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Transport of the Highly Charged *myo*-Inositol Hexakisphosphate Molecule across the Red Blood Cell Membrane: A Phase Transfer and Biological Study

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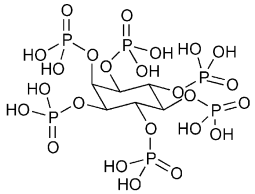
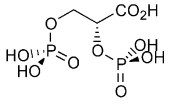
Abstract—To address the problem of delivering highly charged small molecules, such as phytic acid ($InsP_6$ or IHP), across biological membranes, we investigated an approach based on a non-covalent interaction between transport molecule(s) and IHP. Thus, we synthesized a collection of compounds containing IHP ionically bound to lipophilic (but non-lipidic) ammonium or poly-ammonium cations. First, we assessed the ability of these water-soluble salts to cross a biological membrane by measuring the partition coefficients between human serum and 1-octanol. In view of the ability of IHP to act as potent effector for oxygen release, the O_2–hemoglobin dissociation curves were then measured for the most efficient salts on whole blood. From both the biological and the physical properties of IHP-ammoniums salts we determined that cycloalkylamines (or poly-amines) were the best transport molecules, especially cycloheptyl- and cyclooctylamine. Indeed, the octanol/serum partition coefficient of IHP undecacycloctylammonium salt, is superior to 1, which is very favorable for potential uptake into the red blood cell membrane. A qualitative correlation was found between the partitioning experiments and the biological evaluations performed on whole blood.

Phytic acid (*myo*-Inositol HexakisPhosphate, $InsP_6$ or IHP) is the most abundant form of phosphate in plants.[1] This organic polyphosphate also has the property to tightly bind to human hemoglobin.[2] Hemoglobin is a tetrameric protein that cooperatively fixes four O_2 molecules and whose affinity for molecular oxygen is allosterically regulated by 2,3-diphosphoglycerate (DPG). By binding to hemoglobin, polyphosphates such as DPG or phytic acid, trigger a decrease of the O_2/hemoglobin affinity and the subsequent release of oxygen. Moreover, IHP interacts with hemoglobin 1000 times more tightly than DPG.[3] This interesting property makes IHP a good pharmaceutical candidate in the case of diseases characterized by a limited oxygen flow to organs or tissues (generally called *ischemia*, or ischemic insult). Heart attacks and stroke are the most widely recognized examples of the damage resulting from ischemia. Furthermore, if brain and nerve cells are deprived of oxygen for too long, irreversible brain damage or cell death may occur. In a general manner, IHP loaded erythrocytes would help to alleviate the ischemic problems as well as diseases or damages requiring repeated transfusions (such as anemia, sickled cell disease, surgery…).



Phytic acid (**$InsP_6$ or IHP**)
(*myo*-**I**nositol **H**exakis **P**hosphate)



DPG
2,3-**dip**hospho-*D*-**g**lyceric acid

However, in physiological conditions, IHP bears at least seven charges, making it very difficult to be transported across cell membranes. Although IHP delivery has already been achieved using a liposome delivery system[4,5] and electroporation techniques,[6] a more classical transport approach would be of much interest, involving in particular a IHP derivative that would be soluble in both aqueous and low polarity media.

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As emphasized in phase transfer catalysis, it has long been recognized that organic and inorganic anions can be efficiently solubilized in organic media when associated to *tri-* or *tetra*-alkyl ammonium counter-cations. IHP is a highly charged polyphosphate insoluble in many organic solvents, but is also capable of multiple ionic bonds with organic ammonium cations. Since pK_a values of all acidic protons of IHP have been measured either by NMR,[7] or by potentiometric methods,[8] it has been established that IHP bears 7 or 8 charges at physiological pH. It also means that IHP can be associated to at least seven lipophilic cations in physiological conditions. Thus, depending on the associated ammonium ions, the lipophilicity can theoretically be shifted to a cell-membrane compatible IHP complex, the ideal case being a poly-ammonium IHP derivative that would be soluble in both water and low polarity media. As a result of the very active research in the development of artificial vectors for gene transfer, a large number of cationic lipids have been described in the literature, and extensively reviewed.[9] In most of the cases these chemical vectors are lipids functionalised with amines,[9] poly-amines,[9] (poly-) guanidiniums,[10,11] and, in rare cases, phosphonium cations.[12] These cationic lipids are designed for gene delivery, and the mechanism by which they transport oligonucleotides across biological membranes may be very different for the delivery of a small poly-anionic molecule such as phytic acid. Moreover, to avoid the technical problems associated with drug delivery mediated by liposomes or vesicles, we decided, as a first approach, to prepare water-soluble lipophilyzed IHP derivatives. Thus, a collection of IHP/ammoniums salts has first been prepared from commercially available non-lipidic amines in order to assess the structural parameters allowing both the transport (by increasing the lipophilicity) and the water solubility of the salts (for the improvement of the bioavailability). Both the biological and physical properties of each salt has been evaluated by the measurement of O_2 dissociation curves, performed on whole blood, and by the determination of 1-octanol/serum partition coefficients, respectively.

IHP-ammonium Salts Preparation

IHP ammonium salts can be efficiently prepared from IHP, dodecasodium form and the corresponding free amine. IHP was first protonated using a cation-exchange resin and then mixed with an ethanolic solution of the desired amine. After evaporation the number of ammonium per IHP molecules was determined by ^{1}H NMR.

Partition Coefficients of IHP-ammoniums Derivatives

Partition coefficients relate to the distribution of a solute between two immiscible liquid phases and are defined as the ratios of concentrations (or molar fraction) of the distributed solute. These data have been used to predict or rationalize numerous drug properties such as quantitative structure–activity relationship,[13] lipophilicity[14] and pharmacokinetic characteristics.[15] 1-Octanol has been selected to mimic biological membranes, and it has been estimated than 1-octanol/water (K_{ow}) partition coefficients of more than 18,000 substances are now available in the literature.[15]

$$K_{os} = \frac{[IHP]_{octanol}}{[IHP]_{serum}}$$

Using ^{31}P NMR, we have determined the partition coefficients of IHP salts between a 1-octanol phase and two different aqueous phases: water and human serum. In Figure 1 are represented the structures of the different ammonium ions preassociated to IHP and the corresponding partition coefficients K_{ow} and K_{os}. These coefficients are measured after equilibration, at a concentration of 30 mM, close to the typical concentration employed for the biological evaluations (the standard concentration used for O_2–dissociation curve measurements was 22 mM).

