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ANTIRADICAL ACTIVITY OF L-GLUTAMINE, L-ASPARAGINE AND L-ASPARTIC ACID DERIVED REDUCED SCHIFF BASE COPPER(II) COMPLEXES

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ABSTRACT. Superoxide dismutases (SOD) are a group of metallo-enzymes, containing one or two coordinated metals, and their main role in the organism is protection against superoxide radicals, one of the reactive oxygen species (ROS). Among these Cu(II)-Zn(II) SOD is present in cytoplasm and acts as a scavenger of superoxide radicals. Cu(II)-complexes may act as low-molecule analogue of Cu-Zn SOD and therefore are being studied as antiradical agents. In this study a series of Cu(II) complexes were synthesizes, containing ligands prepared from salicyl aldehyde and amino acids: L-asparagine, L-glutamine and L-aspartic acid. Complexes prepared from L-asparagine and L-glutamine are novel and were compared with the already reported complex Cu(HSal-L-Asp) hydrate (**6e**). An assay based on the ability to inhibit reduction of iodonitrotetrazolium dye (INT) by superoxide anion-radicals was used to determine antiradical activity of these complex. The prepared complexes - Cu(HSal-L-Asn) acetate (**6a**), Cu(HSal-L-Asn) hydrate (**6b**) and Cu(HSal-L-Gln) acetate (**6c**) proved to be good antiradical agents compared to complex **6e**. The IC_{50} values of the radical transport were 19.2 ± 1.2 μM for **6a**, 53.9 ± 9.4 μM for **6b** and 4.11 ± 0.37 mM for **6c**.

Keywords: *copper complexes, reduced Schiff bases, antiradical activity, SOD mimetics, INT method, amino acid derived ligands*

INTRODUCTION

Superoxide radicals and other reactive oxygen species (ROS) are produced by normal cell metabolism and their overproduction may damage proteins, saccharides, cell membrane lipids and nucleic bases. This process

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is also called oxidative stress [1] and it's connected to many diseases (infertility, cardiovascular diseases, diabetes, neoplasia, ischaemia and reperfusion, asthma etc.) [2-5]. In the last 20-30 years, plant extracts or compounds isolated from plants, mostly flavonoids and polyphenolic acids, were studied as antiradical agents [6-8]. Also, new synthetic compounds including Schiff bases of various benzaldehydes and amines or amino acids [8-15] were studied mostly *in vitro* by various methods for their antiradical and/or anti-inflammatory activity [16]. Main used methods used for radical scavenging are the DPPH method (using 2,2-diphenyl-1-picrylhydrazyl) or ABTS method (using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) [17,18].

Metal complexes (Cu(II), Fe(III), Zn(II), Mn(II), Co(II) etc.) containing these antiradical agents as ligands were reported to yield even better antiradical activities [19], but also act as compounds with cytostatic [20], antidiabetic [21] or anti-inflammatory compounds [22]. The idea of using metal complexes as potential agents in therapy of oxidative stress was inspired by the enzyme group of Superoxide dismutases (SOD), which contain one or two different metal ions (Fe^{3+}, Mn^{2+}, Cu^{2+}-Zn^{2+}) in their active center. SOD enzymes act as protectors against oxidative stress, because they catalyze the dismutation reaction of free superoxide anion-radicals to oxygen molecules and peroxide anions [23]. For radical scavenging models, the copper(II) and zinc(II) containing SOD1 enzyme is most interesting, since it's present in the cell cytoplasm [24]. Metal complexes of small molecule ligands with radical scavenging ability are often called SOD mimetics (or mimics). A lot of attention was given to flavonoid metal complexes, especially highly active copper(II) complexes [25].

But Schiff bases, being good chelating agents of Cu(II), Ni(II), Fe(III), Zn(II), Co(II) and other ions, also became popular ligands for antiradical or antioxidant complexes [19,26,27]. In Schiff bases two moieties can be combined – an aldehyde, usually a benzaldehyde derived from phenolic acid derivatives, for example salicylic or gallic acid, and an amine with chelating groups in the structure for binding to the central ion of the complex. These chelating groups can be nitrogen in heterocycles [28,29] or with carboxylic (amino acids) and other groups [10,16,25,30]. Besides antiradical activity, some of these Schiff base complexes showed also antimicrobial [31], cytotoxic [32], antifungal [33], DNA-binding [30] or catecholase activity [34]. Vančo et al. studied the SOD mimetic abilities of Schiff base ligands derived from L-alanine, β-alanine and γ-aminobutyric acid with activities 28.2 – 68.8% inhibition at $1.43.10^{-3}$ M using the INT method [21]. Another structural variation of the Schiff base ligands is the reduction of the C=N bond to form secondary amines or so called reduced Schiff bases, which are more stable and easier to isolate then the Schiff bases themselves, especially in aqueous systems. This reduction causes significant changes in the chelating abilities of the ligand, which also affects the antiradical, catecholase mimetic or other activities [35,36,37].

