Pyridinium Ylides as Potential Inhibitors for Glutamate Racemase

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Cobalt (II), nickel (II) and copper (II) complexes of 2 - (4, 4' –bipyridin – 1 – ium – 1 - yl) - 1- (4 - methoxyphenyl) - 3 -(naphtalen – 1 - ylamino) - 1, 3 dioxopropan – 2 – ide ligand (Y) were synthesized and analyzed by ^{1}H NMR, FT-IR, UV-Vis, ESI-MS, Fluorescence Spectroscopy and thermal analysis. RacEa peptide is part of the catalitic site of the glutamate racemase which is an attractive antimicrobial drug target. The Affinity-MS was used for studing the interaction between the ligand Y or the nickel complex of ligand Y and the synthetic RacEa peptide. Only the ligand showed binding to the synthetic RacEa peptide in the presence of dithiothreitol (DTT).

Keywords: N-ylides, metal complexes, RacEa peptide, Glutamate racemase inhibitors.

The zwitterionic organic compounds, with the carbanion covalently bound to a positively charged nitrogen are known as nitrogen-ylides [1]. The ylidic carbanion center and the presence of additional donor atoms, as oxygen, sulfur and nitrogen atoms from the substituents groups, allows nitrogen-ylides to behave as bidentate ligands for transition metals [2].

Glutamate racemase (GR) is a source of D-glutamate in bacteria through the racemization of L-glutamate [3, 4]. D-glutamate is an essential component of the peptidoglycan layer from bacterial cell walls; therefore Glutamate racemase is an attractive target for antibacterial drug development [5-8]. The transition metal ions as cobalt, nickel, copper are the key intermediates for chemical reactions of metalloproteins [9]. The nitrogen-ylides and their metal complexes are known as good candidates for therapeutic drugs [10].

Because of the specific structure, 2–(4,4'–bipyridin–1–ium–1–yl)–1–(4– methoxyphenyl)–3–(naphtalen–1–ylamino)–1,3–dioxopropan–2–ide ligand (**Y**) can be an interesting candidate for mimic the glutamate carbanion in binding to GR (fig. 1) [11].

Therefore, our aim was to synthesize the ligand **Y** and its complexes with transitional metals and test them by Affinity-Mass Spectrometry as inhibitors for Glutamate Racemase (GR). Mass spectrometric affinity-based approaches have been successfully employed for the identification of epitope and paratope structures from antibody-antigen complexes [12-18], as well as for the identification of carbohydrate binding structures in lectins [19, 20].

Experimental part

Materials and methods

All syntheses and handling were carried out in air. All chemicals were purchased from Aldrich and used without further purification. The infra-red spectra of the ligand and complexes in the region 400 - 4000cm^{-1} were recorded using an Jasco FTIR-660 Plus spectrometer, using KBr pellets. The ^{1}H NMR spectra of the ligand and complexes were performed using a Brucker AM 350 spectrometer. Chemical shifts are reported in δ units (ppm) relative to tetramethylsilane as internal standard. Spectra were obtained in d-DMSO 400 MHz and chemical shifts calculated in ppm with respect to TMS (δ = 0). The electronic UV-Vis spectra of all compounds were measured on a Jasco V-550 (Ubest) spectrometer at room temperature, λ (1100-190nm), using quartz cuvettes. Mass spectra of the compounds were recorded with a Bruker Esquire 3000 (Bremen, Germany) Ion trap Mass Spectrometer coupled with an ESI Dionex UltiMate 3000 Binary Semipreparative LC system. The interaction between compounds and peptide RacEa was done by Micromass Tof Spec 2E MALDI-TOF-MS.

