Study of chiral aza-macrocyclic ligands involved in important biological processes

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Abstract. Azamacrocyclic ligands able to coordinate metal ions in a selective way are used in a wide variety of applications, such as in metal extraction or as models of protein binding sites. Keeping in mind the importance of this family of compounds, we studied synthetic methods to obtain an enantiomerically pure hexaaza tetramine macrocycle $M_1$, resulting from the condensation of two units of pyridine-2,6-dicarbaldehyde and $(R,R)$-1,2-diaminocyclohexane and the subsequent in situ reduction. The condensation conducted in the presence of Ba(II), which acts as a templating agent, represents the more efficient way of synthesis, in terms of yield and selectivity. The need to get the macrocycle of interest was dictated by the birth of a collaboration with the University "La Sapienza" of Rome. In fact, the target macromolecule is the first of many structural variations to be carried out on a starting prototype $M$, that proved extraordinarily affinity to potassium ion, in the presence of an acidic species in gas phase. Knowing the important role of this ion at physiological level, it was decided to determine the structural features of the macrocycle, in order to investigate possible useful applications.

Keywords. Chiral macrocycles; Schiff bases; Polyamines; Template synthesis; Gas phase studies.

Introduction. Inspire by nature, during the last 50 years, chemists have started to synthesize azamacrocycles intentionally. Indeed this class of compounds has a plethora of applications both alone\textsuperscript{1,2} and/or in their metal ion complexes.\textsuperscript{3} Moreover optically active polyazamacrocycles are important compounds in organic,\textsuperscript{4} supramolecular,\textsuperscript{5} medicinal\textsuperscript{6} and bioorganic chemistry.\textsuperscript{7} Here we report the study for the optimization of a synthetic methodology of the chiral hexaazamacrocycle $M_1$ (Scheme 1). The macrocyclization reaction represents the step that takes most of the efforts, in order to obtain imine macrocycle 4, that can be easily transformed into the target reduced macromolecule $M_1$. 
Basically two synthetic methods were investigated: (a) the metal-free synthesis and (b) the metal-template synthesis. The first strategy consists in a polycondensation between primary diamines and dialdehydes, in which the outcome structure is governed by several factors, such as the structure and the stoichiometric ratio of the substrates, the solvents, and the concentration of the reaction mixture. However, to synthesize macrocyclic systems, the metal-free method does not always allow to achieve acceptable yields and selectivity. So an alternative approach is necessary for the synthesis of these macromolecules, especially when the control of the reaction conditions and the geometry of the components isn’t enough to guide to a specific product. For this reason we explored the second strategy, which involves the in situ action of a metal center. It plays a very important role in the formation of the macrocycle. This ion, in fact, may be able to direct the course of the reaction and the effect that emerges is called "metal-template effect".

To designing a metal-template synthesis, it is necessary to choose carefully the metal ion. The coordination of ligands to metal ions involves electronic factors (HSAB theory) as well as geometric relationship (preferential coordination geometry and ionic radius) between these two parts.

The need to obtain the chiral azamacrocycle \( M_1 \) of interest derives from the peculiar behavior already observed for meso compound \( M \) (Fig. 1), synthesized by professor Marcantoni’s research group.

The racemate azamacrocycle \( M \) was studied by the research group of Prof. Speranza and Prof. Filippi, and it showed a particular affinity towards potassium ions in presence of an acidic species, in gas phase analyses. Indeed the ESI-MS spectrum of macrocycle \( M \) only shows an important intensity of the \([M\cdot H]^+\), with a less extent the \([M\cdot Na]^+\), while the \([M\cdot K]^+\) signal is almost not observable. Surprisingly this trend is reverse when inside the sample an acidic species, such as HCl, HF and amino acids, is put: the \([M\cdot K]^+\) signal increases a lot its intensity whilst \([M\cdot H]^+\) and \([M\cdot Na]^+\) intensities are lower. So infrared multiphoton dissociation and collisional induced dissociation were then used to try to measure the azamacrocycle affinity towards potassium ions within the species \([M\cdot K \cdot A]^+\).