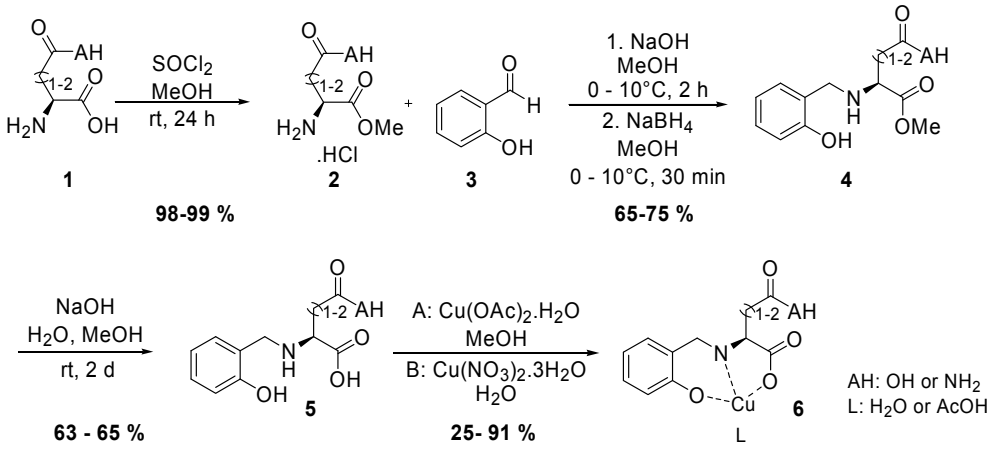
In this research, we synthesized five copper(II) complexes of reduced Schiff base ligands (Figure 1) prepared from salicyl aldehyde (**3**) and the amino acids L-asparagine (**1a**) and L-glutamine (**1b**), which contain both an amide group at the end of their side chain, but differ in the number of carbons in the chain. There is just little information about the two compounds **5a** and **5b**, which we used as ligands, in literature. We found only two complexes of the **5a** containing Ni(II) with only studied structure [38], and a large heptametric La(III) complex able to bind to DNA[39]. There is practically no data available of the L-glutamine derived ligand and its complexes. Therefore, we wanted to prepare this novel complexes and compare the antiradical activities of their copper(II) complexes also with the copper(II) complex of the L-aspartic acid derived ligand (**5c**). This complex was already reported in literature, its structure was studied and it's known to form stable copper(II) complexes, however, its antiradical activity was not yet studied [38,40].

RESULTS AND DISCUSSION

The synthesis (Scheme 1) of the target ligands started from L-asparagine, L-glutamine and L-aspartic acid, which were first turned into methyl ester hydrochlorides (**2**) using thionyl chloride in methanol. This step was necessary to achieve higher purity of the synthesis intermediates and final ligands. Due to esterification, the polarity of the compounds decreased and this allowed a much easier and more reliable separation of the compounds from inorganic material which was present in the second synthesis step. In this second step the methyl esters reacted with salicyl aldehyde (**3**). The use of sodium hydroxide served for neutralization of the hydrochloride salt. The condensation reaction lead to the Schiff base (imine) formation, which was not isolated because of possible decomposition. A reduction reaction with $NaBH_4$ followed the condensation as a one-pot synthesis. This process produced a stable secondary amine (4), a reduced Schiff base. Last step of the ligand synthesis was a mild hydrolysis of the methyl ester under basic conditions and after neutralization with 10% HCl we obtained the ligands **5a-c** as white powders. All the intermediates and the ligands of the synthesis were characterized by spectroscopy methods (IR, ^{1}H and ^{13}C NMR) and elemental analysis was measured from all ligands **5a-c** (see in exp. section).

For complexation reactions, we used either method A with copper(II) acetate monohydrate or method B with copper(II) nitrate trihydrate for L-asparagine and L- glutamine derived ligands (5a and 5b) and in case of the L-aspartic acid derived ligand **5c** only method B was used. Complexes **6a-e** were isolated as fine green or blue powders, where L was either an acetic acid molecule (in

case of method A) or water molecule (in case of method B). The presence of acetic acid or water was determined by elemental analysis and they are either coordinated directly to copper(II) or adsorbed on the ligand by electrostatic interactions or hydrogen bond. Green powders were obtained from all 5 complexation reactions and they were characterized by IR spectra and elemental analysis. However, the product of complexation of HSal-L-Gln (**5b**) and copper(II) nitrate trihydrate yielded a powder of insufficient elemental analysis result and for this reason could not be used in the antiradical activity assays.



1 **2** **3** **4** **5** **6**

98-99 % **65-75 %** **63 - 65 %** **25- 91 %**

Scheme 1

The other four complexes with good elemental analysis were used in antiradical activity determination assays. The method used in the assay is a spectrophotometric detection of single electron transfer from KO_2, which served as a radical source, to 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (iodonitrotetrazolium dye, INT). The electron transfer leads to formation of blue formazan, which is detected as absorbance at 500 nm. The decline of the INT-formazan concentration is expressed as % of inhibition and it is linearly proportional to antiradical (SOD-mimic) activity of the measured compound.