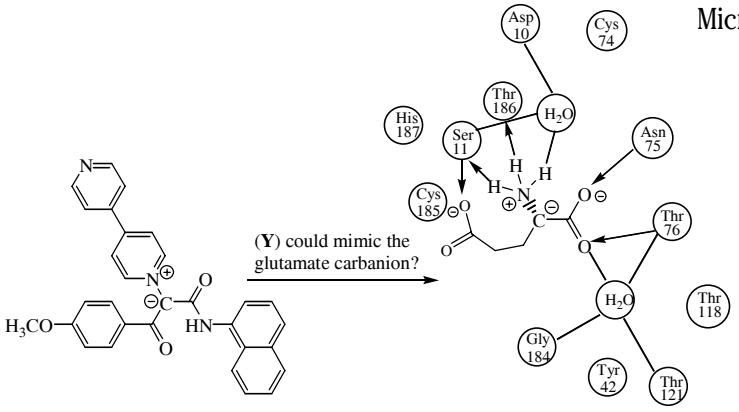

Fig. 1. The structure of 2-(4,4'-bipyridin-1-ium-1-yl)-1-(4-methoxyphenyl)-3-(naphtalen–1-ylamino)-1,3 dioxopropan–2–ide ligand (a), compared with ligand map for RacE-Glu-carbanion „reactive" form with glutamate carbanion (b) (adapted from [11]).

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Synthesis of the ligand

The ligand (**Y**) named 2-(4,4'-bipyridin-1-ium-1-yl)-1-(4-methoxyphenyl)-3-(naphtalen-1-ylamino)-1,3-dioxopropan -2-ide was synthesized as previously reported [21].

The mixture of 1mmol 4-(4 '-bipyridyl) pyridinium salt and 4mL aqueous 0.2N NaOH was stirred for 15 min at room temperature and then the suspension was centrifuged, decanted and the obtained solid washed with water till the *p*H is neutral. After that the dried ylide obtained was dissolved in 4mL DMF and 1.1mmol naphtyl isocyanate was added. The resulting solution was stirred under nitrogen for 5-6 h at room temperature. The precipitate was filtered and dried. Orange powder of the ligand (**Y**) with melting point 235°C was obtained in 50% yield.

*Synthesis of cobalt (II) complex (**1**)*

All the complexes were synthesized using methods from literature [22, 23]. Thus, cobalt (II) complex was obtained by mixing the solution of 2-(4,4'-bipyridin-1-ium-1-yl)-1-(4-methoxyphenyl)-3-(naphtalen-1-ylamino)-1,3-dioxopropan-2-ide **Y** (38mg, 0.1mmol) in dichloromethane (5mL) and the solution of cobalt chloride $CoCl_2 \cdot 6H_2O$ (9.56 mg, 0.05mmol) in ethanol (5mL). Triethylamine (14μL, 0.1mmol) was added to the mixture to create the basic medium for the reaction. The mixture was stirred for two hours at room temperature then filtered and allowed to crystallize. Brown crystalline powder of the cobalt complex (**1**) was obtained in 43% yield.

*Synthesis of nickel (II) complex (**2**)*

The nickel complex **2** was prepared by a similar procedure to that of **1**, using $NiCl_2 \cdot 6H_2O$ (8.5mg, 0.05mmol) and ligand **Y** (32mg, 0.1mmol). The brown-red powder of the nickel complex in 73% yield was obtained.

*Synthesis of copper (II) complex (**3**)*

The copper complex **3** also, was obtained by a similar procedure to that of **1**, using $CuCl_2 \cdot 2H_2O$ (6.3mg, 0.05mmol) and ligand **Y** (35mg, 0.1mmol). The dark-brown powder of the copper complex in 65% yield was obtained.

UV-Vis titration of Y with nickel chloride

The stock solutions were 1.37mM for ligand **Y** and 0.29mM for anhydrous $NiCl_2$ in DMSO. Titrations involved a concentration of 0.77mM ligand **Y** and nickel chloride concentrations varied in the range 0.55mM - 22.22mM.

The UV-Vis spectra of **Y** and metallic complexes were measured in solution of 10^{-4}M methanol:water 1:1 (v:v).

