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Inositol tripyrophosphate: a new membrane permeant allosteric effector of haemoglobin

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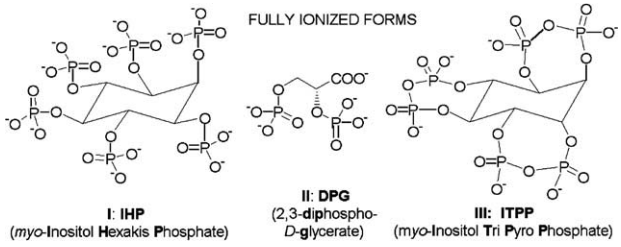
Abstract—Nine inositol tripyrophosphate (ITPP) salts have been synthesized. Their ability to act as allosteric effectors of haemoglobin (Hb) has been measured in vitro with free Hb and whole blood. All the synthesized compounds bound to free Hb and were also able to cross, to a certain extent, the plasma membrane of the red blood cells (RBCs) in whole blood samples, lowering the affinity of Hb for oxygen. The oxy-haemoglobin dissociation curves were significantly shifted towards higher values of oxygen partial pressures, both for free Hb and for intracellular Hb in whole blood.

Polyphosphorylated inositols, in particular inositol penta phosphate, are found in the erythrocytes of birds and reptiles.[1] *myo*-Inositol hexakis phosphate, (IHP), **I**, was the first inositol phosphate discovered in the seeds of green plants and is the most abundant inositol phosphate in the vegetable kingdom.[2] However, it was not until more than 60 years later that high performance liquid chromatography separation techniques revealed what has turned out to be the ubiquitous presence of IHP in animal cells.[3]

Haemoglobin (Hb) is a tetrameric protein that cooperatively binds four O_2 molecules and whose affinity for molecular oxygen is regulated in human red blood cells (RBCs), among other factors, by 2,3-diphospho-D-glycerate **II** (DPG), an allosteric effector of Hb. IHP may displace Hb-bound 2,3-DPG, binding to the allosteric pocket with higher affinity.[4,5] By binding to Hb, IHP triggers a decrease of the O_2/Hb affinity and subsequently leads, when loaded into circulating RBCs to increased, regulated release of oxygen upon tissue demand.[6] Physiological experiments on piglets, having received exchange transfusion with IHP-loaded RBCs, have shown significant beneficial effects of a reduction of the affinity of Hb to oxygen.[6,7] However, under physiological conditions, IHP bearing at least seven charges, is unable to cross the RBC's plasma membrane.

In the 1980s, IHP encapsulation in RBCs was achieved using a controlled lysis and resealing technique[7] and later on, by continuous flow electroporation.[8] We have recently reported a study of IHP uptake by RBCs using a two phase liquid distribution system involving IHP derivatives that would be soluble in both, aqueous and low polarity media.[9] In the course of investigating



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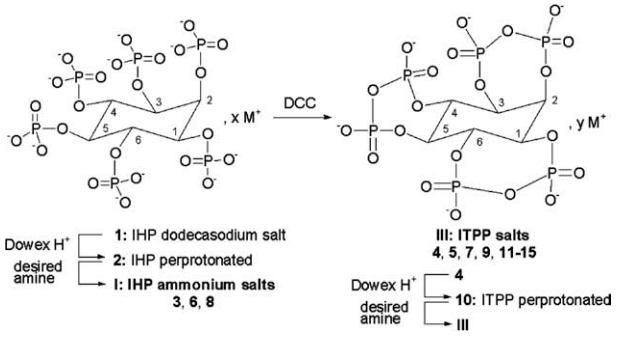
prodrug approaches for IHP delivery throughout lipid membranes, we have found that cyclic pyrophosphates were able to be delivered into RBCs despite the fact that they still bear a considerable amount of charge.

Our initial findings prompted us to consider the inositol tripyrophosphate (ITPP) **III** as an attractive candidate, and investigate its ability to act as an allosteric effector of Hb, in vitro. ITPP **III** (Na salt), was, for several years, the only known seven-membered cyclic pyrophosphate. The purpose of its synthesis was the identification via spectroscopic methods, of the original structure of phytic acid,[10] and it found no further use afterwards.