Both IR-MPD and CID gave unusual results. It is easy to understand that less energy is required to break non-covalent interactions...
with respect to covalent ones. So initially, removal of potassium or the acidic species was supposed to happen, but raising irradiation or collisional energy leads just to uncontrolled fragmentation. This result was surprising, so that great interest aroused in determining the causes of this particular behavior. On the other hand, this analysis couldn’t furnish substantial elucidation because of missed fragmentation.

To help the right interpretation of this data, theoretical studies have been performed by a biophysicist at University of La Sapienza, in Rome. Results of preliminary calculations have been obtained through a simulating annealing with classical force field; simulated annealing is a strategy that is used to solve optimization problems and it is carried out heating at high temperature (1000 K or even more) the structures of interest for a certain time and then cool them. This was done in 1000 conformations of these structures. This procedure allows to overcome the energies related to the torsional barriers, in order to obtain a more detailed conformational investigation. Moreover, classical forced field means that the molecular structure is represented by balls (atoms) and springs (bonds), so it is not possible to simulate the creation and breaking of bonds or proton transfer. Structures with the lowest energy (typically chosen within a range of 5 kcal-mol\(^{-1}\)), obtained with this procedure, were then chosen to perform static quantum calculation, that is without considering the time evolution and temperature. The method used is the ab intio density functional theory (DFT).

The results of these preliminary calculations shows that the protonated host has a major stability when protonation occurs on aliphatic nitrogen with respect to the protonation on pyridine one. The energy difference is about 6 kcal-mol\(^{-1}\) and it is easily justified by the greater basicity of aliphatic amine than that of aromatic ones. This behavior was observed for the complex [M\(\cdot\)K\(\cdot\)HCl]\(^+\) (Fig. 2).

![Fig. 2](image)

**Fig. 2** [M\(\cdot\)K\(\cdot\)HCl]\(^+\) complex view (bond length expressed in angstrom). The most stable structure is found to bind hydrogen chloride, that is dissociated in H\(^+\) and Cl\(^-\). Its coordination is more stable on aliphatic nitrogen with respect to that one on aromatic nitrogen, with an extent of 1.9 kcal-mol\(^{-1}\).

On the contrary in [M\(\cdot\)K\(\cdot\)H\(_2\)O]\(^+\) and [M\(\cdot\)K\(\cdot\)HF]\(^+\) complexes the dissociation isn’t observed (Fig. 3).

![Fig. 3](image)

**Fig. 3** [M\(\cdot\)K\(\cdot\)H\(_2\)O]\(^+\) complex (above) and [M\(\cdot\)K\(\cdot\)HF]\(^+\) complex (below) view (bond length expressed in angstrom).

However in every case, the potassium ion is closely coordinate to the aromatic nitrogen (Fig. 4).
Till now gas phase studies, together with theoretical ones, gave just preliminary results and further deepened investigation are required. However the preamble is of great interest, so that collaboration between organic, inorganic and computational chemists is strongly motivated, in order to shed light on the reason of the particular affinity of macrocycle $M$ toward potassium ions.

The general idea of the collaboration is to make systematic structural variations on macrocycle $M$, that represents the starting point of our project, in order to synthesize different azamacrocycles, which will be investigate in the same manner. In fact, in this way, the moieties and the structural features, that are responsible of the strong binding with potassium, should be determined.

**Results and Discussion.** The first structural variation, as previously said, is represented by the synthesis of the enantiomerically pure hexaaazamacrocycle $M_1$ (Fig. 1). The reason of this choice resides on the fact that great conformational variation of the internal cavity could be induced using enantiopure diamines as reagents. In fact, observing the two imino type diastereoisomers (Fig. 5), from a structural point of view, one could note that the spatial dispositions are different. They adopt two different conformations, that could strongly influence the coordination to a metal ion. The meso compound could adopt a chair conformation, while the enatiopure form arranges itself according a twist conformation.