The solubility often is an issue in case of copper(II) complexes. Good solubility of the complexes in the initial solutions provides also reliable data of the measured % of inhibition. In our case, all measured complexes were soluble in the initial solution with concentration 1.10^{-2} M. Solutions of this concentrations were used in preliminary antiradical assays, which served to select complexes with higher activity. Complexes with good % of inhibition were **6b** with 98.24 ± 0.23% at $1.43.10^{-3}$M and **6a** with 100.33 ± 0.37% at half concentration compared

to **6b**, at $5.71.10^{-4}$M (Table 1). Less active was complex **6c** with only 42.16 ± 3.30% and the least active was the complex **6e** prepared from L-aspartic acid derived ligand displayed only 16.81 ± 0.63 % activity, which is even lower than the inhibition % reported for their complexes reported by Vančo et al. [21]. Therefore, complex **6e** could not be used in further assays. Two carboxylic groups present in the molecule **5c** provide for be better chelating abilities of the ligand compared to the amide containing **5a** and **5b**. High stability of complex **6e** seems to be the reason for rather low antiradical activity, rending the complex unable to interact with free radicals in the solution. The other three complexes proved to be much better antiradical agents already in these preliminary assays.

Table 1. % of inhibition in initial antiradical activity measurements

complex	% of inhibition	concentration [M]
6a	100.33 ± 0.37	$5.71.10^{-4}$
6b	98.24 ± 0.23	$1.43.10^{-3}$
6c	42.16 ± 3.30	$1.43.10^{-3}$
6e	16.81 ± 0.63	$1.43.10^{-3}$

For the IC_{50} determination a series of sample solutions was prepared with concentrations decreasing from the initial value to 4.10^{-4} M for **6b** and **6c**. The concentrations range of **6a** had to be broaden to 4.10^{-5} M to obtain also inhibition values below 50%. The measured concentrations were obtained by addition of INT and KO_2 solutions and DMSO (see in exp. section). Complex **6a** and **6b** are rather active as can be seen in the Figure 1, but the **6b** complex was achieving lower % of inhibition at all concentrations. The % of inhibition had a linear trend depending on the measured concentration and for each complex a linear trend function was proposed (Figure 1). IC_{50} values were calculated from these functions as complex concentration at 50% of inhibition (Table 2). The best value was calculated for HSal-L-Asn Cu(II) acetate (**6a**) with 19.2 ± 1.2 μM, followed by HSal-L-Asn Cu(II) hydrate (**6b**) with 53.9 ± 9.4μM. HSal-L-Gln Cu(II) acetate (**6c**) exhibited much lower activity with IC_{50} equal to 4.11 ± 0.37 mM. This decrease of activity of complex **6c** derived from L-glutamine could have two explanations, again one could be the stability of the complex. But the precipitation of the complex during the preparation took a longer time compared to **6a** and **6b** and the yield of the complexation of **6c** is also the lowest of all. It is more probable that the radical transfer to

copper central ion is disturbed in **6c** by the longer side chain. As standard for scavenging of superoxide anion-radicals served cystamine with IC_{50} = 1.7 ± 0.1 mM.

Table 2. IC_{50} values of prepared complexes

complex	**6a**	**6b**	**6c**
IC_{50}	19.2 ± 1.2 μM	53.9 ± 9.4 μM	4.11 ± 0.37 mM

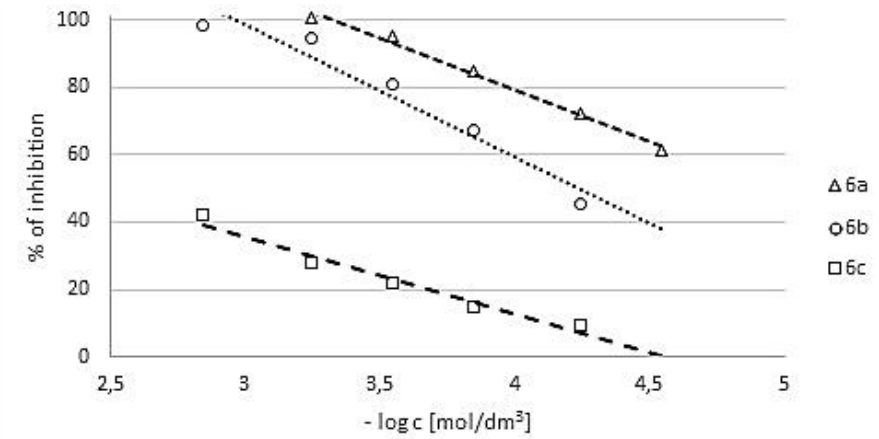


Figure 1. The linear relation between % of inhibition and negative logarithm of measured concentration of the complexes **6a-c**. The linear trend line function was used in IC_{50} value calculation.

