Synthesis, characterization and low-toxicity study of a Magnesium(II) complex containing an Isovanillate group

Rafael C. Marchi[a], Eldevan S. Silva[a], Josenilton J. Santos[a], Izonete C. Guiloski[b], Hugo Cesar R. de Jesus[a], Inara de Aguiar[a], Flavio V. C. Kock[a], Tiago Venâncio[a], Maria Fátima G. F. da Silva[a], João Batista Fernandes[a], Maria A. B. F. Vital[b], Leonardo Castro Souza[b], Helena C. Silva de Assis[b], Leif H. Skibsted[c*], Rose M. Carlos[a*]

[a]Departamento de Química, Universidade Federal de São Carlos, Rodovia Washington Luís, km 235 CP 676, CEP 13565-905, São Carlos, SP, Brazil.

[b]Departamento de Farmacologia, Universidade Federal do Paraná, Setor de Ciências Biológicas, 81531-980, Curitiba, PR, Brazil.

[c]Department of Food Science (UCPH FOOD), University of Copenhagen, Rolighedsvej 26, DK-1958 Frederiksberg, Denmark.

**Corresponding author. +45 35333221*

Email address: ls@life.ku.dk

**Corresponding author. +55 16 33518780*

Email address: rosem@ufscar.br

Experimental Session

Impact of complex on aquatic life. Toxicity studies were conducted with Zebrafish at the Pharmacology Department of the Federal University of Parana - UFPR, Curitiba, Parana state, Brazil. Specimens were divided into four groups of 10 fish each. Three groups were exposed to 0.2, 1, and 2 mg L^{-1} of complex and one group was left without complex as a control. The Zebrafish specimens (0.35 ± 0.01 g wet body mass; 3.4 ± 0.04 cm total body length; mean ± SD) were purchased from “Aquaritiba Aquários e Peixes Ornamentais Ltda”, Curitiba, Brazil, and transported to the Pharmacology Department of the UFPR, Brazil. The fish were divided in tanks of 20.0 L and acclimated at 25 ± 1 °C with dechlorinated water under constant aeration for eight days and maintained on a constant 12:12 h (light:dark) cycle. They were fed with commercial fish Alcon® diet with 30.0% protein. This project was approved by Ethics Committee by Animal Experimentation of the Federal University of Parana under protocol number 943. The bioassay was static with duration of 96 hours. After exposure, all fish specimens were anesthetized in water containing benzocaine ($0.1gL^{-1}$), euthanized by medullar sectioning, measured (total body length) and weighed (wet body mass). After that, they had the digestive tract (liver + intestine), and brain dissected and stored at‑80 °C for biochemical analysis. The samples (digestive tract) were homogenized in phosphate buffer (0.1 M) at pH 7.0 and centrifuged at 10.000 g at 4°C for 30 min. Brain was homogenized in phosphate buffer (0.1 M) at pH 7.5 and centrifuged at 10.000 g at 4°C for 20 min. Superoxide dismutase (SOD) activity was based on the ability of SOD to inhibit autoxidation of pyrogallol. The sample was added to buffer solution (Tris-base 1.0M/EDTA 5.0 mM pH 8.0) containing pyrogallol (15.0 mM) and incubated in a microtube for 30 min. After that, the reaction was stopped with hydrochloric acid 1.0

M. Reading was performed at 440 nm. Activity was expressed as U/mg protein[1]. Catalase (CAT) activity was determined by monitoring the H_2O_2 decomposition[2]. The sample and the reaction solution (buffer Tris-base 1.0M/EDTA 5.0 mM pH 8.0, hydrogen peroxide 20.0 mM, and MilliQ water) were added to a microplate. Reading was performed 240 nm. Activity was expressed as µmol/min/mg protein. Glutathione-S-transferase (GST) activity was determined using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates[3]. The sample and reaction solution [GSH 3.0 mM, CDNB 3.0 mM] were added to a microplate[3]. Reading was performed at 340 nm and the activity was expressed as nmol CDNB/min/mg protein[3]. Lipoperoxidation (LPO) was conducted by ferrous oxidation–xylenol assay[1,4]. The supernatant of the samples was re-suspended in methanol at a 1:2 (v/v) and centrifuged for 10 minutes at 10.000 xg at 4 °C. After that, 100.0 µL of supernatant were mixed with 900.0 µL of reaction solution (100.0 µM xylenol orange, 25.0 mM $H_2SO_{4(aq)}$, 4.0 µM butylated hydroxytoluene (BHT)) and 250.0 µM $FeSO_4NH_4$ (ammonium ferrous sulfate) and added in this specific order to 90% grade methanol. After 30 min of reaction at room temperature, absorbance was measured at 570 nm and LPO was expressed as nmol hydroperoxides/mg protein. Acetylcholinesterase (AChE) activity in the brain was determined by the following procedures: Sample and 0.75 mM of 5.5-Dithiobis-2-nitrobenzoate and 10.0 mM of acetylthiocholine substrate were added to a microplate[5,6]. The activity was measured spectrophotometrically at 405 nm. The protein concentration was determined by the method proposed by Bradford[5,7], with bovine serum albumin as the standard.