Taking into account our previous study[9] on the role of the counter cations associated with IHP, a number of novel ITPP salts have been synthesized, in order to investigate their efficiency for delivery of the highly charged ITPP inside the RBCs. The counter cations were mostly of a cycloalkyl structure, because these ammonium salts were found, through physicochemical partition coefficient studies to be the most efficient delivery systems for IHP, the precursor of ITPP. However, small, biologically important, cations like sodium and calcium could not be excluded, since they are predicted to be less toxic compounds for the oncoming in vivo experiments.

According to the first reported synthesis[10] IHP dodecasodium salt **1**, Scheme 1, had to be transformed via the perprotonated form **2** to the pyridinium salt **3** and then upon heating with DCC in pyridine ITPP pyridinium salt **4**, was formed. Addition of NaOH and recrystallization provided the ITPP sodium salt **5**. A modification of the procedure however, by changing the equivalents of reagents and the time of the reaction led to a more efficient conversion (77% yield). Furthermore, a passage of the pyridinium salt through a Dowex Na^+ column gave directly ITPP–Na **5**, in high purity and excellent yield.[11]



Scheme 1. Synthesis of tripyrophosphates of IHP. For compounds of the type **I**: **1**: $M^+ = Na^+$, $x = 12$; **2**: $M^+ = H^+$, $x = 12$; **3**: $M^+ = Py\ H^+$, $x = 4$; **6**: $M^+ = N,N$-DMCHA H^+, $x = 7$; **8**: $M^+ = TEA\ H^+$, $x = 6$. For compounds of the type **III**: **4**: $M^+ = Py\ H^+$, y = 5; **5**: $M^+ = Na^+$, $y = 6$; **7**: $M^+ = N,N$-DMCHA H^+, $y = 6$; **9**: $M^+ = TEA\ H^+$, $y = 6$; **10**: $M^+ = H^+$, $y = 6$; **11**: $M^+ = CHA\ H^+$, $y = 6$; **12**: $M^+ = COA\ H^+$, $y = 6$; **13**: $M^+ = PAZ\ H^+$, $y = 6$; **14**: $M^+ = PAZ\ H^+$, $y = 3$; **15**: $M^+ = Ca^{2+}$, $y = 3$. Py = pyridine, *N*,*N*-DMCHA = *N*,*N*-dimethylcyclohexylamine, TEA = triethylamine, CHA = cycloheptylamine, COA = cyclooctylamine, PAZ = piperazine.

Besides the improved synthesis of **5**, we have investigated more efficient routes to ITPP, via reactions with various IHP salts, that would lead to elevated yields and diminish the use of excess amount of base at the dehydration step. The route for the synthesis of several salts of structure **I** proceeds in a similar way to the pyridinium salt, **3**. Thus, conversion of **1** into compound **2**, finally leads to the corresponding IHP salt after reaction[9] with the proper amine, (Scheme 1). Among all the salts we have tried, the tertiary ammonium ones, like the *N*,*N*-dimethylcyclohexyl-ammonium salt **6**, and the triethylammonium salt **8**, led to reliable and reproducible results. In particular, an increased conversion of **6** or **8** occurred, under CH_3CN or CH_3CN/H_2O reflux in the presence of DCC affording compounds **7** and **9** in 82% and 94% yields, respectively. Additionally, a reaction mixture of DCC and **8** in $EtOH/H_2O$ resulted in the corresponding ITPP triethylammonium salt **9** (90%), indicating the propensity of IHP to form pyrophosphates. It is worthwhile to mention that DCC/ROH systems have been reported[12,13] to lead to alkyl esters of nucleoside phosphates and dinucleosides.

Each one of the above preparation procedures depended on the individual characteristics of the ammonium salts (most importantly solubilities) and the behaviour of DCC in the corresponding systems. However, all three ITPP salts, **4**, **7** or **9** could be converted into other ammonium salts by passing through ion-exchange columns. Representatively, we indicate the transformation of ITPP pyridinium salt **4** into the ammonium salts **7**, **9**, **11**–**14**, which also corresponds to the synthetic procedure used for the biologically tested compounds. According to the procedure depicted on Scheme 1, compound **4** was passed through an ion-exchange Dowex H^+ to give the perprotonated ITPP compound **10**, which was subsequently reacted with the proper amine without being isolated, due to its susceptibility to hydrolysis under acidic conditions.