Moreover, these ligands are able to adjust their conformation to match the size of coordinated metal ion. This adjustment is based on combination of various degree of bending and helical twisting of the macrocycle.$^{8,9}$ Basing on the assumption that also the corresponding hexaza macrocycle amines have different behaviors on coordination of metal ions, this structural variation has been approved to be the first one to try.
Metal-free synthesis of entiomerically pure azamacrocycle

The first step was to synthesize the dialdehyde, which can be generated in various ways, either starting from 2,6-lutidine and from the corresponding alcohol (2,6-dihydroxymethylpyridine). The higher yields, generally, are obtained from the alcohol, so the latter have been chosen as the starting material (Scheme 2). It could be oxidized through two hypothetical reaction pathways: a) Swern oxidation,\textsuperscript{10} b) Oxidation by SeO\textsubscript{2}.\textsuperscript{11}

\begin{center}
\textbf{Scheme 2} Possible synthetic pathway of 2,6-diformylpyridine.
\end{center}

The second pathway, however, represents the favored choice because of the bad smell that diffuses carrying out the Swern oxidation. The dialdehyde 2 have been obtained as white crystals with a yield of 80%. Its formation is confirmed by GC-MS: the gaschromatogram in fact contains only one peak (R.T. = 6.93 min), whose pattern of fragmentation corresponds to that one of the desired aldehyde. The IR spectrum is also useful to confirm the formation of the carbonyl group with the presence of a strong band at 1711 cm\textsuperscript{-1}. Also \textsuperscript{1}H NMR spectrum underlines the formation of formyl moiety by the singlet at 10.17 ppm, relative to the -CHO proton and the lack of signal relative to hydroxyl proton. Often the synthesis does not conduct to the pure product, but a purification could be carried out by column chromatography. This synthesis has been performed from time to time because of the instability of the 2,6-diformylpyridine towards oxidation. That’s also the reason why it is not purchased directly.

Once that the starting material has been synthesized, the condensation reaction between dialdehyde and diamine has been carried out in order to obtain the [2+2] macrocycle of interest. Considering the relevant factors and the approach used to synthesize the meso form M, this reaction was carried out under conditions of high dilution and in a protic polar solvent such as methanol, using the starting materials in a stoichiometric ratio 1:1. These measures promote the cyclization reactions rather than those of the formation of oligomers. The control of the progress of the reaction has been effectuated by ESI-MS analysis because of thermal instability of the obtained products and so the impossibility to use other common analysis such as gas chromatography or gas chromatography interfaced with electronic ionization mass spectroscopy. At the end of reaction the formation of two macrocycles was observed (Scheme 3).

\begin{center}
\textbf{Scheme 3} Condensation reaction of 2,6-diformylpyridine with \((R,R)\)-1,2-diaminocyclohexane.
\end{center}

The ESI-MS spectrum of the reaction mixture showed a signal m/z = 427, relative to the
protonated macrocycle 4, but also an intense signal with m/z = 640, corresponding to the protonated macrocycle 6, that results from the condensation of three dialdehyde units and three diamine units. The formation of [3+3] product is confirmed by a work of Gregolinski et al.\textsuperscript{12}

Before the adoption of another strategy of synthesis, the reduction of the imine products has been carried out in order to shelve the equilibrium between them and to determine the relative concentration. Another reason of this attempt was to verify if, in the reduction step, a perturbation of the equilibrium could be provoked and just one of the macrocyclic amine formed, as in the case of the meso compound M. Indeed, once a macrocyclic imine has formed through the condensation between dicarbonyl compounds and diamines, it is able to undergo reduction of imine moieties, leading to the formation of the corresponding macrocyclic amines, that are more stable towards hydrolytic decomposition. The reduction can be carried out using several reagents, but the most used is surely sodium borohydride, that could be directly added to the reaction mixture after the macrocyclization step. The commercial availability, the easy handling and simpleness of synthesis make the sodium borohydride the best choice. Due to the esothermic reaction, the released heat could perturb the equilibrium established in the macrocyclization step, so in some particular cases an ice bath is used during the addition of the reagent. Unlike in the synthesis of the meso amine M, macrocyclic amines M\textsubscript{1} and 7 were obtained as a mixture (Scheme 4), which was analyzed by \textsuperscript{1}H NMR in order to evaluate the integration of signal and stabilize the relative concentration, that otherwise has not been possible with gas chromatographic analysis. Unfortunately this strategy hadn’t a positive response because of the similar nature of the two products: they have same structural features and they differ only in ring size. This leads to the superimposition of signals, preventing the right interpretation of the integrations.