CONCLUSIONS

In a three-step synthesis three ligands derived from L-asparagine, L-glutamine and L-aspartic acid were prepared. These ligands were used in complex formation reactions with copper(II) acetate hydrate in methanol and/or copper(II) nitrate trihydrate. From these complexation reactions four complexes with good elemental analysis were obtained and used in an antiradical activity assay using INT spectrophotometric method. The complex **6e** prepared from L-aspartic acid derived ligand had too low activity to be used in IC_{50} determination. The IC_{50} values for the novel complexes were in μM range for complexes **6a** and **6b**, both prepared from L-asparagine, and in mM range in case of complex **6c**, with the L-glutamine derivative as ligand. Complex **6c** also less active then cystamine used as a standard.

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EXPERIMENTAL SECTION

All chemicals for syntheses were reagent grade and were used as they received (Sigma-Aldrich), with exception of methanol which was dried using pre-drying with calcium oxide, reflux with magnesium activated with iodine and distillation.

All NMR spectra were measured on a Varian Gemini 2000 spectrometer at working frequencies 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR. Spectra were measured in DMSO-d_6 or D_2O, using TMS as internal standard. For the NMR signal assigning the numbering on aromatic ring starts from the position of the main chain. Infrared spectra were recorded on Nicolet 6700 FT-IR spectrophotometer in range 500 – 4000 cm^{-1} and the samples were in solid state. Elemental analysis was measured by Flash 2000 CHNS-O Analyser (Thermo Scientific).

Antiradical activity measurements were done on Synergy HT BioTek spectrophotometer. DMSO(analytical reagent grade) was used as solvent, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT) and KO_2 were bought from Alfa-Aesar. INT initial solution was prepared in a sodium tetrahydridoborate buffer solution with pH adjusted to 7.1 with HCl.

General procedure for preparation of methyl ester hydrochlorides of amino acids (2a-c). To a solution of the amino acid **1** (22.45 mmol, 1 mol equiv) in 15 ml (20 ml in case of L-aspartic acid reaction) of dry methanol in a two-neck flask thionyl chloride (2.3 ml, 31.43 mmol, 1.4 mol equiv in case of L-Asn and L-Gln and 3.5 ml, 47.83 mmol, 2.1 mol equiv in case of L-Asp) was added drop-wise over 5 – 10 minutes. Because of the exothermic reaction, the vapors had to be cooled by reflux condenser. After the addition was complete, the temperature was left to decrease to room temperature and the mixture was stirred for 24 hours. After the reaction was complete, the solvent was removed by evaporation (RVE). The white or light yellow solid was washed with n-hexane (twice 50 ml), and evaporated with another portion of n-hexane. The product was dried at low pressure.

Methyl-L-asparagine hydrochloride (2a). Was isolated as light yellow solid in 4.06 g (99 % yield). M.p. 122-126°C.^{1}H NMR (300 MHz, D_2O, ppm) δ: 4.37 (dd, 1H, *J* = 4.8 and 6.0 Hz, CH), 3.71 (s, 3H, CH_3), 3.02 – 3.06 (m, 2H, CH_2). ^{13}C NMR (75 MHz, D_2O, ppm) δ: 171.5 and 169.2 (2x COO), 53.3 (CH_3), 49.0 (CH), 33.5 (CH_2). IR (solid, cm^{-1}): 3420 (m, N-H), 3126 (s), 3041 (s), 2826 (m), 1739 (s, C=O), 1623 (w), 1506 (w), 1399 (s), 1254 (m), 1151 (w), 1072 (w), 955 (w), 886 (w).

Methyl-L-glutamine hydrochloride (2b). Was isolated as light yellow solid in 4.30 g (98 % yield). M.p. 141-144°C.^{1}H NMR (300 MHz, D_2O, ppm) δ: 4.06 (dd, 1H, *J* = 6.6 and 7.2 Hz, CH), 3.68 (s, 3H, CH_3), 2.46 – 2.52 (m, 2H, CH$\underline{CH_2}$), 2.05- 2.15 (m, 2H, $\underline{CH_2}CONH_2$). ^{13}C NMR (75 MHz, D_2O, ppm) δ: 174.8 and 170.1 (2x CO), 53.5 (CH_3), 48.8 (CH), 29.2 ($\underline{CH_2}CONH_2$), 24.7 (CH$\underline{CH_2}$). IR (solid, cm^{-1}): 3412 (m, N-H), 3127 (s), 3040 (s), 2858 (m), 2722 (w), 1721 (s, C=O), 1671 (s, C=O), 1611 (w), 1058 (m), 1425 (w), 1399 (s), 1274 (m), 1253 (m), 1213 (s), 1147 (w), 1081 (w), 1000 (w), 862 (w), 823 (m), 676 (w), 635 (w).