Fluorescence

All compounds were analyzed using λ_{emis} = 380nm in MeOH, (10^{-4}M concentrations).

Synthesis of RacEa peptide

RacEa peptide (fig. 2) (part of the catalitic site of GR) was synthesized using standard protocols for Boc chemistry on solid supports (tBoc-amino acid-OCH_2-PAM MBHA Rink Amide resins from Neosystem Laboratories).

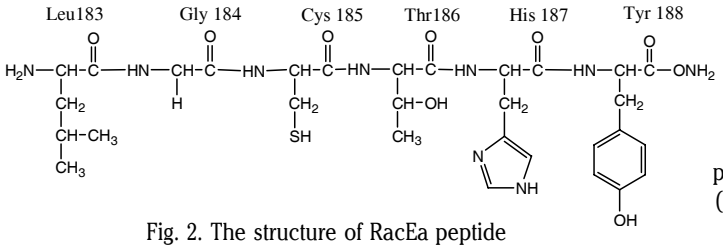

Fig. 2. The structure of RacEa peptide

The synthesis was performed on an ABIMED Economy Peptide Synthesizer EPS 221 Applied 433A peptide synthesizer using PyBoP-L-amino acids purchased from Senn Chemicals. Side chain protecting groups were removed and simultaneously the peptide was cleaved from the resin by treatment with HF for 1h at 4°C and then solution containing 95% TFA, 2.5% H_2O and 2.5% triisopropylsilane TIPS. The synthetic peptide LGCTHY was analyzed by reversed-phase high-performance liquid chromatography HPLC using a Lichrospher (Merck, NJ) C18 silica gel column. The peptide was purified using gradient of 0.1% TFA and 80% MeCN/H_2O, with the flow of 2 mL/min, in 60 min, to obtain 98% purity [15]. After purification, the peptide was analyzed by matrix-assisted laser desorption-ionization–time-of-flight (MALDI–TOF) mass spectrometry using MeCN: 0.1% TFA in ratio 2:1 (v:v) as solvents.

Results and discussions

The structure of the ligand (Y) (fig.1) was proved by analytical and spectroscopic data [21].

UV-Vis spectroscopy

The complexation of ligand **Y** with Ni was quantitatively studied using UV-Vis titrations. Thus, by UV- Vis titration of ligand **Y** with nickel chloride in dimethylsulfoxide (DMSO), we observed that all UV-Vis absorption bands of the ligand are shifted (fig. 3), but the strongest shift was observed for the absorption band from 475nm to 420nm in the nickel complex.

For Ni, Co and Cu complexes of ylide **Y**, the UV-Vis spectra were recorded and compared with the spectrum of the ligand (table 1). Thus, UV-Vis spectrum of the ligand presents four absorption bands. The UV absorption band at λ_3 = 331nm can be assigned to π-π* transitions while the