This procedure has yielded, in quantitative yields and in high purity, seven novel ITPP salts of general structure **III**, bearing *N*,*N*-dimethylcyclohexyl-ammonium **7**, triethylammonium **9**, cycloheptyl-ammonium **11**, cyclooctyl-ammonium **12** and piperazinium **13**, as counter cations ($y = 6$). In the case of piperazine, the tripiperazinium salt **14** was synthesized as well. Addition of equimolar amounts of $CaCl_2$ to an aqueous solution of compound **5**, gave the calcium derivative **15**, which was obtained contaminated with NaCl, and tested without purification. All salts appear as racemic mixtures, due to the *meso*-form of the precursor IHP. Separation of the two enantiomers and evaluation of their biological activity will be presented in due time.

The ITPP uptake into RBCs can be determined indirectly by measuring the P_{50} value of intracellular Hb in the whole blood samples after incubation with ITPP (P_{50} = partial pressure of oxygen under which 50% of Hb is saturated with O_2). A shift of the dissociation curve to the right, that is, to the higher O_2 partial pressures, indicates a loss of affinity of Hb for O_2 due to the interaction with ITPP when compared to a control experiment performed without ITPP.

All nine compounds (sodium and pyridinium salts included) were examined in vitro with free Hb or whole blood, respectively. All samples were prepared according to standard procedures.[14] The results obtained with free Hb and whole blood from different species are summarized in Tables 1 and 2. The P_{50} control values of fresh Hb and fresh whole blood from all three species are significantly higher than the values for samples, which were stored before use, due to the loss of 2,3-DPG[15] from the allosteric pocket of Hb. The measurements listed in Tables 1 and 2 were mostly performed with older blood or free Hb; the P_{50} values of the control samples are therefore low.

Compounds **5** and **15**, distinguished only by their counter cation, sodium or calcium, did not show major differences in the capacity of shifting the P_{50} value. The ^{31}P NMR spectrum of the calcium salt **15** is markedly different from the spectra of all other salts (sodium as well as organic cations), which are rather similar. This is indicative of binding of the divalent calcium ions to the ITPP polyanion. Both compounds yielded the highest P_{50} shifts ever observed with whole blood. The reason why compounds **5** and **15** are more efficient in shifting the P_{50} value of whole blood than the other compounds tested, maybe due at least in part to the interaction of the sodium and calcium cations with the ITPP polyanion.

Comparing the P_{50} values in free Hb and whole blood of compound **5** and its precursor IHP, we point out that ITPP, **5**, with free Hb shows a shift of ~225%, and with whole blood a strong shift of ~39%. On the contrary, IHP (sodium salt) causes a strong shift of P_{50} in free Hb (up to 190%), but not in whole human blood (<2%). Representative Hb–O_2 dissociation curves are depicted in Figure 1a–d. Binding of ITPP to both, free Hb and RBCs while shifting the P_{50} values in the

Table 1. P_{50} values of free Hb after incubation with compounds **4**, **5**, **7**, **11**–**14** and **15**, in vitro

Compound	P_{50} (Torr) free Hb	P_{50} (Torr) Hb + compound	P_{50} increase (%) + SD
4	(H) 15.3	31.6	107 ± 22
	(M) 25.0	50.0	100 ± 18
5	(H) 15.3	49.8	225 ± 19
	(M) 24.9	69.7	180 ± 25
	(P) 22.0	68.1	209 ± 39
7	(M) 24.9	45.1	81 ± 15
11	(M) 24.9	43.8	76 ± 13
12	(M) 24.9	30.6	23 ± 5
13	(M) 23.4	67.7	189 ± 43
14	(M) 23.4	82.9	254 ± 49
15	(H) 12.3	33.1	170 ± 32
	(M) 26.9	61.9	130 ± 30

H = human; M = murine; P = porcine free Hb. Concentration of the compound solution was 60 mM. Means of P_{50} shifts in % are shown. SD = standard deviation. Compounds **4**, **7**, **11**, **12**, **14** and **15**: three P_{50} values each were used for the calculation of means; compound **5**: with human blood: five values, murine blood: ten values and porcine blood: three values were used for the calculation of the means of P_{50} shifts in %.