![Scheme 4 One-pot synthesis of hexaaza macrocyclic amines.](image)

Nevertheless, separation of the macrocyclic amines M\textsubscript{1} and 7 has been tried, in order to recover the product of interest. The first methodological approach has been consisted in chromatography, although in literature no clear way to elute this type of compound was found. Several mobile phases were investigate through thin layer chromatography in order to find effective conditions for a valuable separation. The difficulty was in finding a means to allow macrocycles M\textsubscript{1} and 7 to run along the TLC. In fact these species are extremely basic and has strong interaction with silica that is acidic. These interactions led to stripes rather than precise spots. Putting triethylamine or formic acid into the eluent or dabbing the silica did not lead to a real improvement, so this attempt to separate the two polyamines was abandoned.
Searching another solution in literature, a purification of [3+3] macrocyclic amine via precipitation in dichloromethane/acetonitrile mixture was found. The idea was to precipitate the [3+3] macrocycle and recovering the filtrate. Unfortunately, although several successive precipitations were performed, a sufficient level of purity of [2+2] macrocycle wasn’t reached.

Metal-free synthesis was then tried again, leaving the reaction mixture of the macrocyclization step to reflux for a prolonged period of time. This idea arose from a work of Kunhert et al. In fact they have synthesized macrocyclic systems similar to those of our interest, starting from (R,R)-1,2-diaminocyclohexane and 5-methyl-1,3-benzenedicarboxaldehyde. They found that [3+3] cyclocondensation product have formed under kinetic control, while [2+2] cyclocondensation products points towards these macrocycles as the products of thermodynamic control (Scheme 5).

Scheme 5 Example of conversion of the kinetic [3+3] product to the thermodynamic one.

So, the possibility to convert totally the [3+3] side product (6) in [2+2] product of interest (4) was considered as a chance to try (Scheme 6).

Scheme 6 Failed attempt to convert [3+3] product into the [2+2] one.

Nevertheless, in ESI-MS spectrum, no changes in signal intensity was observed, regarding both the [3+3] and [2+2] cyclocondensation products, after several hours of reflux.

Furthermore, also some experiments were conducted with microwaves assistance instead the classical heating, in order to investigate if the use of microwaves could accelerate the rate of formation of [2+2] cyclocondensation product. Both dichloromethane and methanol were used as solvents (Scheme 7): in fact different temperatures could be reached in shorter time lapses. Moreover, methanol and dichloromethane, having different polarity, were supposed to be able to stabilize the two macrocycles 4 and 6 with different extent.

Scheme 7 Microwave assisted macrocyclization between diamine and dialdehyde in a) DCM, MW, 100°C, 6 bar and b) MeOH, MW, 160°C, 16 bar.
At the end of several attempts to find a way to obtain the macrocycle $\mathbf{4}$ pure with a metal-free synthesis, unfortunately no effective conditions have been found both for the purification step and for the selective synthesis. This conclusion led to the complete change of the strategy of synthesis.

**Metal-template synthesis of enantiomerically pure azamacrocycle**

The template synthesis has been the alternative means to take into account, in order to obtain selectively the enantiopure hexaazamacroyclic imine $\mathbf{4}$ and successively, with the reduction of imine moieties, the enantiomerically pure macrocyclic amine $\mathbf{M_1}$. In fact, often metal-free synthesis offers different advantages with respect to the template one, but, on the other hand, in some case it does not allow to reach efficient results, in terms of yield and selectivity, as in the case of our study. On the contrary, template synthesis could permit to obtain selectively the product of interest among the several possible products, cyclic or acyclic ones, simplifying the process of synthesis and increasing the yield.

Taking into account the guidelines mentioned above, a metal ions screening has been performed in order to investigate the appropriate choice, as reported in Scheme 8. In literature barium (II) has been found as good templating agent, to together with lanthanides (III).

![Scheme 8 Template syntheses of hexaaza tetratimine macrocycle 4 performed using different metal salts.](image-url)
The metal ion screening was based on the idea of using the same procedure and the same reaction conditions for all the different trials, in which only metal ion has been changed, in order to observe just its effect on the pathway of synthesis. Synthesis procedures have been inspired by a work of Busto and coworkers: they consisted in dissolving the two starting materials in a mixture of methanol and dichloromethane. The reaction mixture was stirred for 15 minutes before metal salt addition. After about 15 hours the macrocyclization was complete and reduction was carried out by adding sodium borohydride in large excess. Metal salts have been chosen also on the base of the counter ion: in fact generally nitrate or chloride salts are found in literature in this type of reactions. Describing the general trend of all template reactions, surely that one carried out using dry barium chloride as templating agent has given the best result (Scheme 9).