Impact of complex on terrestrial life. In this study, Wistar male rats, 4 months old, weighing 230 - 270 g were obtained from the central bioterium of the Federal University of Parana - UFPR. The rats were housed in groups of 5 per cage, with controlled temperature and humidity, and maintained on a constant 12:12 h (light:dark) cycle, starting at 7 a.m. Food and water were provided *ad libitum*. All procedures were approved by the Ethics Committee for Animal Experiments at the Biological Sciences Department of the Federal University of Parana (protocol number 924). The male Wistar rats were divided into four experimental groups. Three groups received saline solution (5% m/v control) containing 3 mg Kg^{-1} per daily dose of complex intraperitoneally for 21 days. The control group received only the saline solution. The motor-activity and anxiety related behaviors of the rats were tested on day 22 in an open field, forced swimming, and elevated plus maze tests, respectively. These behavioral tests were conducted as described previously[7-12].

After completion of the behavioral studies on day 23, the rats were anesthetized and euthanized by decapitation. Their brains, livers, and kidneys were rapidly removed and stored at -80 ^{o}C. The neurotoxicity of complex in the cerebral regions: frontal cortex, hippocampus, and striatum was evaluated according to the acethylcholinesterase activity using the Ellman method[6]. The digestive-tracts (liver and kidney) were removed and the oxidative parameters were evaluated according to the catalase,[2] glutathione S-transferase[3], and superoxide dismutase[1] activities. All statistical analyses were performed using the GraphPad Prism 6.01 software.

Diffusion ordered Spectroscopy on Nuclear Magnetic Resonance, DOSY-NMR. To obtain the DOSY-NMR mapsa pulse sequence with eddy current minimizing, employing a gradient length of 1400 us (litlle delta), 5 ms of longitudinal eddy current delay (LED) and 200 us of recovery time between gradients were applied. A total diffusion time of 60 ms (big delta)was used. Sixteen different gradient amplitudes were used; therefore, the strength of the gradients was kept varied from 1.07 $G.cm^{-1}$ and 53.5 $G.cm^{-1}$. For a good signal to noise ratio 16 scans were coadded, separated by 3 s of recycle delay. FID signal were aquired by collecting 32k data points. For data acquisition and processing the software Topspin (version 3.5 pl 7)was employed.

Mass Spectrometry. ESI-QTOF-MS (electrospray ionization quadrupole time-of-flight mass spectrometry) measurements were performed in the positive mode on a Xevo G2 instrument (Waters Co). The full-scan MS data were produced across the mass range of 50-1200 Da. For the MS/MS experiments by collision-induced dissociation (CID), argon was used as collision gas and collision energy was increased until sufficient fragmentation of the precursor was observed. Samples were dissolved in methanol and introduced into the ESI source via direct infusion through a 250 uL glass syringe and were purged with >1mL of pure methanol between each sample. The ESI source conditions were as follows: capillary voltage 1.5 kV, sample cone voltage 30 V, cone gas flow (N2) 50 L/Hr, desolvatation gas flow 750 L/Hr, source and desolvation temperatures were set to 100 °C and 300 °C, respectively. Data were analyzed with use of MassLynx 4.1 software.

DFT calculations. All calculations were performed using the Gaussian 09 (G09) software package D.01 edition,[13] employing the DFT method with Becke's three-parameter hybrid functional[14] and Lee-Yang-Parr's gradient-corrected correlation functional, (B3LYP)[15] in combination with 6-31+G* basis set.[16] The ground-state geometry of the Mg complex was optimized in gas phase. SCF-tight convergence criteria were used for all optimizations. Harmonic vibrational frequencies were computed at the same level of theory to characterize the stationary points as true minima, representing equilibrium structures on the potential energy surfaces.

Cyclic voltammetry. Cyclic voltammetry was conducted with a bipotentiostat/galvanostat μStat400. A screen-printed electrochemical array formed of a working (d = 4 mm) and counter electrode made of glassy carbon and silver as reference electrode was used for measurements. The measurements were taken at room temperature in pure water and in a buffered solution (50 m M, pH 4.5, 7.4 and 10.0) containing 0.1 M of KCl as a supporting electrolyte and 5 mg of the compound.

Superoxide Radical Scavenging Assay. The superoxide radical ($O_2^{-\bullet}$) was generated by the system VitB2/Light/NBT (Halve et al., 2008). The IC_{50} (50% inhibitory concentration) values were calculated by linear regression of plots, where the abscissa *x* is the concentration of compound tested in μ $molL^{-1}$ and the ordinate *y* is the average percent of inhibition of the tested compounds.

Singlet Oxygen Assay. Singlet oxygen formation was monitored with 2,2,6,6-tetramethyl-4-piperidinol (TEMP) as a spin trap and electronic paramagnetic resonance spectroscopy (ESR).[17] Singlet oxygen $^1O_2$ was generated by riboflavin exposure in monochrome light at 420 nm. Singlet oxygen was generated in 1.0 mL of distilled water containing TEMP (0.1 $molL^{-1}$) and riboflavin (3.8 × 10-5 $molL^{-1}$). phenMgIso complex samples were prepared at different concentrations (1.6-28 μg mL^{-1}). The samples were irradiated at room temperature in a plastic cuvette for 30 min. IC50 values were calculated by nonlinear regression from the integration (relative area) of all radical EPR signals as a function of the concentration studied.

References

1. Gao, R.; Yuan, Z.; Zhao, Z.; Gao, X. Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. *Bioelectrochemistry Bioenerg.* **1998**, *45*, 41–45.

2. Aebi, H. Oxygen Radicals in Biological Systems. *Methods Enzymol.* **1984**, *105*, 121–126.

3. Keen, H., Jakoby, B. Mechanism for the Several Activities of the Glutathione 251. **1976**.

4. Jiang, Z.Y.; Hunt, J. V.; Wolff, S.P. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal. Biochem.* **1992**, *202*, 384–389.

5. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

6. Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961**, *7*, 88–95.

7. Delalande, C.D.R. **1978**. BEHAVIOURAL DESPAIR IN RATS : A NEW MODEL SENSITIVE TO ANTIDEPRESSANT TREATMENTS 1 .

8. Montgomery, K., n.d. THE RELATION BETWEEN FEAR INDUCED . BY NOVEL STTMULATfON AND EXPLORATORY BEHAVIOR the fear drive as well as the exploratory drive . novel stimuli evoke the exploratory drive.

9. Ramos, A., Mellerin, Y., Mormède, P., Chaouloff, F. A genetic and multifactorial analysis of anxiety-related behaviours in Lewis and SHR intercrosses. *Behav. Brain Res.* **1998**, *96*, 195–205.

10. Rénéric, J.P., Bouvard, M., Stinus, L. In the rat forced swimming test, NA-system mediated interactions may prevent the 5-HT properties of some subacute antidepressant treatments being expressed. *Eur. Neuropsychopharmacol.* **2002**, *12*, 159–171.

11. Rodgers, R.J., Cao, B.J., Dalvi, A., Holmes, A. Animal models of anxiety: An ethological perspective. *Brazilian J. Med. Biol. Res.* **1997**, *30*, 289–304.

12. Santiago, R.M., Barbieiro, J., Lima, M.M.S., Dombrowski, P.A., Andreatini, R., Vital, M.A.B.F. Depressive-like behaviors alterations induced by intranigral MPTP, 6-OHDA, LPS and rotenone models of Parkinson’s disease are predominantly associated with serotonin and dopamine. *Prog. Neuro-Psychopharmacology Biol. Psychiatry.* **2010**, *34*, 1104–1114.

13. Frisch, M. J.; et al. Gaussian 09; Gaussian, Inc.: Wallingford, CT, 2009.

14. Lee, C.; Yang, W.; Parr, R. G. Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Phys. Rev. B.* **1988**, *37*, 785.

15. A.D. Becke, Density-functional thermochemistry. III. The role of exact exchange, J. Chem.Phys, 98 (1993) 5648-5652.

16. V. A. Rassolov, M. A. Ratner, J. A. Pople, P. C. Redfern, and L. A. Curtiss, “6-31G* Basis Set for Third-Row Atoms,” *J. Comp. Chem.*, **22** (2001) 976-84.

17. Lü, J. Lin, P. H. Yao Q., Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems Chen, *J. Cell. Mol. Med.* **2010**, *14*, 840-860.