Choice of the Aqueous Phases

Because of the large number of comparable data described in the literature we first measured 1-octanol/water partition coefficients K_{ow}. To be closer to physiological conditions, we determined the partition coefficients K_{os} between 1-octanol and human serum. Interestingly, we observed that K_{os} values are systematically lower (by a factor of 2–5) than K_{ow} values. Therefore, we only measured K_{os}'s when K_{ow} values were significant. To illustrate this observation we may compare the partitions of two IHP derivatives, **3** and **4**, in different aqueous systems: water, artificial serum (for its composition, see the experimental part) without and with albumin, and human serum. Table 1 summarizes these results.

This simple series of results emphasizes the problems that can be encountered when experiments are transposed from water to real physiological conditions. Indeed, the proteins present in serum, such as albumin,

Table 1. Partition coefficients (at 18 mM) of IHP salts **3** and **4** with different aqueous phases

Compds	K_{ow} (water)	K_{oa} (artificial serum without albumin)	K_{oas} (artificial serum with albumin)	K_{os} (human serum)
3 IHP, 9 cycloheptylammonium	0.411	0.088	0.041	0.031
4 IHP, 11 cyclooctylammonium	8.98	2.16	1.81	1.85

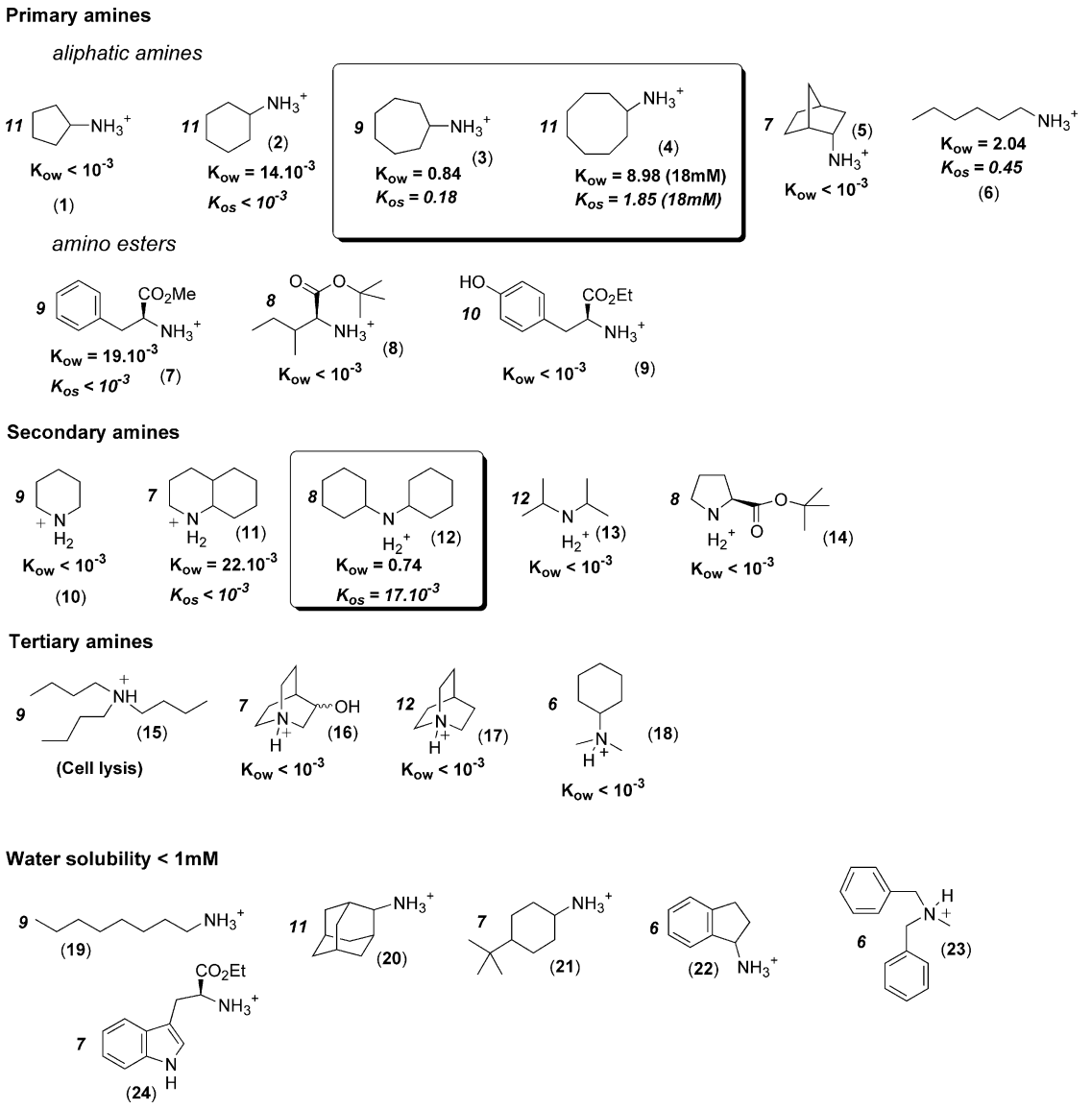


Figure 1. Partition coefficients of IHP ammonium salts (at 30 mM); K_{ow} = octanol/water partition coefficient, K_{os} = octanol/serum partition coefficient; the figures indicate the number of ion units per IHP molecule in the salt used in these experiments. Compound numbers are given in brackets, counter-cation numbers are written in italics.

as well as the cations that can be exchanged after equilibration, may play critical roles during a drug delivery process. For instance, albumin has been shown to participate in cholesterol transport in blood, thus demonstrating its propensity to bind lipophilic compounds.[16] The partition coefficients depicted in Table 1 show that albumin only slightly decreases the ability of a given organic ammonium to transport a polyphosphate into an apolar phase. This conclusion may be correlated to the results of a comparative study between octanol/water an octanol/buffer partition coefficients, correlated to diffusion across brain microvessel endothelium (Fig. 1).[17]

This first series of experiments also showed that artificial serum, that can be easily prepared from commercially available chemicals and BSA is a very good model of human serum.