After work up, the enantiopure hexaaza tetramine macrocycle \( M_1 \) has been obtained pure, as light brown oil, without need of purification and with high yield (85%). Its identity has been determined by ESI-MS analysis and NMR analyses. The ESI-MS spectrum shows exclusively the protonated and diprotonated macrocycle \( M_1 \) adducts and the adduct with sodium.

Also \(^1\text{H} \) NMR and \(^{13}\text{C} \) NMR, both recorded in CDCl\(_3\), give evidence of the presence of a single macrocycle form. Both spectrums don’t present signal relative to nuclei belonging imine moieties, suggesting that the reduction step proceeded easily to completion. This results is extremely important, not only because the selective formation of [2+2] macrocycle adduct, but also because of the obtained high yield. In fact, the synthesis of chiral polyazamacrocycles, that incorporated trans-cyclohexane-1,2-diamine, are not trivial, due to the low yields usually associated to the key macrocyclization step.

Using BaI\(_2\)-H\(_2\)O as templating agent in the same reaction conditions, the results changed (Scheme 10).

ESI-MS spectrum of the crude product shows an intense signal with m/z = 435 that is referred to the protonated macrocycle \( M_1 \). Unfortunately, the synthesis wasn’t selective and two intense signals with m/z = 453 and m/z = 497 are present. Some assumptions has been proposed to interpret the identity of chemical species responsible of these signals. Regarding signal with m/z = 453, it was hypothesized that could represent both the protonated macrocycle \( M_1 \) coordinated with a water molecule, both the acyclic [2+2] product 10 (Fig. 6).
Regarding signal with m/z = 497, the only by-product supposed, that could be associated to this one (Fig. 7), derives from incomplete reduction.

In doing these hypotheses, confirmation was search in NMR spectrum. $^1$H NMR spectrum suggests the presence of more than one species. However, certain conclusions couldn’t be develop because of the complexity of the spectrum, also due to signal superimposition. Separation of all by-products should be performed in order to identify their nature individually. The lack in efficiency of the process, with respect to the previous one, could derive both from the nature of the counter ion or, probably, from the presence of two water molecule of crystallization.

After barium, cerium (III) has been investigated as possible templating agent. In particular Ce(NO$_3$)$_3$·6H$_2$O and CeCl$_3$·7H$_2$O have been chosen. The first because it is usually used in such kind of reactions, the latter because of several characteristics that make it a good candidate to act as a promoter. Firstly, of course, relates to its low toxicity and its low cost, which allows use on a large scale, and therefore also at the industrial level. As a second feature, however, we find its good stability and activity in the presence of both air and water. In reality, the system CeCl$_3$·7H$_2$O, if used as the sole initiator of reaction, seems to have a low activation processes in which it is involved. In fact, it has been observed that the addition of a salt of iodine in the system, in particular NaI, is essential for its implementation and, therefore, for a good outcome of the reaction.

The synthesis in which Ce(NO$_3$)$_3$·6H$_2$O has been used as templating agent (Scheme 11), led to the formation of the target molecule.

As the previous synthesis, also in this case the ESI-MS shows a signal with m/z = 453, which origins has been discussed above, but also a more intense signal with m/z = 357. It has been associated to an acyclic product of the type [1+2], i.e. generated by the condensation of one diamine unit and two dialdehyde units (Fig. 8), followed by the reduction of both imine moieties and formyl groups.
The formation of acyclic by-products was also observed in synthesis using CeCl₃·7H₂O, activated by the promoter NaI (Scheme 12).