Dimethyl-L-aspartate hydrochloride (2c). Was isolated as white powder in 4.04 g (98 % yield). M.p. 112-114°C.^{1}H NMR (300 MHz, D_2O, ppm) δ: 4.52 (dd, 1H, *J* = 6.3 Hz and 5.1 Hz, CH), 3.85 (s, 3H, CH_3), 3.76 (s, 3H, CH_3), 3.19 (m, 2H, CH_2).^{13}C NMR (75 MHz, D_2O, ppm) δ: 174.5 and 172.3 (2x $\underline{C}OOCH_3$), 56.8 and 55.9 (2x COO$\underline{CH_3}$), 52.1 (CH), 36.6 (CH_2). IR (solid, cm^{-1}): 3399 (s, OH), 3212 (m), 2959 (s), 1743 (s), 1621 (m), 1515 (m), 1442 (s), 1410 (w), 1379 (m), 1249 (s), 1216 (s), 1155 (m), 1078 (m), 1016 (m), 951 (w), 888 (w), 843 (w), 798 (w), 667 (m), 641 (m), 621 (m).

General procedure for preparation of *N*-(2-Hydroxy-benzyl)-amino acid methyl esters (4a-c). To a solution of the amino acid methyl ester hydrochloride **2** (12.45 mmol, 1.3 mol equiv) in 20 ml of dry methanol sodium hydroxide (0.50 g, 12.45 mmol, 1.3 mol equiv) was added at room temperature and the mixture was stirred until the hydroxide was consumed completely. During this reaction sodium chloride is formed, which can be observed as white powder-like precipitate. After 15-30 minutes of stirring, the mixture was cooled down using an ice bath to 0 – 10 °C and a solution of salicyl aldehyde (**3**) (1.00ml, 9.58 mmol, 1 mol equiv) in 5 ml of dry methanol was added drop-wise over 5-10 minutes. A color change from almost colorless to yellow was observed. The mixture was stirred at 0 – 10°C until the reaction was complete – about 1 - 2 hours (TLC control: n-hexane : ethyl acetate = 8 : 1). Afterwards, solid $NaBH_4$ (440 mg, 11.50 mmol, 1.2 mol equiv) was added portion-wise and the solution was stirred until the yellow color disappeared (15 – 30 min). Solvent was removed by evaporation to dryness. The product was obtained from the solid by washing the solid with diethyl ether and evaporation. The product was further dried at low pressure.

***N*-(2-Hydroxy-benzyl)-L-asparagine methyl ester (4a).** Was isolated as an oil in 1.81 g (75 %). ^{1}H NMR (300 MHz, DMSO-d_6, ppm) δ: 7.04 – 7.13 (m, 2H, C4-H and C6-H), 6.73 – 6.76 (m, 2H, C3-H and C5-H), 3.78 (d, 2H, *J*(H,H´) = 14.7 Hz, Ar-C<u>H</u>H), 3.62 – 3.68 (m, 2H, Ar-CH<u>H</u> and NH<u>CH</u>), 3.59 (s, 3H, COO<u>CH$_3$</u>), 2.56 – 2.77 (m, 2H, CH<u>CH$_2$</u>). ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ: 173.2 and 170.9 (2x CO), 156.1 (C2), 128.9 and 127.9 (C4 and C6), 124.9 (C1), 118.7 (C5), 115.1(C3), 56.5 (CH), 51.8 (COO<u>CH$_3$</u>), 47.3 (Ar-<u>CH$_2$</u>), 36.9 (CH<u>CH$_2$</u>). IR (solid, cm^{-1}): 3430 (w, N-H), 3094 (w), 2954 (m), 2818 (m), 2754 (w), 1733 (s, C=O), 1603 (w), 1589 (s), 1490 (m), 1459 (m), 1438 (m), 1375 (s), 1253 (s), 1207 (s), 1178 (m), 1114 (w), 1039 (w), 1004 (m), 846 (m), 757 (s), 659 (w).

***N*-(2-Hydroxy-benzyl)-L-glutamine methyl ester (4b).** Was isolated as an oil in 1.66 g (65 %). ^{1}H NMR (300 MHz, DMSO-d_6, ppm) δ: 7.01 – 7.12 (m, 2H, C4-H and C6-H), 6.72 – 6.82 (m, 2H, C3-H and C5-H), 4.62 (d, 1H, *J*(H,H´) = 14.7 Hz, Ar-C<u>H</u>H), 4.07 – 4.11 (m, 1H, CH), 4.01 (d, 1H, *J*(H,H´) = 14.7 Hz, Ar-CH<u>H</u>), 3.63 (s, 3H, COO<u>CH$_3$</u>), 2.25 – 2.32 (m, 2H, CH<u>CH$_2$</u>), 1.91 – 1.96 (m, 2H, <u>CH$_2$</u>CONH$_2$). ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ: 174.5 and 172.4 (2x CO), 155.5 (C2), 129.7 and 128.7 (C4 and C6), 122.1 (C1), 119.0 (C5), 115.1(C3), 58.7 (CH), 52.1 (COO<u>CH$_3$</u>), 40.3 (Ar-<u>CH$_2$</u>), 28.9 (CH<u>CH$_2$</u>), 22.3 (<u>CH$_2$</u>CONH$_2$). IR (solid, cm^{-1}): 3420 (m, N-H), 3097 (m), 2954 (m), 2741 (w), 1746 (s, C=O), 1659 (s, C=O), 1607 (w), 1598 (w), 1488 (w), 1457 (s), 1436 (w), 1382 (w), 1357 (w), 1331 (w), 1279 (m), 1245 (s), 1207 (s), 1174 (s), 1109 (m), 1036 (w), 999 (w), 868 (w), 763 (s), 677 (w).