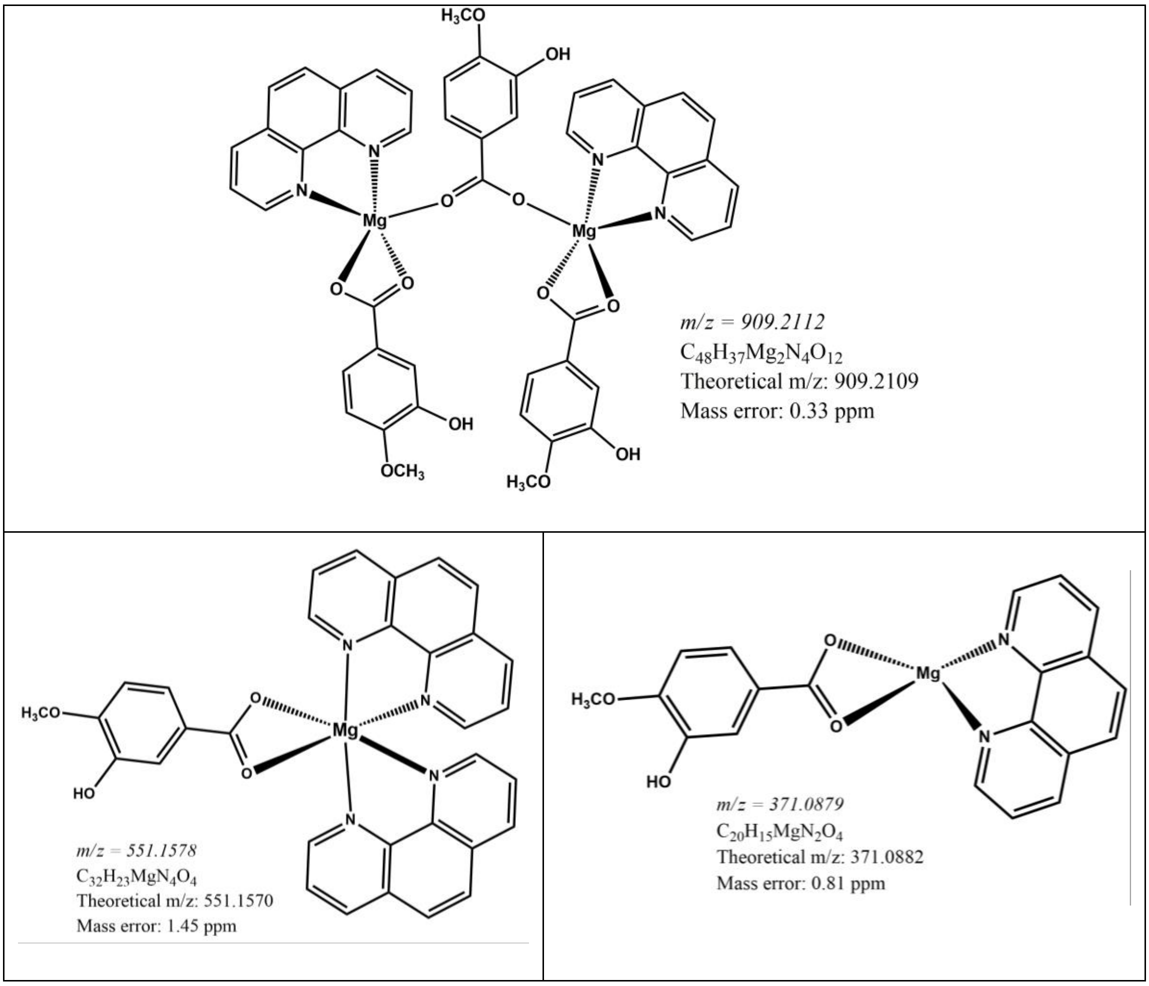


Figure S1. The structures observed in solution; the binuclear complex corresponding to Mg-bi (top) and the structures of the hexacoordinated (Mg-hex) and tetracoordinated (Mg-tet) complexes (bottom).

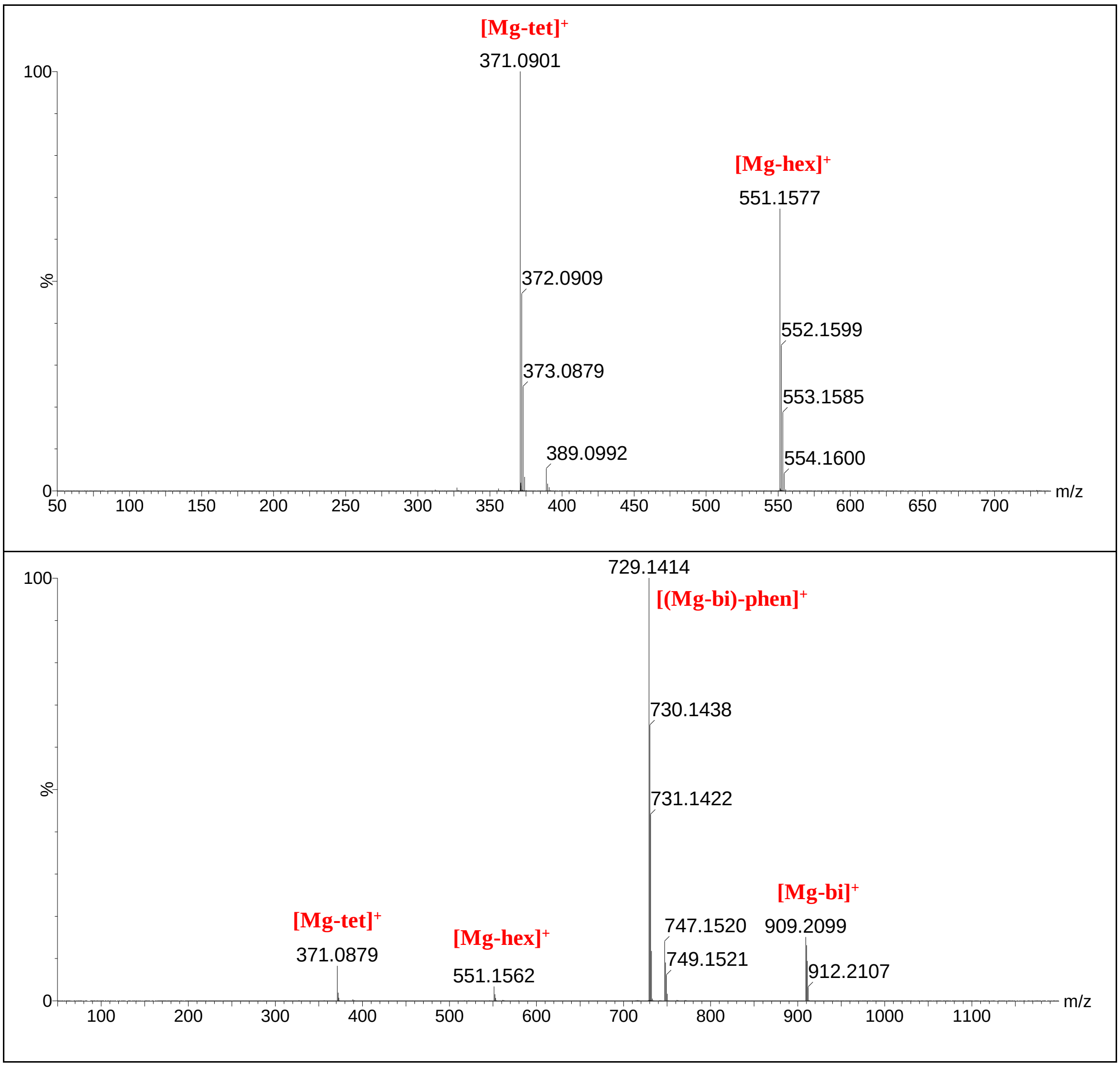


Figure S2. ESI(+)-MS/MS of the m/z 551.15 (top) and of m/z 909.21 (bottom) in methanol.