Analysis of the Results

The analysis of this first series of partition coefficients already leads to interesting conclusions: *cyclic aliphatic amines* seem to display the best characteristics with regard to both lipophilicity and water solubility. Indeed, we observed a very significant increase in the K_{ow} values from cyclopentyl- ($<10^{-3}$) to cyclooctyl-ammoniums (9.98). Furthermore, the IHP cyclooctylammonium salt is still reasonably soluble in water (the aqueous solubility limit is between 25 and 30 mM), whereas the corresponding *n*-octylammonium IHP salt presents a solubility limit below 1 mM (thus rendering impossible its K_{ow} measurement as well as its biological evaluation). Interestingly, *hydrophobic amino-acids*, even esterified, do not possess satisfactory transport properties into the octanol phase, and will not be considered suitable for further studies aiming to IHP delivery into

red blood cells. Thus, from this first library we selected cycloalkyl groups for a further design of carrier molecules.

From a quantitative point of view, it is remarkable that a hexaphosphate could reach K_{os} values around or above 1 (compound **3** $K_{os} = 0.84$, compound **4** $K_{os} = 1.85$) indicating that more than 40% of the starting polyanion diffused from human serum to the octanolic phase after equilibration. As a comparison, several studies described buffer/1-octanol partition coefficients of uncharged antiviral nucleosides or nucleotides to predict their ability to diffuse across cell membranes. For instance, the K_{ow} value of 3′-azido-3′-deoxythymidine (AZT) was determined to be 1.26 (1-octanol/100 mM sodium phosphate, pH 7.0) while the K_{ow} of thymidine in the same conditions was 0.064.[18] More recently, the partitioning properties of uncharged phosphonic acid esters of 2′,3′-dideoxythymidine gave K_{ow} values between 0.23 and 2.06.[19]

However, new questions arise from this first study concerning the importance of the number of lipophilic amines associated to IHP and the variation of IHP distribution in apolar phases as a function of the polyanion concentration. To answer them, we measured K_{ow} and K_{os} variations for the two best IHP salts, **3** (9 cycloheptylammoniums) and **4** (11 cyclooctylammoniums) as a function of concentration. The resulting curves are depicted in Figure 2.

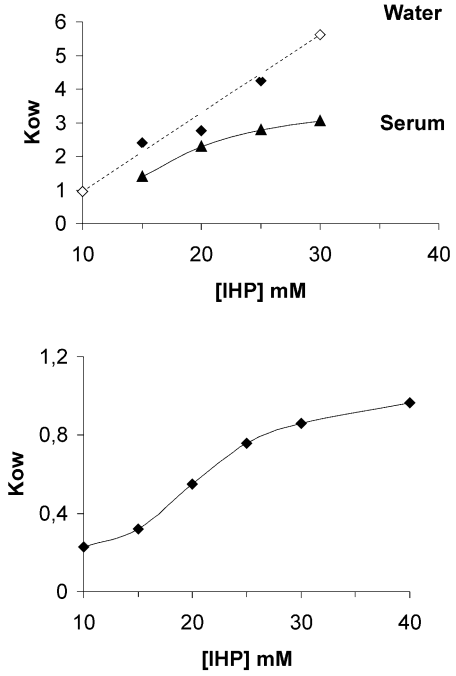


Figure 2. K_{ow} dependence on IHP starting concentration. (top) Compound **4** (IHP, 11 cyclooctyl-ammonium) was dissolved in 1-octanol at various concentrations and agitated with an equal volume of water or human serum. (bottom) Compound **3** (IHP, 9 cycloheptyl-ammonium) was dissolved in octanol at various concentrations and agitated with an equal volume of water.

K_{os} Variation as a Function of Cyclooctylammonium Concentration

The results (Fig. 3) show two important characteristics of the IHP transfer into an apolar phase: at a constant IHP concentration (22 mM), 8 equivalents of cyclooctylammoniums are required to reach a K_{os} value equal to 1, corresponding to an identical distribution between human serum and octanol. Secondly, cyclooctylammonium ions, initially present in their hydrochloride form in the organic phase, are able to extract IHP, exclusively present as a sodium salt in human serum at the beginning of the experiment as a result of chloride/polyphosphate exchange between 1-octanol and human serum.

To improve the polyphosphate transfer efficiency we reasoned that multiple interactions between a polyanion and a single (or two) transport molecule(s) will increase the binding strength between the two partners, thus preventing or limiting the exchange, under physiological conditions, with other cations present in high concentration in serum. For this reason, lipophilic polyamines seem to be the best candidates for transporting polyphosphates, such as IHP or DPG, across non-polar biological membranes. The 1-octanol/water partition measurements showed that amines bearing cycloalkyl groups display the best transport properties. Thus, we optimized two general synthetic procedures in order to obtain a new library of polyamines bearing cycloalkyl groups: a reductive amination procedure leading to acyclic tri- or tetra-amines, and a coupled acylation/borane-reduction procedure for the preparation of macrocyclic polyamines.

Synthesis of Lipophilized Polyamines

A first procedure used for the derivatization of primary amines with different cyclic ketones (Scheme 1), proved efficient, giving good to excellent yields, and versatile enough to allow reactions with hindered ketones such as 2-decalone. The two central tetra-amine cores employed are TREN [tris-(2-aminoethy)-amine] and BAP [*N*,*N*′-bis-(3-aminopropyl)-piperazine]. BAP is a linear tetra-amine presenting two primary amines susceptible to be alkylated, whereas TREN is a branched molecule presenting three possible *N*-alkylation sites through reductive