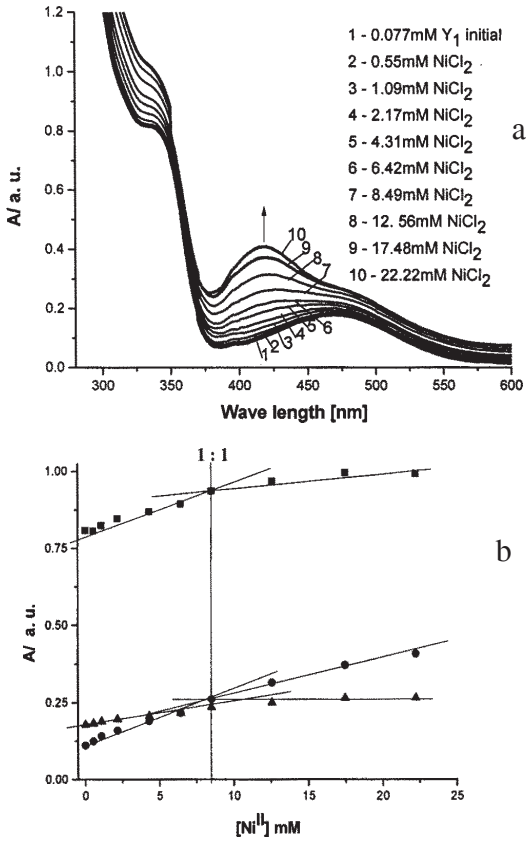

Fig. 3. UV-visible absorbance spectra for $7.7x10^{-2}$mM of **Y** in the presence of 0.55 – 22.22mM of $NiCl_2$ (a) and absorbance at 340nm (squares), 420nm (circles) and 475nm (triangles) as a function of $NiCl_2$ concentration, all in DMSO at 25°C (b)

No.	Compound	λ_1 [nm] ($\lg \varepsilon_1$)	λ_2 [nm] ($\lg \varepsilon_2$)	λ_3 [nm] ($\lg \varepsilon_3$)	λ_4 [nm] ($\lg \varepsilon_4$)
	(Y)	226 (5.02)	266 (4.95)	331 (4.74)	475 (4.17)
1	$[Co(Y)_2Cl_2]$	223(4.52)	263 (4.43)	336 (4.08)	445 (3.05)
2	$[Ni(Y)_2Cl_2]$	259 (3.92)	288 (3.49)	347 (3.19)	420 (2.78)
3	$[Cu(Y)_2Cl_2]$	221 (5.11)	265 (4.95)	338 (4.81)	440 (3.12)

Tabel 1
ABSORPTION BANDS IN REGION UV – Vis OF LIGAND AND COMPLEXES

absorption band in visible at $\nu\lambda_4$ (440nm) is assigned to n-π^* transitions [22]. For complexes **1** and **3**, we observed a broadening of the absorption bands around λ_3 = 334nm, and the disappearance of the band from 440nm, caused by metal coordination. The d - d bands of Co and Cu complexes are weak because of their low solubility in methanol while the nickel complex shows a strong change in position and intensity of λ_2 and λ_3 bands.

Fluorescence

From fluorescence spectra of complexes **1**-**3**, it was observed that only $[Cu(Y)_2Cl_2]$ (**3**) and $[Ni(Y)_2Cl_2]$ (**2**) complexes present fluorescence with the absorption maxima at λ = 465 nm and λ = 460 nm, respectively.

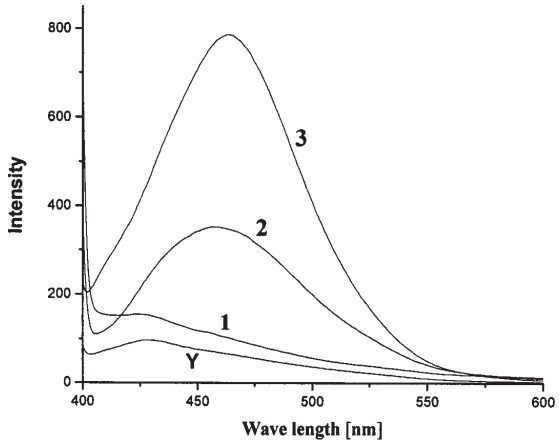

Fig. 4. Fluorescense absorbtion spectra of the ligand **(Y)**, $[Co(Y)_2Cl_2]$ - **(1),** $[Ni(Y)_2Cl_2]$ - **(2),** $[Cu(Y)_2Cl_2]$ - **(3)** compounds in MeOH

IR spectra

The characteristic bands of the ligand **Y** were modified in position and intensity, by coordination with cobalt (II), nickel (II) and copper (II) metals. The absorption band of N-H vibration appears at 1630cm^{-1} in the ligand, but at 1623cm^{-1} in the copper (II) complex. The band corresponding to $\nu_{C=O}$ carbonyl group which appears at 1485cm^{-1} in the ligand are shifted at 1475cm^{-1} for cobalt and copper complexes. New absorption bands of complexes **1**-**3** in the region 502-401cm^{-1}, assigned to $\nu_{(M-N)}$ and $\nu_{(M-O)}$ show the coordination of the carbonyl oxygen and amide nitrogen from the ligand **Y** [23]. Significant IR bands of ligand **Y** and its metal complexes are presented in table 2.