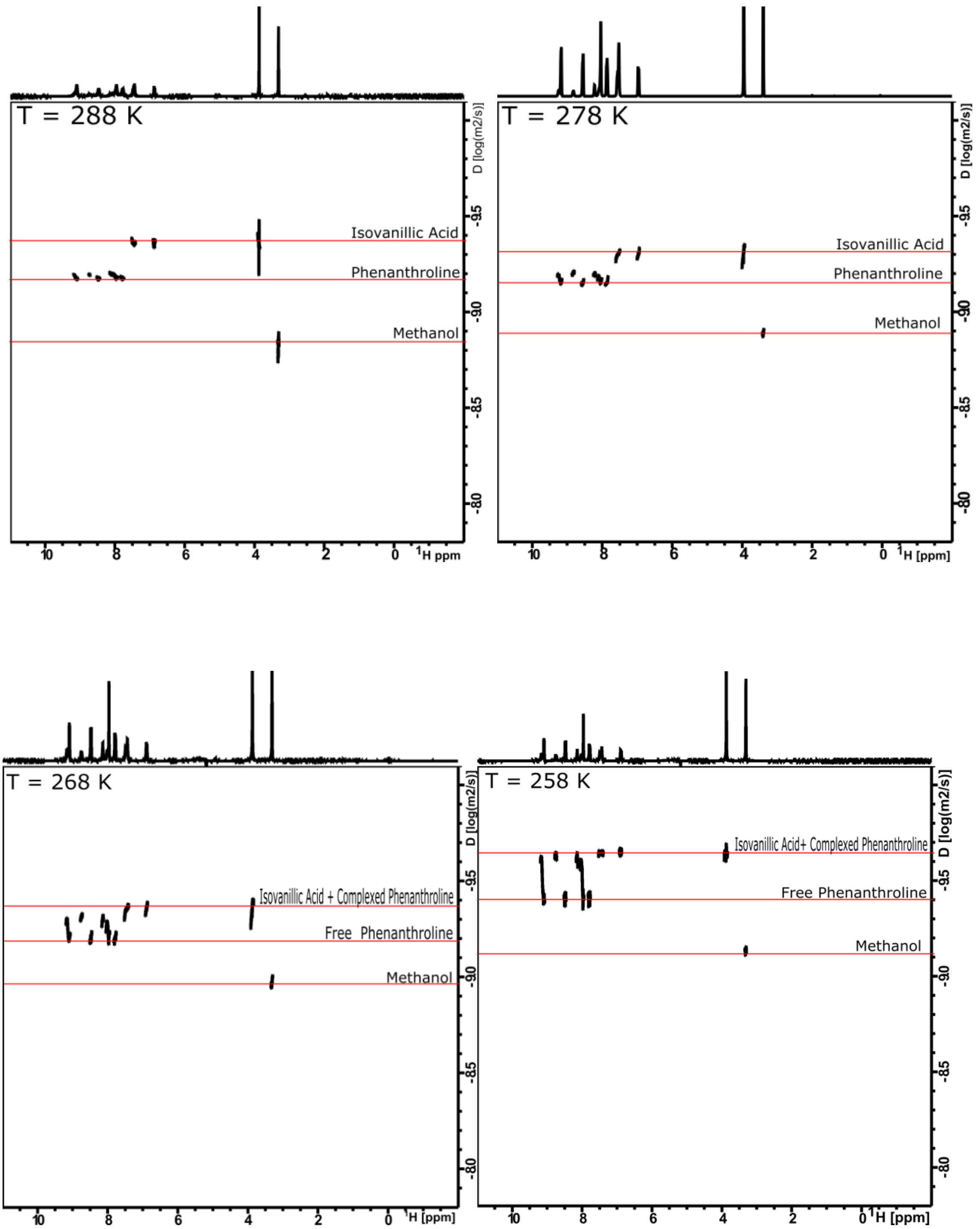


Figure S3. 1H-DOSY-NMR map obtained for the Mg-complex, at 288 K (top left-hand side), at 278 K (top right-hand side), at 268 K (bottom left-hand side) and at 258 K (bottom right-hand side).