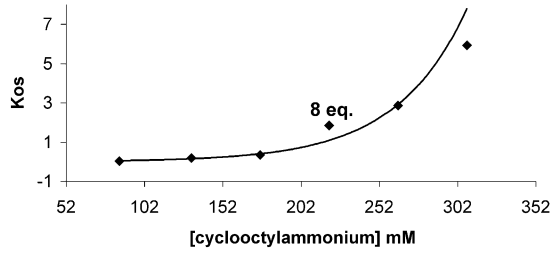
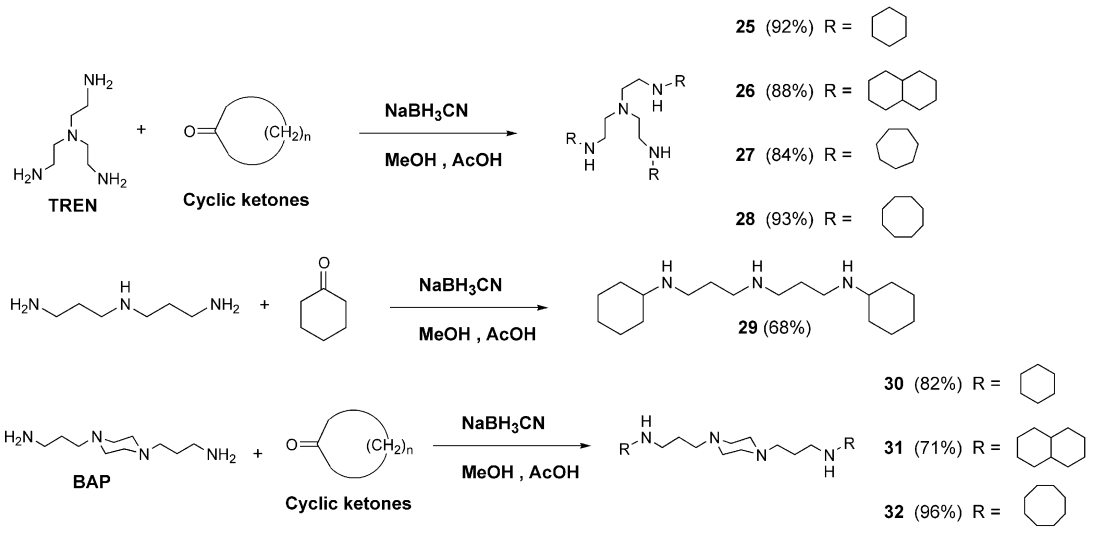


Figure 3. IHP uptake in 1-octanol by cyclooctylammonium ions. IHP, dodecasodium form, was dissolved in human serum ([IHP] = 22 mM, pH = 7.4). Each sample was agitated 2 days with an equal volume of octanolic solution of cyclooctylamine,HCl at different concentrations. Partition coefficients K_{os} were measured by ^{31}P NMR.



Scheme 1. Poly-(cycloalkyl-amines) obtained by reductive amination.

amination. Seven new polyamines were thus obtained from these two central cores, bearing either cyclohexyl groups (**25**, **29** and **30**), cycloheptyl group (**27**), cyclooctyl- (**28** and **32**), or 2-decalinyl groups (**26** and **31**).

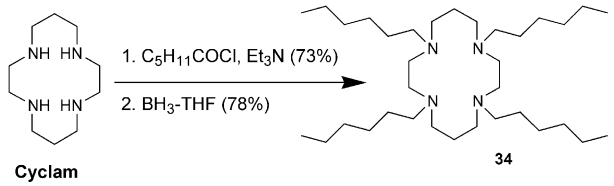
We then prepared a lipophilic polyamine derived from Cyclam, a well studied and commercially available 14-membered ring tetra-aza macrocycle.

This macrocycle was chosen in order to take advantage of the strengthened interaction of polyammonium ions, especially of macrocyclic type, with polyanions such as polyphosphates.[20,21] One of the best procedures to obtain tetra-*N*-alkyl-cyclam derivatives consists in a per-acylation of the four secondary amines of cyclam, followed by reduction of the resulting tetra-amide using an excess of borane-THF complex (Scheme 2).[22] Taking into account the lack of solubility of IHP *n*-octyl-ammonium salts in water, we chose to introduce shorter chains, *n*-hexyl lipophilic groups, for compound **34**.

Partition Coefficient of IHP Associated with Polyamines

A second library of IHP salts of di-, tri- and tetra-*cycloalkyl*ammonium ions was prepared using the standard procedure. The structures are listed in Figure 4. Compounds **35**, **36**, **37**, **38**, **44** and **45** have been obtained from commercially available diamines.

The partition coefficients K_{ow} and K_{os} were measured by ^{31}P NMR. Once again, the partitioning experiments were performed only with water-soluble salts. Polyamines bearing aromatic or decalinyl groups generally lead to insoluble salts. With its three lipophilized amines, the TREN core structure (**42**) gave rise to the most favorable uptake into 1-octanol, with both water and human serum. At a concentration of 30 mM, the K_{ow} value for compound **42** is 2.71 (meaning that 73% of starting IHP was found in 1-octanol after equilibration) while the K_{os} value is 0.64 (39% of the total amount of IHP stays in the apolar phase), which is an excellent distribution for a hexaphosphate under nearly physiological conditions. To compare with the partition experiments of the first library (IHP/*mono*ammonium salts), it should be emphasized that, in the case of compound **42**, only two transfer molecules are required to efficiently solubilize IHP into 1-octanol whereas at least eight cycloalkylamines were necessary to reach similar K_{os} values.



Scheme 2. Synthesis of a lipophilic macrocyclic polyamine.

Biological Evaluation and Comparison with the Physico-chemical Data

The IHP transport into red blood cells can be indirectly determined by measuring the partial pressure of oxygen of whole blood. Indeed, by binding to intracellular hemoglobin, IHP triggers an oxygen release from the erythrocytes. This release can be measured by standard hemoglobin–O_2 dissociation curves: P_{50} values refer to the partial pressure of molecular oxygen for which half of the hemoglobin molecules are bound to O_2. A shift to the right of the dissociation curve indicates a loss of affinity of hemoglobin for O_2 due to the interaction with phytic acid (when compared to a control experiment performed without IHP). The extent of IHP delivery into erythrocytes may be considered as proportional to the P_{50} shift. A 30 mM mother solution of a given IHP salt was first prepared, the pH was adjusted to 7.2 and the osmolality measured. This solution was then mixed to the proper volume of whole blood (final IHP concentration: 22 mM) and the dissociation curve was measured after homogenization. To properly assess

