-NMR studies.

Myo-inositol 1,4,5-tris(phosphate) (Ins(1,4,5)P_3) **1** is an important second messenger. Its binding to specific receptors on the endoplasmic reticulum membrane induces the mobilization of intracellular calcium [1,2]. Published structure-activity relationships indicate that both phosphates in position 4 and 5 are of prime importance for the activity, while the presence of the phosphate in position 1 appears less important, playing an amplifying role[3].



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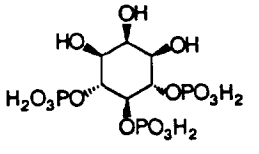

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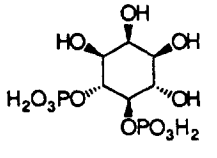

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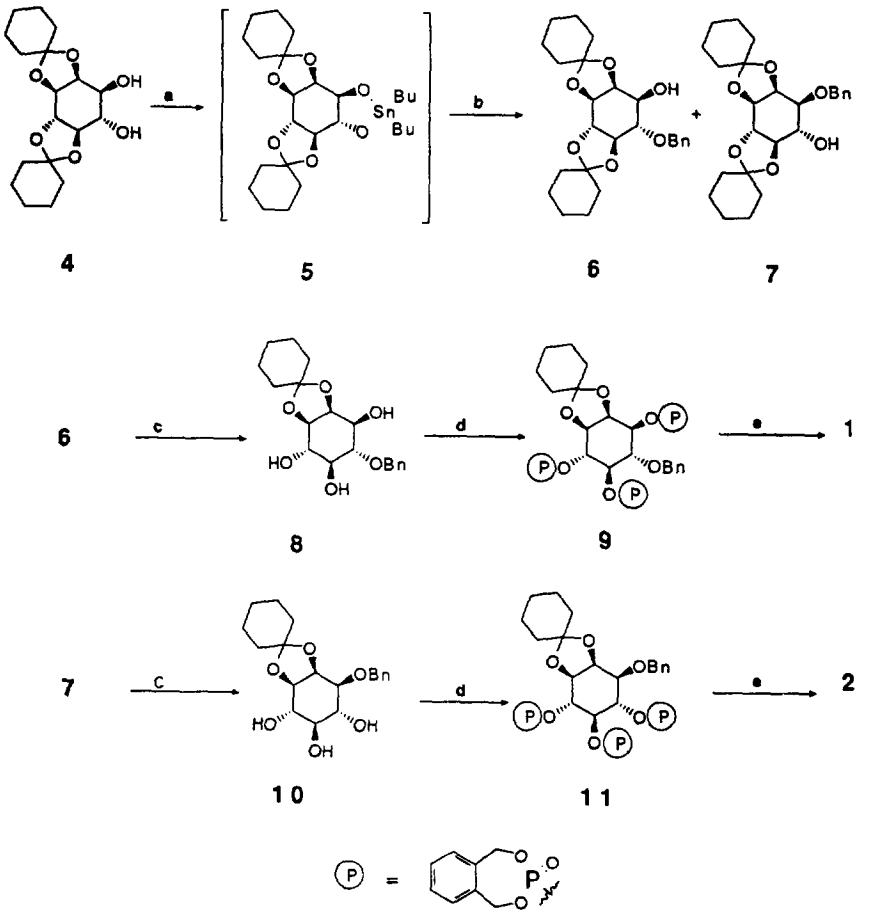
We report here the synthesis and the binding affinity of a new analogue of Ins(1,4,5)P_3 **1**, which retain the vicinal diphosphates in positions 4 and 5. The phosphate in position 1 was moved to position 6 to explore regioselectivity and the amplifier property of the phosphate in position 1. This modification led to Ins(4,5,6)P_3 **2**. The binding results are analysed in relation to the ionization state of the entire molecule and of each phosphate by means of potentiometric analysis and ^{31}P-NMR studies.

Scheme 1 shows the synthesis of Ins(1,4,5)P_3 **1** and Ins(4,5,6)P_3 **2**. The starting material was (±) 1,2-5,6-di-*O*-cyclohexylidene-*myo*-inositol (**4**) [4]. Monobenzylation of the diol **4** proceeded via the stannylidene



Scheme 1: a: Bu_2SnO; b: CsF, BrBn; c: ethyleneglycol, Amberlyst 77H; d: N,N,-diethylamino-1,5-dihydro-2,4,3-benzodioxaphosphepane, 1*H*-tetrazole, *m*CPBA; e: H_2, Pd/C.