ESI- MS analysis

The nitrogen-ylides can be proton acceptors, electron acceptors or electron donors; therefore their behaviour under chemical ionization conditions in a mass spectrometer is of interest. The ESI-Ion Trap-MS spectrum of ligand **Y** from (fig. 5) shows a molecular peak for $[YH]^+$ at m/z = 474.

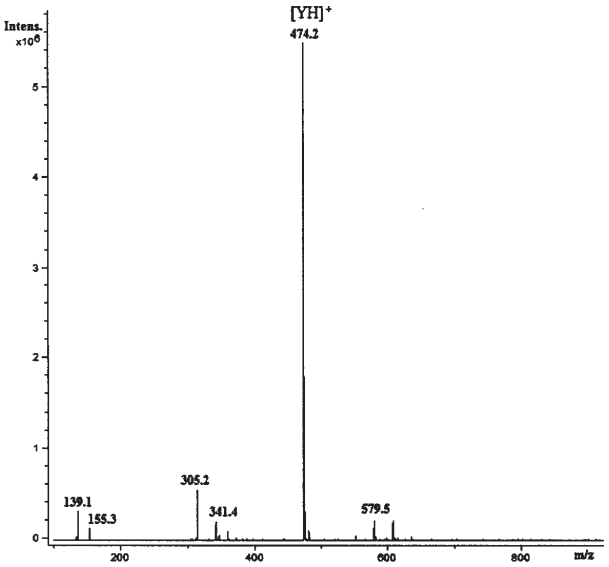

Fig. 5. ESI - IonTrap-MS of ligand Y

In the mass spectrum of cobalt (II) complex (**1**) it was observed a peak with low intensity at m/z = 1078 (fig. 6) which corresponds to $H[Co(Y)_2Cl_2]^+$.

Thermal analysis

The thermogravimetric curves TG, DTG and DTA of complex $[Co(Y)_2Cl_2]$ (**1**) are presented in figure 7 and the decomposition steps in table 3. The complex **1** decomposed in four exothermic steps.

The first mass loss of 3.09% in temperature range 40–120°C, was assigned to removal of two crystallization water molecules (table 3). The next step with the mass loss estimated at 30.53%, in temperature range 120–260°C, was assigned to partial decomposition of the complex, and elimination of two bipyridyl groups ($2C_{11}H_8N_2$). In the third step, in the range 260-390°C the mass loss of 21.45% was

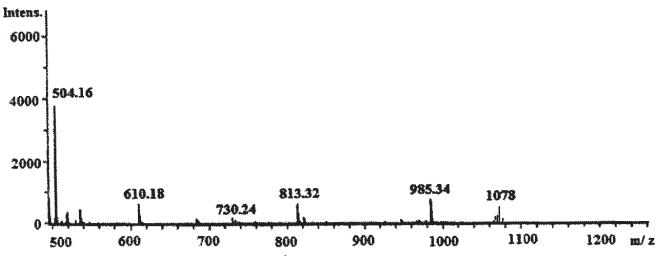

Fig. 6. ESI-MS spectra of $[Co(Y)_2Cl_2]$ complex

No.	Compound	ν_{H2O}	δ_{N-H}	$\nu_{C=O}$	ν_{M-N}	ν_{M-O}
	(Y)	3391	1630	1485	-	-
1	$[Co(Y)_2Cl_2]$	3387	1623	1475	492	421
2	$[Ni(Y)_2Cl_2]$	3410	1628	1502*	462	401
3	$[Cu(Y)_2Cl_2]$	3387	1629	1475	502	419