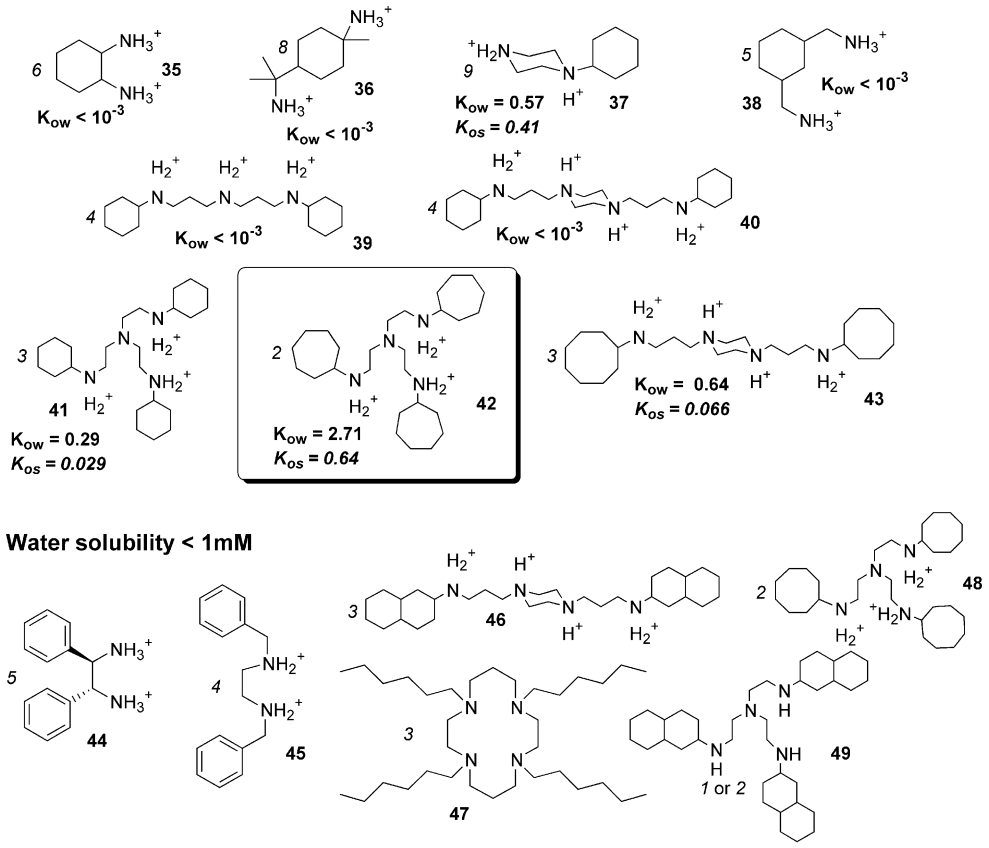


Figure 4. Partition coefficients of IHP/poly-*cycloalkyl*ammoniums salts (at 30 mM); K_{ow} = octanol/water partition coefficient, K_{os} = octanol/serum partition coefficient; the figures indicate the number of ion units per IHP molecule in the salt used in these experiments. Counter-cation numbers are written in italics.

which amines or polyamines could trigger an IHP transport, we only compared data corresponding to experiments displaying similar osmolality values, in the range of 200–240 mOsM.

From the biological evaluation described above (see Table 2), several conclusions can be drawn:

- the best results were obtained with two IHP *cycloalkylammonium* salts **2** (undeca cyclohexylammonium) and **3** (nona cycloheptyl-ammonium). At 220 mOsM, these IHP salts triggered a 50% shift of the P_{50} value (compared to the control experiment);
- *amino-esters* did not display transfer properties;
- tributyl-ammonium salts and isoleucine-*t*Bu ester provoked a fast hemolysis of red blood cells. This lysis might be due either to detergent properties of the salts or to the blockage of potassium channels.

It is interesting to note that there is a good correlation between the biophysical study (1-octanol/serum partition coefficients) and the biological evaluation (P_{50} shift

Table 2. Representative data from P_{50} shift measurements performed on whole blood under low osmolality conditions

Compound	P_{50}[a] (Torr)	P_{50} control[b] (Torr)	Shift (%)	Osmolality[c] (mOsM)
1 (11 cyclopentylammonium)	38	39	3	173
2 (11 cyclohexylammonium)	26.8	42	57	220
3 (9 cycloheptylammonium)	23	31.5	37	224
IHP, 6 tributylammonium	Cell lysis	—	—	
8 (8 *i*Leu-CO2*t*Bu)	Cell lysis	—	—	
17 (12 quinuclidinium)	32.5	34.5	6	240
22 (6 indanammonium)	25	28.5	14	220
5 (7, norbornylammonium)	38.5	38.5	0	200
11 (7 decahydroquinolinium)	24.5	27	10	221
9 (10 Tyr-CO_2Et)	23	28.5	24	247
18 (6 *N*,*N*-dimethylammonium)	41.5	42.5	2	225
43 (3 tetraamine **32**)	25	25	0	221

[a]Measured on whole blood with [IHP] = 22 mM, pH = 7.2 at 37 °C.
[b]Without IHP derivative.
[c]Measured after pH adjustment.

measurements), since both techniques lead to the conclusion that cycloalkylamines display the highest efficiency for both partitioning of IHP into an octanolic phase and transport of IHP across the erythrocyte membrane.

Conclusion

The octanol/water and octanol/serum partition studies described in this report provide significant information regarding both polyanion uptake into apolar phases and design of lipophilic polyamines as polyphosphate transport molecules. Lipophilic ammonium ions bearing cycloalkyl residues are of particular interest and increasing their ring size leads to a remarkable improvement of IHP distribution in an octanol phase. At the same time, the water solubility of IHP associated to cycloalkylamines is preserved compared to linear *n*-alkylamines, which is a valuable feature for the development of a drug delivery strategy. The biological tests performed on the two IHP-ammonium cations libraries are globally in good, qualitative, agreement with the physico-chemical results obtained from the phase distribution studies. Further improvements in transfer efficiencies may yield improved vectors for the delivery of effectors such as IHP into red blood cells.

Experimental

Materials and procedures

All chemicals (starting amines or diamines, IHP, cycloalkanones...) were purchased from Sigma, Aldrich or Fluka and were used without further purification. The resin Dowex 50WX8–200 was purchased from Aldrich and washed with distilled water before the first use. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded with Bruker AC-200 or AC-300 spectrometers. Mass spectra were determined by the Service commun de spectrométrie de masse at the Institut de Chimie, Université Louis Pasteur. Human serum was purchased at the Centre de Transfusion Sanguine de Strasbourg, stored at −18 °C and used such as received. Artificial serum was based on blood composition: $[Na^+] = 140$ mM, $[Mg^{2+}] = 1$ mM, $[K^+] = 5$ mM, $[Ca^{2+}] = 1$ mM, $[Cl^-] = 106$ mM, $[PO_4^{3-}] = 1$ mM, $[SO_4^{2-}] = 0.5$ mM, $[CO_3^{2-}] = 30$ mM, $[Br^-] = 0.5$ mM, bovine serum albumin (fraction V, >98%) 30 g·L^{-1}, pH = 7.41. Blood oxygen dissociation of samples were determined using a Hemox Analyzer Model B (TCS Medical Products Company, New Hope, PA, USA).