The recurring fail on macrocycle closure, encountered especially with cerium salts, led to suppose that something went wrong during the coordination of starting materials to the ion templating agent: in fact, as said previously, the formation of a macrocycle happens because of the juxtaposition of diamine and dialdehyde units on coordination to the metal center. So possible explanation of the reason why the cyclization does not go to completion could be represented by the ineffective disposition of starting materials that prevents the condensation or by the lack of vacant coordination sites that should be available for the reactants. The latter represents a better hypothesis: in fact in most cases macrocyclization occurs, so effective prerequisites for the macrocycle closure are present. The explanation could reside on the difficult coordination of starting materials, due to the presence of other ligands, such as water molecules. This hypothesis suggested to try the template synthesis of the macrocycle M₁ using cerium trichloride dry in order to see if it was more active, but the formation of the target molecule wasn’t observed. Other explanations of this behavior should be search.

Negative results have been obtained using CeCl₃·7H₂O, activated by CuI/I₂. Theoretically the system CuI/I₂ should break the oligomer structure of cerium trichloride and the cerium ion should act as template reagent. However, the macrocycle M₁ wasn’t formed (Scheme 13).

In both reactions that include the use of copper (II) a signal with m/z = 274 has been observed, that could be associated to a [1+1]
product (Fig. 9), coordinated to a potassium ion.

Furthermore, optically active [2+2] macrocycle, adopting a twisted conformation, generates an atypical coordination sphere to host just one transition metal ion, while it can coordinate easier Ln(III) ions. So also the preferred coordination geometry of a metal ion and the possibility of adjustment of the macrocycle to this one influences greatly the course of the reaction. In fact it was observed that Ag(I), although it has an ionic radius similar to that one of Ba(II), doesn’t allow the formation of macrocycle M₁, but the ESI-MS spectrum evidences the presence of the [3+3] macrocycle 7, associated at an intense signal with m/z = 652.

At this point, knowing that purification of macrocyclic species was extremely difficult, a third and last attempt to isolate and determine the relative yield of the obtained macrocyclic adduct M₁, especially in all template reactions, has consisted in finding a valuable method to adopt using column chromatography technique. Working in a reverse phase, several mobile phases were tried, such as several mixtures of water and methanol and methanol solution of formic acid (1%). Also in this case a problem arose: the macrocycle M₁ interacts so strongly with the stationary phase of the used column that it was not able to come out of the column. The use of a specific column for highly polar compounds was needed in order to performed the effective separation of crude.

**Conclusion.** Through this work, we have learned the plethora of factors that influence macrocyclization reactions and how to control them, in order to direct selectively the synthesis to the target products. In fact, the synthesis of azamacrocycle through the formation of Schiff bases, has been a perfect example of how many ways can be accessed, using relative simple, difunctionalized starting materials. We’ve also learned how chirality plays an extremely important role, particularly in synthesis: indeed, using an enatiopure diamine as reactant instead a racemic mixture, the results, in trying to obtain hexaaza tetramine macrocycle [2+2], are totally different and mostly dependent on dynamic equilibria establishment and conformational stability of final adducts. In summary, the synthesis of chiral hexaaza tetramine macrocycle has been carried out in high yields (85%) by a templated one-pot two-steps process, starting from \((R,R)-1,2\)-diamincyclohexane and 2,6-diformylpyridine and using BaCl₂ as templating agent. Also cerium has showed a templating potential relative to the formation of the [2+2] product of interest, although the synthesis isn’t already optimized and needs further investigation, while transition metal ions failed as templating agents.

Future prospect are directed towards the development of an column chromatography method able to allow effective purification of the target macrocycle. Also the monitoring of template synthesis is an interesting tool to understand more deeply the process of macrocyclization. From the point of view of gas phase studies, surely the enantiopure macrocycle will be tested as the same as the meso form, in order investigate its particular
behavior and to planned further structural variations.

**Experimental Section.**

**Materials.** Reactions are monitored through thin layer chromatography on Merck silica gel plates Kieselgel 60 F254, through GC on a gaschromatograph 6850 Agilent Technologies, with capillary column (0.32 mm x 30 m) and stationary phase OV1 Agilent of 0.40-0.45 μm and through a FID detector. Mass spectrum are obtained by a gaschromatograph interfaced with a mass spectrometer Hewlett-Packard GC/MS 6890N that works with the EI method (70eV), or by an HPLC Mass spectrometer Hewlett-Packard 1100 MSD series model G1946A, with a column C18 Lichrospher 100 and mass spectrometer API-ES, in positive mode.