Table 2
SIGNIFICANT IR BANDS (4000-400cm^{-1}) OF LIGAND (Y) AND ITS COMPLEXES

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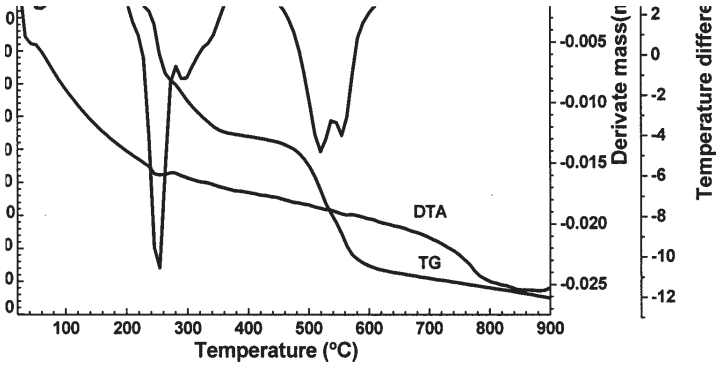

Fig. 7. Thermogram of $[Co(Y)_2Cl_2]$ complex

Temperature range (°C)	Mass loss found (calculated)	Assignments
35 - 120	3.09 (3.15)	$2H_20$
120 - 260	30.53 (30.67)	$2C_{11}H_8N_2$
260 - 390	21.45 (21.49)	$2C_8H_7O$
390 - 670	38.02 (37.96)	$2C_{11}H_7NOCl$
Residue	6.91 (6.73)	CoO

Table 3
THERMOANALYTIC RESULTS FOR COBALT (II) COMPLEX

assigned to eliminate two -C-$C_6H_4OCH_3$, ($2C_8H_7O$) groups. The fourth step in range 390 – 640°C with mass loss of 38.02% can be assigned to two molecule of two –CH-NH-naphtyl groups and two atoms of chloride ($2C_{11}H_7NOCl$). Over 670°C the mass loss is small and can be attributed to the transition of Co_2O_3 in CoO that remains as residue (6.91%).

*Peptide interaction with ylide ligand **Y***

The GR enzyme in *Bacillus Subtilis* contains 272 aminoacids [24]. The sequence proposed to be synthesized was the peptide RacEa (L183-Y188) which contains several amino acids from the catalytic site of GR [25]. The RacEa peptide was synthesized by solid phase peptide synthesis (Fmoc-SPPS) and purified by RP-HPLC. The MALDI-TOF MS spectrum showed only the signal for the RacEa peptide product (fig. 8).

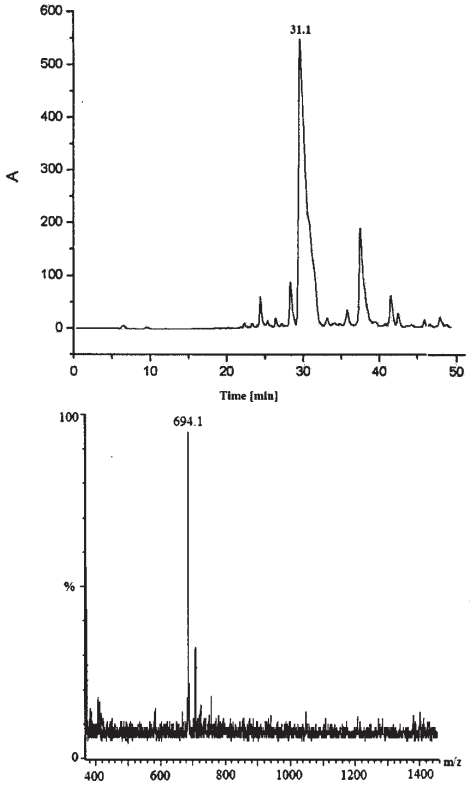

Fig. 8. HPLC chromatogram of synthetic peptide RacEa (a) and MALDI-TOF spectra of purified RacEa peptide (b)