Blood preparation

Whole blood was collected from one subject. The blood was stored in a Vacutainer and stored at 4–8 °C.

Bioassay conditions

Effector stocks were prepared at 100–120 mM using water or Bis–Tris buffer (20 mM Bis–Tris, 140 mM NaCl, pH = 7.45). Effector characteristics prior to incubation were: $c = 30$ mM, pH = 7.1–7.4 (at 37 °C), osmolarity = 170–340 mOsM. 75 μL of whole blood was incubated 2 min at 37 °C with 300 μL of a 30 mM solution of IHP derivative. After incubation with/without effector, the blood cells were washed four times with HBS-BSA (20 mM HEPES, 130 mM NaCl, pH = 7.42 at 37 °C) by centrifugal pelleting to remove exogenous effector and evaluate hemolysis. 20 μL were used for measurement of the hemoglobin–O_2 dissociation curve at 37 °C. Blood oxygen dissociation of samples were determined using a Hemox Analyzer Model B: the control sample contained 2.5 mL buffer HBS (20 mM HEPES, 130 mM NaCl, 20 μL BSA per 5 mL buffer, pH = 7.2–7.4 at 37 °C) and 25 μL of whole blood; the effector sample contained 2.5 mL HBS and 20 μL of pelleted blood cells incubated with the IHP derivative. The P_{50} values were calculated from the dissociation curves compared to the same day control sample.

Partition coefficients

IHP derivatives were dissolved in 1 mL of aqueous phase at a concentration of 30 mM in an eppendorf. 1 mL of 1-octanol was then added and each sample was shaken at 36 rpm during 12 h. The equilibrated biphasic solutions were centrifuged for 2 h at 3000 rpm. 350 μL of the octanolic phase was first taken off with a syringe and transferred into a NMR tube. 350 μL of the aqueous phase was then taken off. ^{31}P NMR spectra were performed on a Bruker AC-300 spectrometer. An external standard (triphenylphosphine oxide, 60 mM in DMSO-d_6) was placed inside the NMR tubes to allow both the locking process of the spectrometer and an accurate integration of IHP peaks. Partition coefficients were determined as the ratio of IHP integrations, relative to the external standard, in the octanol and aqueous phases. The detection limits of this technique do not allow partition coefficient measurements below 10^{-3}.

Preparation of the IHP polyammoniums derivatives

A 100 mM IHP dodecasodium salt solution was applied on a cation exchange column (Dowex 50WX8–200, H^+ form) and eluted with distilled water. The fractions containing the perprotonated IHP were collected and poured into an ethanolic solution of the desired amine. The solution was then concentrated to dryness in vacuo. The product was then dissolved in ethanol or in a 1/1 toluene/EtOH mixture, concentrated and dried in vacuo. The IHP-ammonium salts were characterized by ^{1}H and ^{31}P NMR. The final salt composition was determined by ^{1}H NMR.

Tris-(*N*-cyclohexyl-2-aminoethyl)-amine (25). To a solution of TREN [tris-(2-aminoethyl)-amine, 2.24 g, 15.3 mmol], cyclohexanone [15 mL, 156.4 mmol, 10.2 equiv], in 60 mL methanol and 2.7 mL acetic acid, was added, at 0 °C, sodium cyanoborohydride (3 g, 79.6 mmol, 9.4 equiv) in three portions. The solution was stirred 16 h at room temperature, then fresh sodium

cyanoborohydride (2 g, 52.9 mmol, 6.3 equiv) was added. After 20 h, the reaction was quenched with 20 mL NaOH, 1 N. The mixture was stirred 30 min, diluted with 30 mL H_2O and washed three times with 70 mL CH_2Cl_2. The combined organic phases were concentrated, dissolved in 20 mL ethanol, and 10 mL of HCl 12 N were added. After concentration, the resulting white solid was abundantly rinsed with Et_2O and recrystallized in a 95/5 EtOH/H_2O mixture. Yield: 92%. White solid, $C_{24}H_{48}N_4Cl_4$, $M_r = 538.7$. ^{1}H NMR (200 MHz, D_2O) δ 3.15 (m, 8H), 2.89 (m, 6H), 2.1–1.0 (m, 30H). ^{13}C NMR (50 MHz, D_2O) δ 57.80, 49.09, 41.19, 28.95, 24.54, 24.06. MS (elec. ionization): 100% = 280.1 [M-(*N*-cyclohex.-methyl-amine)], 50% = 268.1 [M-(*N*-cyclohex.-ethyl-amine)], 10% = 393.3 (M + 1).

Tris-(*N*-(2-decalinyl)-2-aminoethyl)-amine (26). To a solution of TREN [tris-(2-aminoethyl)-amine, 0.7 mL, 4.7 mmol], 2-decalone (mixture of isomers, 2.17 g, 14.3 mmol, 3.2 equiv), in 15 mL methanol and 1 mL acetic acid, was added, at 0 °C, sodium cyanoborohydride (1.1 g, 17.5 mmol, 3.7 equiv) in three portions. The solution was stirred overnight at room temperature, then 0.3 g sodium cyanoborohydride was added. After 6 h, the reaction was quenched with 10 mL NaOH, 1 N. The mixture was stirred 30 min, diluted with 30 mL H_2O and washed three times with 70 mL CH_2Cl_2. The combined organic phases were concentrated, dissolved in 20 mL ethanol, and 10 mL of HCl 12 N were added. After concentration, the resulting white solid was abundantly rinsed with Et_2O. Yield: 88%. White solid, $C_{36}H_{70}N_4Cl_4$, $M_r = 700.6$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 9.5 and 9.4 (2 bs, 4H), 4.16 (m, 5H), 3.78 (m, 6H), 3.01 (m, 4H), 1.90–1.40 (m, 48H). MS (FAB, positive mode): M + 1 = 555.4.