***N*-(2-Hydroxy-benzyl)-L-aspartate dimethyl ester (4c).** Was isolated as a colorless oil in 2.30 g (95 %). 1H NMR (300 MHz, DMSO-d_6, ppm) δ: 7.03 – 7.12 (m, 2H, C4-H and C6-H), 6.69 – 6.75 (m, 2H, C3-H and C5-H), 3.77 and 3.65 (d, 1H and 1H, *J*(H,H´) = 13.5 Hz, Ar-<u>CH$_2$</u>), 3.62 and 3.58 (s, 6H, 2x COO<u>CH$_3$</u>), 3.61 (m, 1H, CH), 2.69 (m, 2H, CH<u>CH$_2$</u>). 13C NMR (75 MHz, DMSO-d_6, ppm) δ: 173.1 and 170.9 (2x <u>COO</u>CH$_3$), 156.1 (C2), 128.9 and 127.9 (C4 and C6), 124.9(C1), 118.7(C5), 115.1(C3), 56.5(CH), 51.8 and 51.5 (2x COO<u>CH$_3$</u>), 47.2(Ar-<u>CH$_2$</u>), 36.9(CH<u>CH$_2$</u>). IR (solid, cm^{-1}): 3309(m, NH), 2954(m), 1734(s, C=O), 1616(w), 1588(m), 1490(m), 1456(w), 1437(m), 1369(m), 1252(s), 1203(s), 1171(s), 1104(w), 1036(w), 1001(m), 935(w), 844(w), 756(s), 722(w), 647(w), 639(w), 622(w).

General procedure for preparation of *N*-(2-Hydroxy-benzyl)-amino acid ligands (5a-c). To a stirred solution of the methyl ester **4**(4.51 mmol, 1 mol equiv) in 12 ml of methanol and 3 ml of water sodium hydroxide (0.42 g, 10.55 mmol, 2.1 mol equiv) was added and left to stir for 2 – 3 days. The pH of the reaction mixture was altered to 5 – 6 using 10% hydrochloric acid. All solvents

were removed by evaporation. The solid was washed with water (to remove inorganic salts), acetone and diethyl ether. The product was obtained as a powder after drying.

***N*-(2-Hydroxy-benzyl)-L-asparagine (5a).** Yield 698 mg (65 %). M.p. 204-207°C.Elemental analysis calculated for $C_{11}H_{14}N_2O_4$ (MW = 238.24) C 55.46; H 5.92; N 11.76, measured C 55.39; H 6.01; N 11.61. ^{1}H NMR (300 MHz, D_2O, ppm) δ: 7.19 – 7.26 (m, 2H, C4-H and C6-H), 6.83 – 6.88 (m, 2H, C3-H and C5-H), 4.11 – 4.21 (m, 2H, *J*(H,H´) = 17.1 Hz, Ar-$\underline{CH_2}$), 3.64 (dd, 1H, *J* = 4.2 Hz and 9.0 Hz, CH), 2.68 (dd, 1H, *J*= 4.2 Hz and 17.7 Hz, CHC$\underline{H}$H), 2.56 (dd, 1H, *J* = 9.0 Hz and 17.7 Hz, CHCH$\underline{H}$). ^{13}C NMR (75 MHz, D_2O, ppm) δ: 173.1 and 173.0 (2x CO), 156.7 (C2), 129.7 and 128.7 (C4 and C6), 127.3 (C1), 118.6 (C5), 115.4 (C3), 56.6 (CH), 46.8 (Ar-$\underline{CH_2}$), 28.0 (CH$\underline{CH_2}$). IR (solid, cm^{-1}): 3450 (m, N-H), 3151 (m), 2754 (w), 2573 (w), 1593 (s, C=O), 1508 (w), 1464 (m), 1393 (s), 1373 (s), 1333 (m), 1278 (m), 1258 (m), 1203 (w), 1113 (w), 1040 (w), 1017 (w), 861 (w), 758 (s), 664 (m).