Table 2. P_{50} values of whole blood after incubation with compounds **4**, **5**, **7**, **11**–**14** and **15**, in vitro

Compound	P_{50} (Torr) whole blood	P_{50} (Torr) compound + whole blood	P_{50} increase (%) + SD
4	(H) 22.1	24.3	10 ± 4
	(M) 37.9	42.7	13 ± 2
5	(H) 22.1	30.8	39[a] ± 5
	(P) 31.6	44.2	40[a] ± 3
	(M) 36.7	47.4	29[b] ± 3
	(M) 40.1	52.0	30 ± 3
7	(M) 37.9	45.5	20 ± 2
11	(M) 37.9	41.3	9 ± 1
12	(M) 37.9	41.7	10 ± 2
13	(M) 39.2	41.9	7 ± 1
14	(M) 39.2	42.3	8 ± 2
15	(H) 24.8	31.0	25 ± 3
	(M) 40.1	55.3	38[a] ± 4

H = human; M = murine; P = porcine whole blood. Compound concentrations: 30 mM; means of (four single values) P_{50} shifts + SD are shown.

[a] Compound concentration: 60 mM.

[b] Compound concentration: 4 mM.

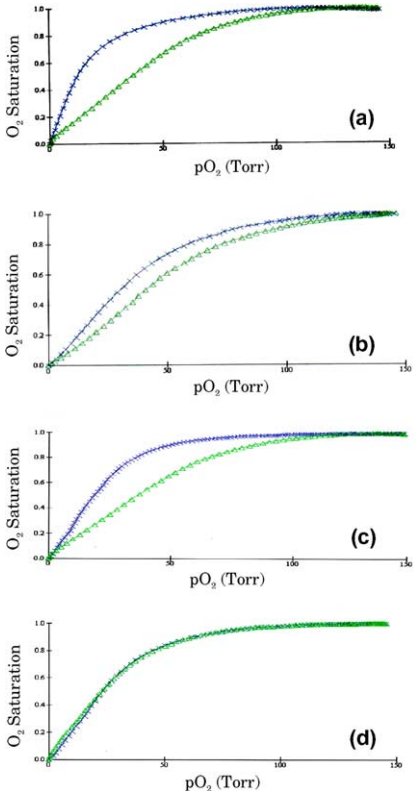


Figure 1. (a) Hb–O_2 dissociation curves: blue (×): free human Hb (2.5 mM), P_{50} = 13.03 Torr, green (Δ): free human Hb + 60 mM **5**, P_{50} = 36.51 Torr (ΔP_{50} = 180%). Hill coefficients (HC): blue: 2.55; green: 1.70. (b) Hb–O_2 dissociation curves: blue (×): whole human blood. P_{50} = 30.57 Torr, green (Δ): whole blood + 30 mM **5**, P_{50} = 41.52 Torr (ΔP_{50} = 36%). HC: blue curve: 2.44; green curve: 1.62. (c) Hb–O_2 dissociation curves: blue curve (×): free human Hb, P_{50} = 18.2 Torr, green (Δ): free human Hb + 30 mM IHP, P_{50} = 45.2 Torr (ΔP_{50} = 148%). HC: blue: 2.35; green: 1.60. (d) Hb–O_2 dissociation curves: blue (×): whole blood, P_{50} = 25.4 Torr, green (Δ): whole blood + 30 mM IHP, P_{50} = 25.8 Torr (ΔP_{50} = 2%). HC: blue: 2.61; green: 2.58.

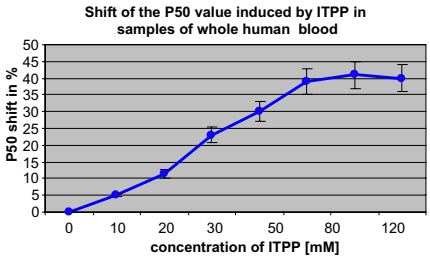


Figure 2. Concentration dependence of P_{50} shifts from compound **5** measured in human whole blood. Means of three values and standard deviation are shown. Concentrations of **5** between 5 mM and 120 mM (final concn) were tested.

oxy-Hb dissociation curves, alters only slightly the sigmoid shape of the curves. In control Hb the Hill coefficient is 2.55 and decreases upon binding of compound **5** to 1.70. In whole blood samples the Hill coefficient goes from 2.44 (control) to 1.62 for RBCs incubated with compound **5**, but the curves retain their sigmoid aspect. Similar changes have been reported when IHP was introduced in RBCs by controlled lysis and resealing.[6,7] As far as the stability of ITPP is concerned, various NMR experiments performed in human serum revealed a considerable stability of the pyrophosphate groups over a period of 72 h.