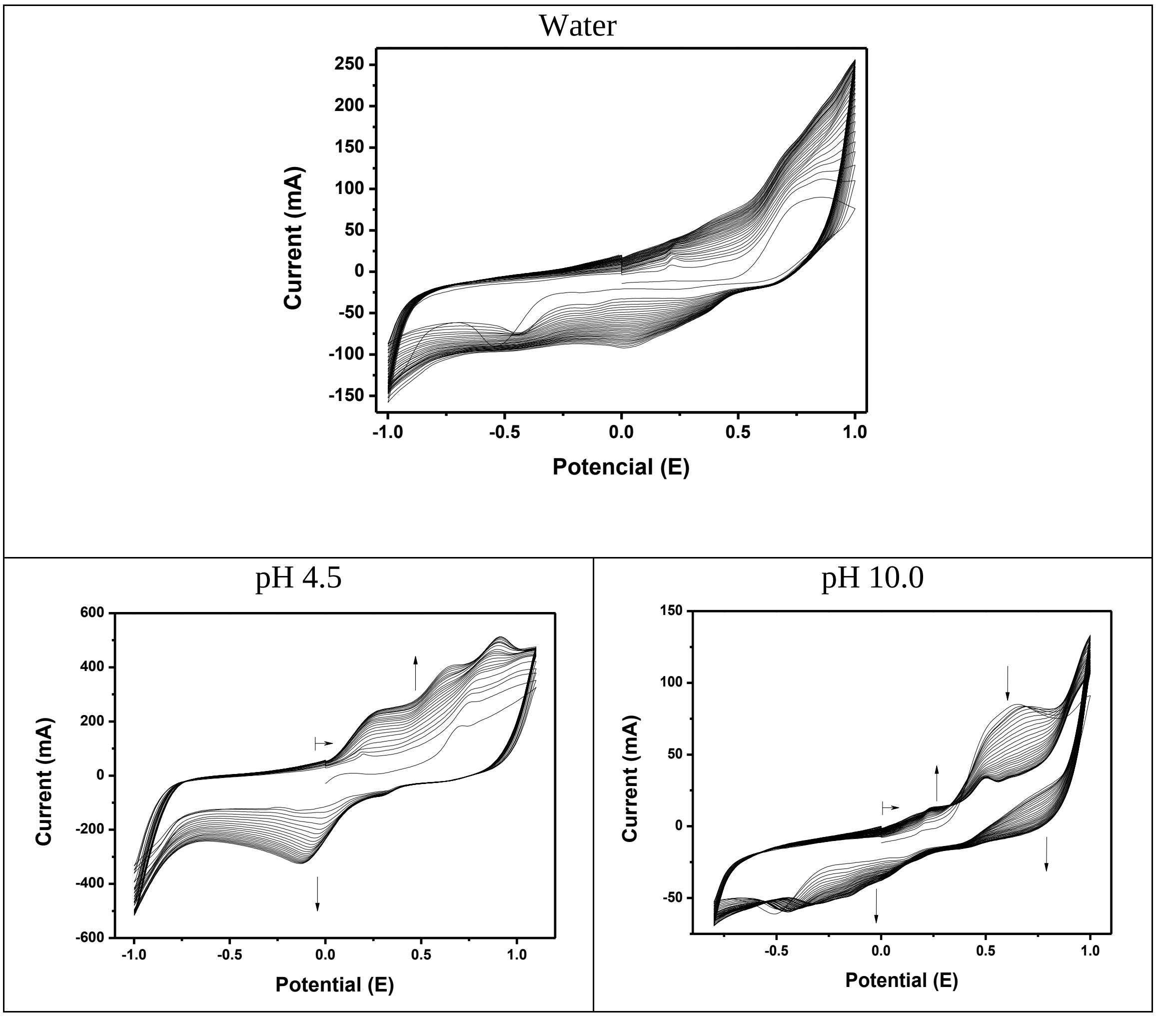


Figure S4. Cyclic voltammograms of 5 mg of complex in pure water, buffered solution at pH 4.5 and at pH 10.0, with 0.1M KCl and v= 100 mV s−1. The reduction peak at −0.50 V is ascribed to blank solution, phosphate buffer in the presence of KCl.