***N*-(2-Hydroxy-benzyl)-L-glutamine (5b).** Yield 716 mg (63 %). M.p. 215-219°C. Elemental analysis calculated for $C_{12}H_{15}N_2O_4$ (MW = 252.27) C 57.13; H 6.39; N 11.10, measured C 57.02; H 6.30; N 10.95. 1H NMR (300 MHz, DMSO-d_6, ppm) δ: 7.13 (ddd, ^{1}H, *J*(3,4 or 4,5) = 9 Hz, *J* (3,4 or 4,5) = 7.8 Hz, *J*(4,6) = 1.5 Hz, C4-H), 7.00 (dd, 1H, J(5,6) = 6 Hz, J(4,6) = 1.5 Hz, C6-H), 6.77 – 6.83 (m, 2H, C3-H and C5-H), 4.72 (d, 1H, J(H,H') = 14.7 Hz, Ar-C$\underline{H}$H), 3.97 (d, 1H, J(H,H´) = 14.7 Hz, Ar-CHH), 3.83 (dd, 1H, J = 4.5 Hz and 9.3 Hz, CH), 2.16 – 2.45 (m, 4H, CH$\underline{CH_2CH_2}$). ^{13}C NMR (75 MHz, DMSO-d_6, ppm) δ: 174.5 and 173.7 (2x CO), 155.8 (C2), 129.5 and 128.4 (C4 and C6), 122.8 (C1), 118.8 (C5), 115.6 (C3), 59.9 (CH), 40.3 (Ar-$\underline{CH_2}$), 29.4 (CH$\underline{CH_2}$), 25.5 ($\underline{CH_2}CONH_2$). IR (solid, cm^{-1}): 3458 (w, N-H), 3183 (m, O-H), 2957 (m), 2736 (w), 1659 (s, C=O), 1596 (s, C=O), 1505 (w), 1489 (w), 1457 (s), 1418 (s), 1357 (w), 1277 (m), 1240 (s), 1183 (w), 1154 (w), 1107 (m), 1041 (w), 957 (w), 850 (w), 756 (s), 665 (w).

***N*-(2-Hydroxy-benzyl)-L-aspartic acid (5c).**Yield 576 mg (76 %). M.p. 218-220°C. Elemental analysis calculated for $C_{11}H_{13}NO_5$ (MW = 239.23) C 55.23; H 5.48; N 5.86, measured C 54.99; H 5.33; N 6.06. 1H NMR (300 MHz, D_2O, ppm) δ: 7.16 – 7.21 (m, 2H, C6-H and C4-H), 6.81 – 6.85 (m, 2H, C3-H and C5-H), 4.16 (d, 1H, *J*(H,H´) = 13.0 Hz, Ar-C$\underline{H}$H), 4.10 (d, 1H, *J*(H,H´) = 13.0 Hz, Ar-CH$\underline{H}$), 3.60 (dd, 1H, *J* = 9.3 Hz and 3.9 Hz, CH), 2.63 (dd, 1H, *J* = 15.6 Hz and 3.9 Hz, CHC$\underline{H}$H), 2.53(1H, dd, *J* = 15.6 Hz and 9.3 Hz, CHCH$\underline{H}$). 13C NMR (75 MHz, D_2O, ppm) δ: 177.3 and 173.2 (2x $\underline{C}$OOH), 155.1 (C2), 131.6 and 131.3 (C4 and C6), 120.4 (C5), 117.6 (C1), 115.4 (C3), 58.7(CH) 46.5(Ar-$\underline{CH_2}$), 35.4(CH$\underline{CH_2}$). IR (solid, cm^{-1}): 3215(m), 3046(m), 2591(m), 2359(w), 1625(s), 1600(s), 1489(w), 1463(m), 1410(s), 1387(s), 1371(s), 1313(m),

1273(w), 1259(m), 1243(m), 1208(w), 1113(w), 1074(w), 1041(m), 978(w), 946(w), 915(w), 884(m), 833(w), 801(w), 786(w), 751(s), 753(s), 714(w), 668(m), 645(w), 616(w).

General procedure for preparation of Cu(II) complexes.

Method A. To a stirred and to reflux heated solution of $Cu(OAc)_2.H_2O$ (168 mg, 0.84 mmol, 1.00 mol equiv) in methanol (10 ml), a solution of the amino acid ligand **5** (0.84 mmol, 1.00 mol equiv) in 10 ml methanol was added. A color change from blue to green is usually observed. The mixture is stirred with heating for 30 minutes and then left to cool without stirring. The cooled solution was allowed to concentrate by slight evaporation.

Method B. To a stirred and heated solution of the amino acid ligand **5** (0.84 mmol, 1.00 mol equiv) and NaOH (34 mg, 0.84 mmol, 1.00 mol equiv) in 10 ml of distilled water a solution of $Cu(NO_3)_3.3H_2O$ (203 mg, 0.84 mmol, 1.00 mol equiv) in 2 ml of distilled water was added. A color change was observed from blue to green. The mixture is left to cool without stirring. The cooled solution was allowed to concentrate by slight evaporation.

HSal-L-Asn Cu(II) acetate complex (6a). Method A provided Cu(HSal-L-Asn) acetate as a green powder in 274 mg (91 %). Elemental analysis calculated for $C_{13}H_{17}CuN_2O_6$ (MW = 359.82) C 43.39; H 4.48; N 7.79, measured C 43.11; H 4.35; N 7.84. IR (solid, cm^{-1}): 3386 (m, N-H), 3250 (m, N-H), 2957 (w), 2784 (w), 1603 (m, N-H), 1582 (s, C=O), 1487 (w), 1456 (m), 1413 (s), 1285 (m), 1244 (m), 1185 (w), 1153 (w), 1108 (w), 1043 (w), 959 (w), 899(w), 852 (w), 757 (m), 676 (s).