Finally, we have also investigated the influence of the concentration of compound **5** on the induction of P_{50} shifts in whole blood and noted a significant dependence on concentrations up to 60 mM; higher concentrations ($\leqslant$120 mM) of compound **5** did not increase the P_{50} shifts further (Fig. 2). One reason for the limitation of P_{50} shift in whole blood might be the high osmolarity of the compound solution. An osmolarity lower than 300 mOsM facilitates the uptake of a compound by RBCs and may even cause lysis of the cells. Higher osmolarities diminish the uptake of a compound through the cell membrane. Solutions of compound **5** at concentrations higher than 60 mM have much higher osmolarities than 300 mOsM. The increase of P_{50} shifts with increasing **5** concentrations up to 60 mM is an additional proof of the uptake of the compound by the RBCs. A comparison of the P_{50} shifts induced by the same concentration of **5** in free Hb with those observed in whole blood strongly suggests that the limiting step is the crossing of the plasma membrane by the compound.

In conclusion, the tripyrophosphate derivative ITPP, **III**, of *myo*-inositol hexakis phosphate IHP, **I**, was found to be powerful, synthetically very easily accessible, novel membrane permeant allosteric effector of haemoglobin. The evaluation of its biological and physiological properties in vivo will be of much interest.

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11. Modified procedure for the synthesis of compounds **4** and **5**: IHP-tetra-pyridinium salt **3** was dissolved in water (30 mL) and pyridine (130 mL) containing *N*,*N*-dicyclohexylcarbodiimide (8 g) was added. The reaction mixture was heated to 65 °C for 18 h and evaporated to dryness. The residue was extracted with water (4 × 10 mL) filtered and the filtrate was evaporated to dryness to give the pentapyridinium salt of *myo*-inositol 1,6:2,3:4,5-tripyrophosphate **4** (3.355 g, 77% yield). ^{31}P NMR (121 MHz, D_2O): δ −8.83 and −13.53 (AB, J = 22.3 Hz, 2P, ax–eq), −9.82 and −10.00 (AB, J = 17.8 Hz, 2P, eq–eq), −10.19 (AB as a singlet, 2P, eq–eq); ^{1}H NMR (300 MHz, D_2O): δ 8.65 (d, J = 5.6 Hz, 2 × 5H), 8.48 (dd, J = 7.9, 7.9 Hz, 1 × 5H), 7.94 (dd, J = 7.0, 7.0 Hz, 2 × 5H), 5.00 (br d, J = 10.5 Hz, 1H), 4.57 (ddd, J = 9.6, 9.6, 5.5 Hz, 1H), 4.43–4.36 (m, 2H), 4.30–4.18 (m, 2H); ^{13}C NMR (75 MHz, D_2O): δ 147.0, 140.9, 127.3, 76.9 (t, J = 6.8 Hz), 76.2–76.0 (m), 75.3–75.0 (m), 73.8 (t, J = 7.4 Hz), 73.4–73.1 (m), 72.9–72.6 (m). Compound **4** dissolved in water and passed through an ion exchange Dowex 50 W × 8 Na^+ column. The eluate was concentrated in vacuum to give hexasodium salt of *myo*-inositol 1,6:2,3:4,5-tripyrophosphate **5**. All data were found identical to the reported ones.
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14. All compounds were soluble in water yielding clear solutions. For the incubation with Hb, solutions of effectors and Hb were mixed in the ratio 1:1 vol/vol, either at the same molarities or up to 240 mM of **5** and 2.5 mM of Hb and measured for P_{50} shifts immediately. Compound solution was adjusted at pH = 6.9–7.1. For the incubation of whole blood with the effectors, the osmolarity of the compound solutions was adjusted at 290–310 mOsM and pH at 7. Effector solutions (up to 240 mM) and whole blood at 1:1 vol/vol ratios were incubated at 37 °C for 60 min. Following incubation the blood cells were washed three times with 20 mM bis–Tris buffer, pH = 7, 308 mOsM. No lysis of the incubated RBC was observed. The oxy-Hb dissociation curves were recorded using the Hemox Analyzer (PD Marketing, West Sussex PO 207 RH, NK) and a HAS software from TCS Scientific (New Hope, PA). Reading solution consisted of an aliquot of RBCs to set instrument parameters and 20 mM Hemox buffer (Osm = 310 ± 10 mOsM, pH 7.1–7.2) at 37 °C (concentration: 40–60 μL RBCs in 3 mL buffer).
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