intermediate **5**. Opening of the stannylene acetal with benzyl bromide, in the presence of two equivalents of cesium fluoride [5,6], led to a mixture of the monobenzylated derivatives **6** and **7** with compound **6** protected in position 4 predominating (70% and 20% for **6** and **7**, respectively). The major compound **6** was used for the preparation of Ins(1,4,5)P_3 **1**. Thus, the less stable *trans*-cyclohexylidene protective group in position 5 and 6

was selectively hydrolyzed by transacetalization using ethylene glycol [7], yielding the triol **8**. Treatment of the triol **8** with N,N-diethylamino-1,5-dihydro-2,4,3-benzodioxaphosphepane in the presence of tetrazole gave an intermediate tris(phosphite), which was oxidized *in situ* to phosphate **9** by means of *m*-chloroperbenzoic acid (*m*CPBA) [8]. The protected phosphate **9** was submitted to hydrogenolysis to remove the benzyl and the xylyl protective groups. The intrinsic acidity of the resulting phosphates catalyzed the hydrolysis of the remaining *cis*-cyclohexylidene group, giving the expected Ins(1,4,5)P_3 **1** (Scheme 1).

The minor compound **7** was used to achieve the synthesis of Ins(4,5,6)P_3. Selective removal of the *trans*-cyclohexylidene group gave the triol **10** which, after phosphorylation and deprotection as described above, gave racemic Ins(4,5,6)P_3 **2**. Te final products were converted to cyclohexylammonium salts to avoid any phosphate group migration or other side reactions (scheme 1).

The inositol analogue was tested on Ins(1,4,5)P_3 receptors prepared from rat brain cerebellum and purified according to Worley [9].

Table 1 reports the binding data observed for the synthesized compounds and for some reference molecules.

Nr	Compound	log K_y			IC_{50} (nM)
		y = 1	y = 2	y = 3	
1	Ins(1,4,5)P_3	7.85	6.40	5.31	10
1	Ins(1,4,5)P_3 [12*]				300
1	Ins(1,4,5)P_3 [12**]				200
2	Ins(4,5,6)P_3	9.81	7.00	5.22	9300
3	Ins(4,5)P_2 [12*]	7.44	5.28		20000
3	Ins(4,5)P_2 [12**]				50000

Table 1: Binding data and protonation constants for the synthesized inositol-phosphates and some reference compounds: *Swiss 3T3 cells; **Guinea pig hepatocytes, see reference 12. The standard deviation of the protonation constants is ± 0.01

For compound **2**, the binding activity stays around 10^{-5}M compared to 10^{-8}M for **1**. Such a loss of binding ability could be explained by the displacement of the phosphate from position 1 to position 6 on the inositol ring which could lead to steric hindrance inhibiting the binding to the receptors. An additional intramolecular hindrance could be due to the presence of two vicinal phosphates next to P5 which tends to lock

P5 in an unfavourable conformation. Furthermore, even if the presence of the phosphate in position 1 seems less important, displacing it to position 6 generates a compound unable to reach the recognition site of P1. As the binding activity declines towards that of Ins(4,5)P_2 3 one could conclude that the migration of the phosphate 1 to position 6 induces the loss of the amplifying property.

On the other hand, Worley *et al.* have shown that the affinity for the Ins(1,4,5)P_3 receptors increases with increasing pH (Figure 1)[9]. Taking into account the acidic functions of the ligand, it can be expected that the ionization states of the Ins(1,4,5)P_3 phosphate groups correlate with its binding affinity. Therefore, we performed some potentiometric studies to determine the potentiometric constants of inositol-phosphates (IP) in different media, particularly in a 0.2M KCl medium which roughly mimics the ionic strength of the cell. Thus, we have shown that the binding probably requires a totally deprotonated Ins(1,4,5)P_3[10]. In addition, ^{31}P-NMR studies indicated that the affinity seems related to the ionization state of the phosphate in position 5 of Ins(1,4,5)P_3 [11] (Figure 2).