The interaction between the ligand 2-(4,4'-bipyridin-1-ium-1-yl)-1-(4-methoxyphenyl)-3-(naphtalen-1-ylamino)-1,3-dioxopropan-2-ide (109μg, 2.3μmol) and the synthetic peptide RacEa (100μg, 2.3μmol) was tested by affinity-MS analysis [26]. Initially, a HiTrap column (3.5 μm filter) filled with (0.2 μmol) NHS-Sepharose TM4 Fast Flow was pre-equilibrated with the binding buffer (0.2M $NaHCO_3$, 0.5M NaCl) at *p*H 8.3. Then, the peptide RacEa was immobilized on NHS-activated Sepharose which is a good matrix for immobilization of peptides and provides a macroporous matrix with high chemical and physical stability and low non-specific adsorption to facilitate a high binding capacity. The RacEa peptideimmobilization is made through the N-terminal amino group. The resulting affinity matrix was washed and equilibrated with binding buffer (5mM NH_4HCO_3, *p*H 7.5). 10 μg ligand **Y** solved in 200μL binding buffer was added in the column and left overnight to rotate at 25°C. The unbound ligand **Y** was eliminated from the column by washing four times using the binding buffer. The affinity-bound ligand **Y** was recovered by elution with MeCN: 0.1% aqueous TFA, in a 2:1 ratio (*p*H 2).

In figure 9, a, b, c) we presented comparatively the MALDI-TOF spectra of supernatant (solution of ylide **Y** in buffer), the last fraction collected after the elimination of unbound ylide (wash) and the fraction collected after the cleavage and elution of the bound ylide on RacEa (elution). In the figurte 9, d, e, f) there are the mass spectra for the same fractions of the negative control of the ligand **Y**. This was done in the same conditions, without immobilizing the peptide RacEa, only in the presence of Sepharose. There is no peak of the ligand **Y** after elution which proves that there are no interactions between the ligand and sepharose.

The spectra of *supernatant* show the molecular peaks at m/z = 475 for the ligand. The last *wash* fractions showed no peak for the ligand **Y**, while the *elution* fractions showed the recovered bound ligand molecular peak with m/z = 474 only for the experiment involving RacEa.

All these spectra show that between synthetic *RacEa* peptide and ligand **Y** occur strong interactions.

In order to prevent the RacEa dimerization through formation of disulfide bridges due to Cys 185, we repeated the same experiment in the presence of dithiothreitol (DTT).

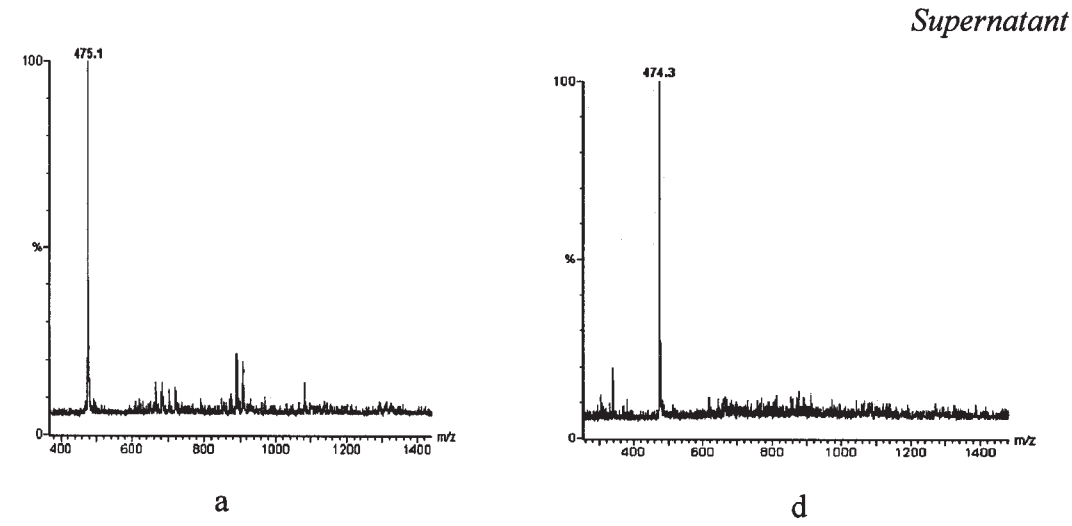

Fig. 9. MALDI-TOF spectra of *supernatant, wash* and *elution* fractions of **Y** with RacEa (a-c) and negative control of the ligand with Sepharose (d-f)