HSal-L-Asn Cu(II) hydrate complex (6b). Method B provided Cu(HSal-L-Asn) hydrate as a green powder in 77 mg (29 %). Elemental analysis calculated for $C_{13}H_{17}CuN_2O_6$ (MW = 317.79) C 41.51; H 4.44; N 8.82, measured C 41.21; H 4.67; N 8.60. IR (solid, cm^{-1}): 3452 (m, N-H), 3220 (m, N-H), 2986 (m), 2768 (w), 1581 (s, C=O), 1482 (w), 1435 (m), 1403 (m), 1335 (s), 1291 (s), 1159 (w), 1096 (m), 1040 (w), 1017 (w), 893 (w), 827 (w), 759 (w), 667 (w).

HSal-L-Gln Cu(II) acetate complex (6c). Method A provided Cu(HSal-L-Gln) acetate as a green powder in 80 mg (25 %). Elemental analysis calculated for $C_{14}H_{19}CuN_2O_6$ (MW = 373.85) C 44.98; H 4.85; N 7.49, measured C 44.84; H 4.96; N 7.62. IR (solid, cm^{-1}): 3392 (m, N-H), 3266 (m, N-H), 2950 (w), 2802 (w), 1600 (m), 1583 (s, C=O), 1488 (w), 1456 (m), 1416 (s), 1286 (m), 1243 (m), 1185 (w), 1153 (w), 1108 (w), 1044 (w), 957 (w), 900(w), 848 (w), 757 (m), 677 (s).

HSal-L-Asp Cu(II) hydrate complex (6e). Method B provided Cu(HSal-L-Asp) hydrate as a blue powder in 112 mg (42 %). Elemental analysis calculated for $C_{11}H_{13}CuNO_6$ (MW = 318.77) C 41.45; H 4.39; N 4.11, measured C 41.06; H 4.38; N 4.04. IR (solid, cm^{-1}): 3514 (w), 3280 (m), 3120 (s), 2966 (s), 1645 (s), 1612 (w), 1574 (s), 1505 (m), 1429 (m), 1448 (w), 1429 (m), 1406 (s), 1383 (w), 1353 (s) 1330 (w), 1317 (s), 1294 (w), 1271 (s), 1232 (m), 1191 (m), 1158 (w), 1109 (m), 1081 (w), 1047 (w), 1013 (m), 987 (w), 952 (w), 896 (w), 872 (w), 852 (m), 828 (w), 777 (s), 766 (s), 732 (w), 660 (s), 645 (w).

Antiradical activity assay. The used method is based on competitive equilibrium between transition of electrons/radicals from KO_2 to 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT) and transition of electrons/radicals to the measured compound. Transition of electrons to INT leads to formation of formazan. Active compounds are, in this fashion, able to inhibit the formation of the blue formazan, detected as light absorption at 500 nm.

For each measurement, an initial solution of the sample of a specific concentration was prepared (1.10^{-2} – 1.10^{-5} M) in DMSO, also initial solutions of INT (4 M) in borate buffer (pH = 7.1) and a saturated solution of KO_2 in DMSO. From these a set of diluted solutions of volume 3.5 ml was made: 1. blank solution containing only INT dye (0.5 ml of INT solution and 3 ml of DMSO), 2. 3-5 control solutions containing INT dye and KO_2 (0.5 ml of INT solution, 0.5 ml of KO_2 solution and 2.5 ml of DMSO), 3. 3 sample solutions containing all three compounds - the complex, INT dye and KO_2 (0.5 ml of INT solution, 0.5 ml of initial complex solution of concentration 1.10^{-2} – 1.10^{-5} M, 0.5 ml of KO_2 solution and 2 ml of DMSO) and 4. sample control solution containing the complex and KO_2 (0.5 ml of complex solution of concentration 1.10^{-2} – 1.10^{-5} M, 0.5 ml of KO_2 solution and 2.5 ml of DMSO). In sample solutions and sample control solution (3. and 4.) the concentration of the sample was diluted 7-times, since each solution contained only 1/7 of the original initial solution. 200 µl of the solutions was moved into a micro-titration plate and the absorbance at 500 nm was measured in parallel in all four types of solutions. The resulting inhibition percent was calculated according the formula: 100 – [(sample absorbance – sample control absorbance) / average absorbance of control solutions] x 100. Result of each measurement was the average value from the 3 sample solutions with a standard deviation [21].

For IC_{50} determination a series of measurements with decreasing sample concentration was evaluated. With decreasing concentration, the % of inhibition was also decreasing in linear trend. A diagram of this linear relationship was created and a linear trend function was calculated. The IC_{50} value was calculated as the concentration with 50% inhibition of INT-formazan formation from the resulting diagram.

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