Tris-(*N*-cycloheptyl-2-aminoethyl)-amine (27). To a solution of TREN [tris-(2-aminoethyl)-amine, 2.52 g, 17.2 mmol], cycloheptanone (7.0 mL, 59.3 mmol, 3.5 equiv), in 60 mL methanol and 3 mL acetic acid, was added, at 0 °C, sodium cyanoborohydride (3.5 g, 55.7 mmol, 3.3 equiv) in three portions. The solution was stirred 16 h at room temperature, then 1 g sodium cyanoborohydride was added. After 20 h, the reaction was quenched with 20 mL NaOH, 1 N. The mixture was stirred 30 min, diluted with 30 mL H_2O and washed three times with 70 mL CH_2Cl_2. The combined organic phases were concentrated, dissolved in 20 mL ethanol, and 10 mL of HCl 12 N were added. After concentration, the resulting white solid was abundantly rinsed with Et_2O and recrystallized in methanol. Yield: 84%. White solid, $C_{27}H_{58}N_4Cl_4$, $M_r = 580$. ^{1}H NMR (200 MHz, D_2O) δ 3.23 (h, $J = 4.6$ Hz, 3H), 3.14 (t, $J = 6.1$ Hz, 6H), 2.89 (t, $J = 6.1$ Hz, 6H), 1.97 (m, 6H), 1.70–1.30 (m, 30H). ^{13}C NMR (50 MHz, D_2O) δ 62.06, 51.07, 43.16, 32.51, 29.16, 25.38. MS (FAB, positive mode): M + 1 = 435.4.

Tris-(*N*-cyclooctyl-2-aminoethyl)-amine (28). To a solution of TREN [tris-(2-aminoethyl)-amine, 2.35 g, 16.7 mmol], cyclooctanone (9.3 g, 73.7 mmol, 4.4 equiv), in 30 mL methanol and 2 mL acetic acid, was added, at 0 °C, sodium cyanoborohydride (2.8 g, 44.6 mmol, 2.7 equiv) in three portions. The solution was stirred 3 days at room temperature (every day, 0.3 g sodium cyanoborohydride was added). The reaction was quenched with 20 mL NaOH, 1 N. The mixture was stirred 30 min, diluted with 30 mL H_2O and washed three times with 70 mL CH_2Cl_2. The combined organic phases were concentrated, dissolved in 20 mL ethanol, and 10 mL of HCl 12 N were added. After concentration, the resulting white solid was abundantly rinsed with Et_2O. Yield : 93%. White solid, $C_{30}H_{64}N_4Cl_4$, $M_r = 622$.^{1}H NMR (200 MHz, $CDCl_3/CD_3OD$ 1/1) δ 3.39 (m, 3H), 3.27 (bt, $J = 4.9$ Hz, 6H), 2.97 (bt, $J = 4.6$ Hz, 6H), 2.10 (m, 6H), 1.85 (m, 12H), 1.70–1.40 (m, 24H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 60.61, 51.97, 40.94, 29.00, 26.28, 25.47, 23.67.MS (FAB, positive mode): M + 1 = 477.5. El. analysis: calcd (%) C 57.87, H 10.36, N 9.00; meas. C 58.14, H 10.55, N 9.17.

Bis-(*N*-cyclohexyl-3-aminopropyl)-amine (29). To a solution of dipropylene triamine (2.1 g, 16.0 mmol), cyclohexanone (5.1 mL, 48.9 mmol, 3.1 equiv), sodium cyanoborohydride (2.9 g, 46.1 mmol, 2.9 equiv) in 40 mL methanol, was added, at 0 °C, 2.7 mL acetic acid. The solution was stirred 24 h at room temperature then quenched with 30 mL saturated sodium carbonate. The mixture was stirred 30 min, diluted with 30 mL H_2O and washed three times with 70 mL CH_2Cl_2. The combined organic phases were concentrated, dissolved in 20 mL ethanol and 10 mL of HCl 12 N were added. After concentration, the resulting white solid was abundantly rinsed with Et_2O and recrystallized in a 95/5 EtOH/H_2O mixture. Yield: 68%. White solid, $C_{18}H_{40}N_3Cl_3$, $M_r = 405.0$. ^{1}H NMR (200 MHz, D_2O) δ 3.15 (m, 10H), 2.21 (m, 8H), 1.79 (m, 4H), 1.63 (bd, 2H), 1.40–1.00 (m, 12H). ^{13}C NMR (50 MHz, D_2O) δ 57.61, 44.82, 41.33, 28.94, 24.57, 23.98, 22.99. MS (EI): 100% = 112.1 (*N*-cyclohexyl-methyl-iminium); 30% = 296.2 (M + 1); El. analysis: calcd (%) C 53.40, H 9.96, N 10.38; meas. C 53.67, H 10.16, N 10.15.

***N*,*N*′-di-[3-Cyclohexylamino-propyl]-piperazine (30).** To a solution of bis-(3-aminopropyl)-piperazine (3.80 g, 19.0 mmol), cyclohexanone (4.0 g, 40.8 mmol, 2.2 equiv), in 40 mL methanol and 2.0 mL acetic acid, was added, at 0 °C, sodium cyanoborohydride (2.0 g, 31.8 mmol, 1.7 eq.) in three portions. The solution was stirred overnight at room temperature. Sodium cyanoborohydride (1.0 g, 15.9 mmol, 0.85 equiv) was added, and the reaction mixture was stirred 6 h at room temperature. The reaction was quenched with 10 mL H_2O and 40 mL satd sodium carbonate. The mixture was stirred 30 min, diluted with 30 mL H_2O and washed three times with 70 mL CH_2Cl_2. The combined organic phases were concentrated, dissolved in 20 mL ethanol, and 10 mL of HCl 12 N were added. After concentration, the resulting white solid was abundantly rinsed with Et_2O and recrystallized in a 9/1 EtOH/H_2O mixture. Yield: 82%. White solid, $C_{22}H_{48}N_4Cl_4$, $M_r = 510$. ^{1}H NMR (200 MHz, D_2O) δ 3.71 (s, 8H), 3.37 (t, $J = 7.9$ Hz, 4H), 3.15 (t, $J = 7.6$ Hz, 6H), 2.15 (m, 8H), 1.78 (m, 4H), 1.60 (bd, 2H), 1.40–1.15 (m, 10H). ^{13}C NMR (50 MHz, D_2O) δ 59.63, 55.66, 50.99, 43.13, 30.85, 26.51, 25.93, 23.06. MS (FAB, positive mode): M + 1 = 465.3.