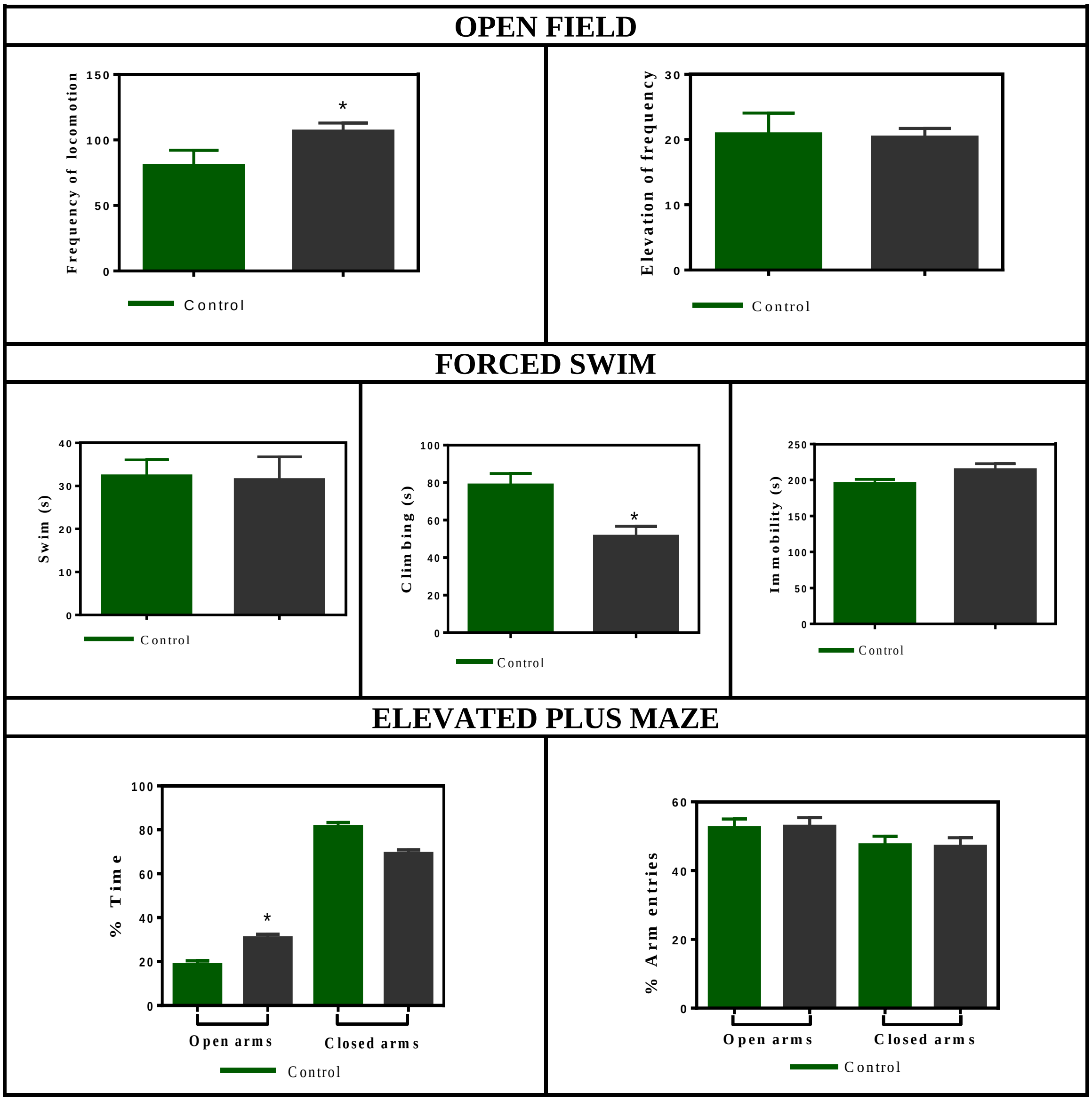


Figure S5. Locomotion and anxiety test in male Wistar rats after 21 days after exposure of 3.0 mgkg-1/day of the complex and control (green bar). Control results are represented in the dotted areas of the graphs. Data are mean ± SEM, n = 6-10. There was no significant difference in the mean value compared to the control ($p < 0.05$). * Significant difference between samples.

Table S2. Optimized structure of tetracoordinated $[Mg(phen)(iso)]^+$ in gas phase, B3LYP/6-31+G* basis set.

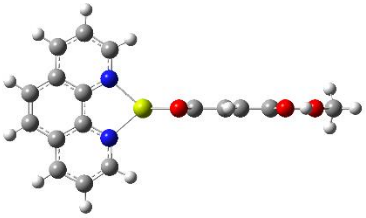
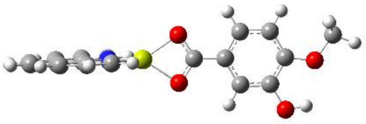
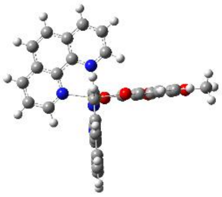
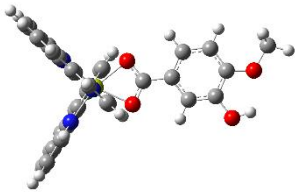
Tetracoordinated structures	
Tetrahedral geommetry	
	
Calculation Type = FREQ Calculation Method = RB3LYP Basis Set = 6-31+G* Charge = 1 Spin = Singlet E(RB3LYP) = -1381.67664236 a.u. RMS Gradient Norm = 0.00000316 a.u. Imaginary Freq = 0 Dipole Moment = 11.5631 Debye Point Group = C1 Sum of electronic and zero-point Energies= -1381.359723 Sum of electronic and thermal Energies= -1381.337405 Sum of electronic and thermal Enthalpies= -1381.336461 Sum of electronic and thermal Free Energies= -1381.413819	

Table S3. Optimized structure of hexacoordinated [Mg(phen)$_2$(iso)]$^+$ in gas phase, B3LYP/6-31+G* basis set.

<table>
<tr><td colspan="2">Hexacoordinated structures</td></tr>
<tr><td colspan="2">Octahedral geommetry</td></tr>
<tr><td></td><td></td></tr>
<tr><td colspan="2">Calculation Type = FREQ
Calculation Method = RB3LYP
Basis Set = 6-31+G*
Charge = 1
Spin = Singlet
E(RB3LYP) = -1953.38180403 a.u.
RMS Gradient Norm = 0.00001425 a.u.
Imaginary Freq = 0
Dipole Moment = 9.0984 Debye
Point Group = C1

Sum of electronic and zero-point Energies= -1952.891850
Sum of electronic and thermal Energies= -1952.858808
Sum of electronic and thermal Enthalpies= -1952.857863
Sum of electronic and thermal Free Energies= -1952.958633</td></tr>
</table>

Table S4. Distribution of Mulliken charges over the atoms for the Mg complexes and free ligands.

Isov. acid (free)		**Mg-tetra**		**Mg-hexa**	
Atom	**charge**	**Atom**	**charge**	**Atom**	**charge**
O (=O)	-0.48	O (=O)	-0.34	O (=O)	-0.25
O (OH)	-0.59	O (OH)	-0.35	O (OH)	-0.25
		Mg	+0.62	Mg	-0.50
Phen (free)		N(phen1)	-0.016	N(phen1)	+0.41
Atom	**charge**	N(phen1)	-0.016	N(phen1)	+0.46
N	-0.47			N(phen2)	+0.47
N	-0.47			N(phen2)	+0.41

Table S5. Gas phase B3LYP/6-31+G* determined xyz coordinates (in Å) for the complex [Mg(phen)(iso)]$^+$, in the GS.