Characterization of products is effectuated through mass spectrometry, infrared spectroscopy and $^1$H and $^{13}$C nuclear magnetic resonance. IR spectrum are obtained with an IR spectrophotometer Perkin-Elmer 1310 in the 4000-600 cm$^{-1}$ range. NMR spectrum are acquired with a spectrometer Varian Mercury Plus 400, operating at 400 MHz, using various deuterated solvents. Chemical shifts are expressed in δ (ppm) regard to the not deuterated solvent. The following abbreviation are used: s = singlet, d = doublet, t = triplet, q = quartet, quint. = quintet, bs = broaded singlet, dd = double doublet, dt = double triplet, tt = triple triplet, m = multiplet. Reactions under microwave irradiations were performed using Biotage Initiator Microwave Reactor with the follow technical feature: temperature range (40–250°C), heating rate (2-5°C/sec), pressure range (0-20 bar), power range (0-400W) with magnetron (2.4 GHz), and variable magnetic stirrer. Substrates, reactants and solvents are acquired from common commercial sources and used as received or, if necessary, purified by distillation.

**2,6-diformylpyridine (2).** A 50 mL two neck flask equipped with a cooling system is treated with a nitrogen flow and the entire system is dried. 2,6-bis(hydromethyl)pyridine (0,400 g, 2.9 mmol) is added to dry dioxane (15 mL), creating a suspension. SeO$_2$ (0,322 g, 2.9 mmol) is added to this suspension and the mixture is refluxed. When the reaction is complete (control with TLC, and CHCl$_3$:MeOH mixture (9:1) as eluent), the reaction mixture is filtered using celite and washing with dioxane. The product is purified by chromatographic column using CHCl$_3$:EtOAc (8:2) as eluent. White crystals are obtained. Yield: 80%. IR (cm$^{-1}$): 3084m, 3018w ($\nu$ C-H); 2861m ($\nu$ C-H, formyl); 1711s ($\nu$ C=O). GC analysis (RT, min): 6,93. MS-MS m/z (%): 135(M$^+$), 107 (100%), 86, 78, 52, 44, 38, 29. $^1$H-NMR (400MHz, CDCl$_3$): δ 8.04-8.08 (m, 1H, γ-pyridine); δ 8.18 (d, 2H, J = 7.27, β-pyridine); δ 10.17 (s, 2H, -CHO).

**Hexaazatetramine macrocycle (M$_{1}$).** (R,R)-1,2-diaminocyclohexane (0.6 mmol, 68 mg) is dissolved in a mixture of methanol (5 mL) and dichloromethane (5 mL). Then pyridine-2,6-dicarbaldehyde is added to the reaction mixture that is stirred for 15 minutes. Then BaCl$_2$ (1.2 mmol, 250 mg) is added and the reaction mixture is stirred for 15 hours at room temperature. Then NaBH$_4$ (2.4 mmol) is added in three portions to the reaction mixture, which is stirred for other 7 hours at room temperature. The reaction is quenched with HCl conc. (0,75 mL). Then NaOH 4N (10 mL) is added. Extraction with CH$_2$Cl$_2$ is done and the combined organic phases are dried over Na$_2$SO$_4$. Filtration, and evaporation under reduced pressure give the pure [2+2] product. Yield: 85%. ESI-MS m/z (%): 435 (MH$^+$), 457 (MNa$^+$), 218 (M+2H$^+$). $^1$H-NMR
(400MHz, CDCl$_3$): δ 0.97-1.09 (m); δ 1.58 (d); δ 2.06-1.97 (m, NCH$_2$); δ 3.64 (s, br, NH); δ 3.74 (d, 4H, NCH$_2$); δ 4.02 (d, 4H, NCH$_2$); δ 6.99 (d, 4H, β-pyridine); δ 7.49 (t, 2H, γ-pyridine). $^{13}$C NMR (400MHz, CDCl$_3$): δ 24.86 (cyclohexyl); δ 32.814 (cyclohexyl); δ 51.51 (NCH$_2$); δ 59.46 (NCH); δ 121.38 (β-pyridine); δ 136.95 (γ-pyridine); δ 160.26 (α-pyridine).

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