***N,N′*-di- [3- (*N*- (2-Decalinyl)- amino)- propyl] piperazine (31).** To a solution of bis-(3-aminopropyl)-piperazine (0.80 mL, 4.0 mmol), 2-decalone (mixture of isomers, 1.23 g, 8.1 mmol, 2.1 equiv), in 20 mL methanol and 1 mL acetic acid, was added, at 0 °C, sodium cyanoborohydride (1.9 g, 30.2 mmol, 7.7 equiv) in three portions. The solution was stirred 60 h at room temperature. The reaction was quenched with 10 mL NaOH, 1 N. The mixture was stirred 30 min, diluted with 30 mL H_2O and washed three times with 70 mL CH_2Cl_2. The combined organic phases were concentrated, dissolved in 20 mL ethanol, and 10 mL of HCl 12 N were added. After concentration, the resulting white solid was abundantly rinsed with Et_2O and recrystallized in a 9/1 EtOH/H_2O mixture. Yield: 71%. White solid, $C_{30}H_{60}N_4Cl_4$, $M_r = 618$. ^{1}H NMR (200 MHz, D_2O) δ 3.27 (s, 8H), 3.38 (t, $J = 7.9$ Hz, 4H), 3.14 (m, 6H), 2.17 (m, 4H), 1.90–1.15 (m, 32H). ^{13}C NMR (50 MHz, D_2O) δ (the spectrum displays two sets of peaks corresponding to two different isomers of the decaline moiety, the major isomer is labelled *M*, the minor one *m*) 60.47(*M*), 56.29(*m*), 55.70(*M*), 50.96(*M*), 43.27(*m*), 43.06(*M*), 37.06(*m*), 36.58(*M*), 36.50(*m*), 36.14(*M*), 33.13(*M*), 32.58(*m*), 31.66(*M*), 30.85(*m*), 30.08(*M*), 28.31(*M*), 27.91(*m*), 26.81(*M*), 25.45(*M*), 23.06(*M*), 22.47(*m*), 22.21(*M*). MS (FAB, positive mode): M + 1 = 473.4. El. analysis: calcd (%) C 58.14, H 9.97, N 8.91; meas. C 58.25, H 9.78, N 9.06.

***N,N′*-di-[3-Cyclooctylamino-propyl]-piperazine (32).** To a solution of bis-(3-aminopropyl)-piperazine (1.33 g, 6.6 mmol), cyclooctanone (4.6 g, 36.4 mmol, 5.5 eq.), in 30 mL methanol and 1.0 mL acetic acid, was added, at 0 °C, sodium cyanoborohydride (2.3 g, 36.6 mmol, 5.5 equiv) in three portions. The solution was stirred 12 h at 50 °C. Sodium cyanoborohydride (1.0 g, 15.9 mmol) was added, and the reaction mixture was stirred 12 h at 50 °C. The reaction was cooled down at room temperature and quenched with 10 mL H_2O and 10 mL NaOH, 1 N. The mixture was stirred 30 min, diluted with 30 mL H_2O and washed three times with 70 mL CH_2Cl_2. The combined organic phases were concentrated, dissolved in 20 mL ethanol, and 10 mL of HCl 12 N were added. After concentration, the resulting white solid was abundantly rinsed with Et_2O. Yield: 96%. White solid, $C_{26}H_{56}N_4Cl_4$, $M_r = 566$. ^{1}H NMR (200 MHz, D_2O) δ 3.72 (s, 8H), 3.38 (dd, $J = 7.9$ Hz $J = 11.1$ Hz, 6H), 3.15 (t, $J = 7.9$ Hz, 4H), 2.19 (m, 4H), 1.99 (m, 4H), 1.80–1.30 (m, 24H). ^{13}C NMR (50 MHz, D_2O) δ 61.47, 55.66, 50.99, 43.61, 30.74, 27.87, 27.14, 25.19, 23.13. MS (FAB, positive mode): M + 1 = 421.5. El. analysis: calcd (%) C 55.12, H 9.96, N 9.89; meas. C 55.36, H 10.23, N 9.93.

***N,N′,N″,N‴*-Tetrahexanoyl-cyclam (33).** Hexanoyl chloride (0.8 mL, 5.82 mmol, 5.2 equiv) was added dropwise, at 0 °C, to a solution of cyclam (224 mg, 1.12 mmol) and 1.0 mL Et_3N in 15 mL anhydrous CH_2Cl_2. This solution was stirred 24 h at room temperature, washed twice with satd $NaHCO_3$, dried over $MgSO_4$, concentrated under vacuum, and chromatographed on silica gel (elution conditions: AcOEt/hexane 1/1 until all the unreacted acyl chloride was totally eluted, then pure AcOEt). The tetra-amide was isolated as a white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.46 (m, 16H), 2.37 (t, $J = 7.3$ Hz, 4H), 2.28 (t, $J = 7.9$ Hz, 4H), 1.88 (m, 4H), 1.64 (m, 8H), 1.30 (m, 16H), 0.87 (m, 12H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.56, 173.83, 48.06, 47.55, 45.71, 33.36, 32.88, 31.66, 28.91, 25.27, 24.94, 22.55, 13.99. MS (FAB, positive mode) M + 1 = 593. El. analysis: calcd (%) C 68.88, H 10.88, N 9.45; meas. C 68.79, H 11.14, N 9.26.

***N,N′,N″,N‴*-Tetrahexyl-cyclam (34).** The general procedure of amide reduction[22] was applied from the cyclam derivative **33**. The tetraamine was isolated as a colorless oil. $C_{34}H_{72}N_4$, $M_r = 536.4$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 2.53 (s, 8H), 2.47 (t, $J = 7.0$ Hz, 4H), 2.37 (dd, $J = 7.6$ Hz $J = 7.0$ Hz, 4H), 1.57 (m, 4H), 1.45–1.15 (m, 32H), 0.88 (t, $J = 6.4$ MHz, 12H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 55.83, 51.64, 50.72, 31.83, 27.42, 27.27, 22.64, 14.00. MS (FAB, positive mode) M + 1 = 537.5 ; El. analysis: calcd (%) C 76.05, H 13.52, N 10.43; meas. C 73.31, H 13.72, N 10.20.

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