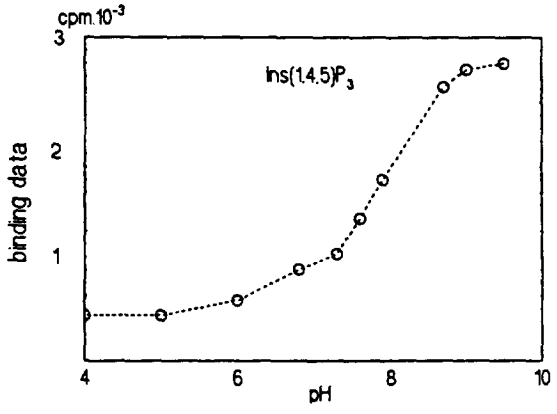


Figure 1: Variations of the affinity of Ins(1,4,5)P_3 1 versus pH according to Worley [9]

If the ionization state could control the binding affinity, it is worthwhile to consider the protonation constants of the new compound compared to those of Ins(1,4,5)P_3. Results of these potentiometric determinations are given in table 1 The difference between the logarithm of the first protonation constant of 2 and 1 is:

$$\Delta \log K_1 = 9.81 - 7.85 = 1.96$$

This indicates that at any pH, compound 2 is about 100 times more protonated than the parent compound 1. In the presence of 3 vicinal phosphates, the first proton is strongly stabilized by all the negative charges of the three phosphates, inducing a conformation of P5 which could differ from that of compound 1 [11]. The distribution curves of the protonated species of 1 and 2 clearly show that, at physiological pH, the relative amount of the monoprotonated species (HL) and the totally deprotonated one (L) markedly differ for these two inositols (Figure 2).

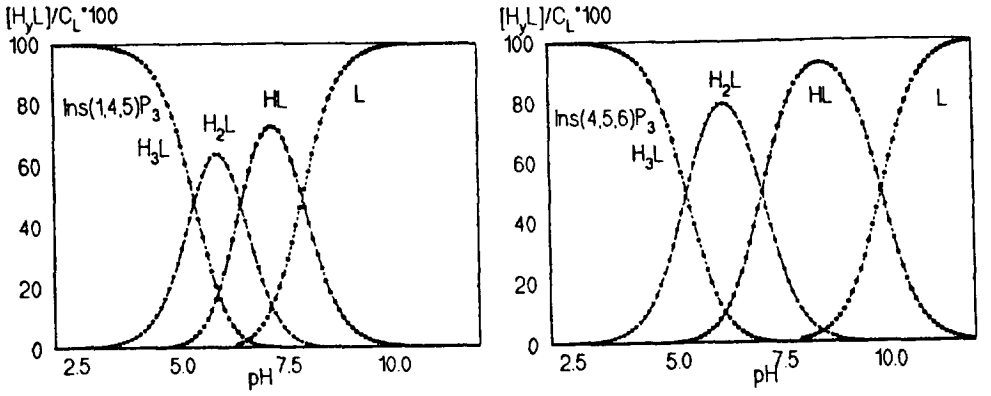


Figure 2: Species distribution of Ins (1,4,5)P_3 **1** and Ins(4,5,6)P_3 **2** vs pH

By considering the ^{31}P-NMR results (Figure 3), it can be seen that the phosphates behave very differently from one inositol to another. This is especially true for the phosphate in position 5 (P5), whose ionization seemed correlated with the binding properties [10].

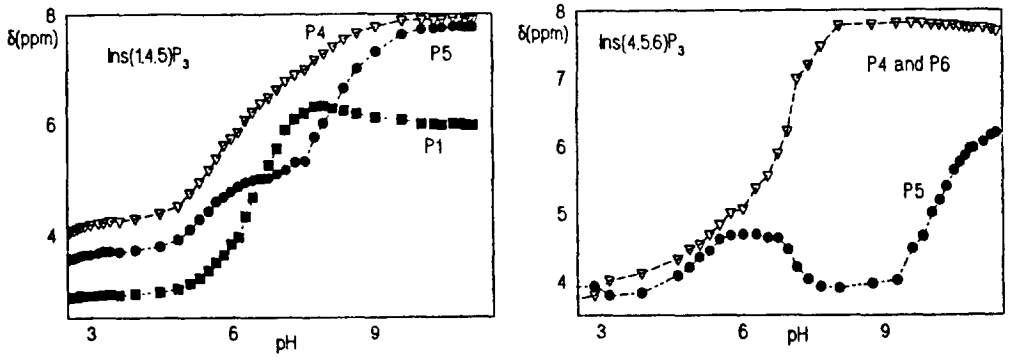


Figure 3: ^{31}P-NMR titration curves of Ins(1,4,5)P_3 **1**, and Ins(4,5,6)P_3 **2**, ,vs pH.

The NMR titration curves indicate that the large increase in basicity of the phosphate groups from **1** and **2** mainly affects P5 leading to an entirely monoprotonated phosphate at physiological pH. The removal of this proton appears to be much more difficult for **2** than for **1.**

More work is in progress to take in account physico-chemical studies such as potentiometric constants and potentiometric micro-constants for the interpretation of the structure-activity relationships in the field of inositol-phosphates.

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