Mg	-0.75922100	-0.00010500	-0.06915000
O	0.88516500	-0.00004600	-1.19066400
O	0.90471100	-0.00013400	1.01935600
C	1.56703800	-0.00008000	-0.09098100
C	3.03266400	-0.00007100	-0.10100000
C	3.72850200	-0.00003600	-1.31831800
C	3.73715800	-0.00009600	1.11919600
C	5.12358200	-0.00002900	-1.32540900
H	3.17676100	-0.00003400	-2.25178500
C	5.12183300	-0.00010800	1.11586300
H	3.20504900	-0.00009700	2.06389500
C	5.82276000	-0.00005800	-0.11478800
H	5.65743200	-0.00001300	-2.26911200
O	5.80109100	-0.00020400	2.29404400
H	6.75591100	-0.00003400	2.10375500
O	7.17591300	-0.00007700	0.02509100
C	8.00020300	0.00036800	-1.14437000
H	9.02752900	0.00058000	-0.77977500
H	7.81993300	-0.89836100	-1.74489600
H	7.81940400	0.89920200	-1.74458300
N	-2.37772200	-1.37072300	-0.04033800
C	-2.35996200	-2.70425900	-0.03906800
C	-3.57907800	-0.72282300	-0.01491100
C	-3.53361900	-3.47738000	-0.01317200
H	-1.38258900	-3.17873700	-0.05895900
C	-4.81109800	-1.42060600	0.01214200
C	-4.75707900	-2.83486200	0.01229500
H	-3.46267000	-4.55978300	-0.01309500
H	-5.68163900	-3.40589800	0.03295400
N	-2.37759400	1.37067200	-0.04028600
C	-2.35970700	2.70420500	-0.03896300
C	-3.57901100	0.72288400	-0.01488400
C	-3.53329300	3.47743600	-0.01303700
H	-1.38228900	3.17859100	-0.05883600
C	-4.81096500	1.42078200	0.01219600
C	-4.75681300	2.83503300	0.01240400
H	-3.46224100	4.55983200	-0.01291800
H	-5.68131900	3.40615400	0.03308600
C	-6.04240700	-0.68190500	0.03852100
C	-6.04234300	0.68219500	0.03854700
H	-6.97825400	1.23323900	0.05914300
H	-6.97836900	-1.23286200	0.05909600

Table S6. Gas phase B3LYP/6-31+G* determined xyz coordinates (in Å) for the complex $[Mg(phen)_2(iso)]^+$, in the GS.

Mg	0.59887900	-0.00511600	-0.03542200
O	-1.14272400	-0.39466400	-1.10990900
O	-1.18559900	0.35006800	0.96958600
N	0.83114400	1.96840300	-1.01617000
C	0.18628300	2.37593500	-2.10655200
C	1.60933900	2.85444700	-0.34296600
C	0.29399300	3.68801200	-2.60700800
H	-0.44665600	1.63477000	-2.58731700
C	1.78225600	4.19503500	-0.76992700
C	1.09599200	4.59499800	-1.94042700
H	-0.25428000	3.96940300	-3.50017900
H	1.19946800	5.61559500	-2.30039100
N	2.05266000	1.11192200	1.25700000
C	2.64219800	0.68729300	2.37079600
C	2.26355000	2.39707500	0.86251300
C	3.47633600	1.50103300	3.15982500
H	2.44503300	-0.34195800	2.65738300
C	3.08317300	3.29596900	1.59192300
C	3.69617300	2.80763500	2.76856700
H	3.92798100	1.09695400	4.05997800
H	4.33103600	3.46556000	3.35665400
C	2.62259900	5.07659800	-0.00856200
C	3.24899800	4.64391400	1.12321600
H	3.88064800	5.31813600	1.69527200
H	2.74738700	6.10064300	-0.35002200
C	-1.81574300	-0.02994200	-0.08055000
C	-3.29859900	-0.04799400	-0.10150300
C	-3.98520000	-0.50895100	-1.23091600
C	-4.01689100	0.39915500	1.02190800
C	-5.38262600	-0.52768300	-1.24739800
H	-3.42271100	-0.85583800	-2.09092800
C	-5.40305700	0.38468600	1.01127700
H	-3.49188200	0.76145900	1.89891000
C	-6.09325800	-0.08235000	-0.13058700
H	-5.90644000	-0.88860500	-2.12592600
O	-6.09443600	0.82056500	2.10253300
H	-7.04630400	0.74073700	1.91510600
O	-7.45342700	-0.04336600	-0.00750900
C	-8.26026500	-0.49451000	-1.09556000
H	-9.29341600	-0.37041300	-0.76940300
H	-8.08037300	0.11147400	-1.99135800
H	-8.06554600	-1.55143400	-1.31236900
N	2.12013500	-1.10060300	-1.26595200
C	2.74469500	-0.66935300	-2.35786200
C	2.34220500	-2.37878000	-0.85502000
C	3.62681000	-1.46939600	-3.10780600
H	2.53798100	0.35424300	-2.65788800
C	3.20896500	-3.26396900	-1.54566300
C	3.85804200	-2.76901000	-2.70000100
H	4.10638200	-1.06038200	-3.99113100
H	4.53014400	-3.41629400	-3.25774500
N	0.82883700	-1.96988000	0.96350400
C	0.15192200	-2.38373800	2.03189300

C	1.65134200	-2.84299500	0.32726500
C	0.26929800	-3.68972700	2.54625600
H	-0.51449600	-1.65337900	2.48311700
C	1.83719900	-4.17640100	0.77078200
C	1.11584400	-4.58326600	1.91772000
H	-0.30602300	-3.97656400	3.42048800
H	1.22835900	-5.59857800	2.28976000
C	3.38543900	-4.60504800	-1.06135800
C	2.72532100	-5.04406600	0.04867000
H	2.85952600	-6.06274200	0.40243600
H	4.05369000	-5.26875500	-1.60335300