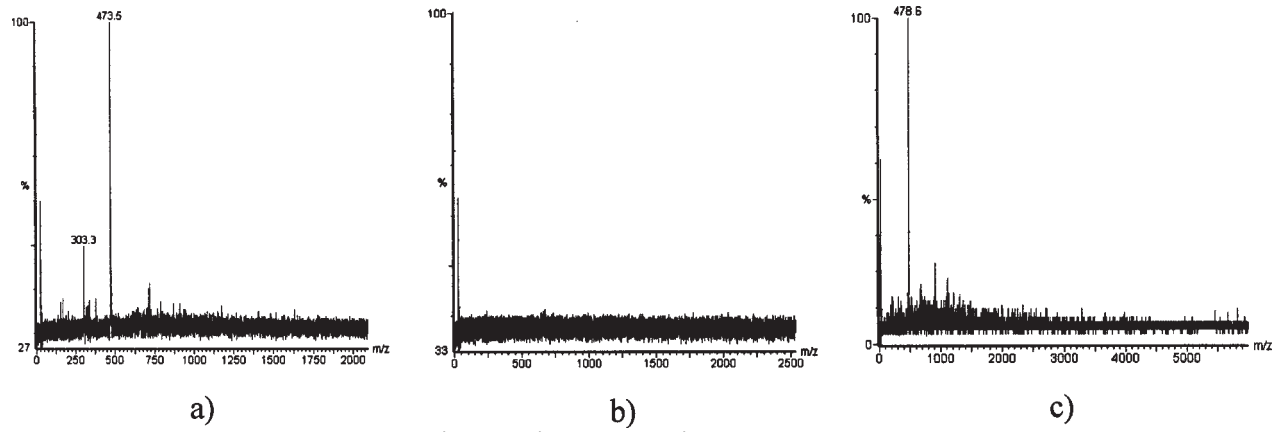

Fig. 10. MALDI-TOF spectra of *supernatant* a), *wash,* b) and *elution,* c) fractions of **Y** interction with RacEa in the presence of DTT

In figure 10 MALDI-TOF spectra of *supernatant*, *wash* and *elution* fractions in the presence of DTT are presented. The spectrum a) showed the peak at m/z = 473 for the ligand *supernatant*. The last *wash* fraction b), showed again no peak of the ligand, while the *elution* fraction c), showed again the peak for the recovered ligand Y. This experiment in the presence of DTT proves that there are indeed strong interactions between RacEa and ligand **Y**. These preliminary results make this class of compounds interesting as inhibitors for GR.

Peptide interaction with nikel complex

Interaction of the nickel complex **2** with RacEa was done by the same procedure as for the free ligand. Nickel complex was not recovered after cleavage and elution step performed in the same conditions (solution of ACN: 0.1% aqueous TFA, ratio 2: 1, *p*H 2), which shows there are no interactions between RacEa peptide and complex **2**. Due to the negative results for the RacEa - $[Ni(Y)_2Cl_2]$ interactions, we have not performed similar experiments for complexes **1** and **3**.

Conclusion

The new complexes of cobalt, nickel, copper with 2-(4,4'-bipyridin-1-ium-1-yl)-1-(4-methoxyphenyl)-3-(naphtalen-1-ylamino)-1,3-dioxopropan-2-ide ligand were obtained in molar ratio 1:2. The interaction of these compounds with the RacEa peptide (LGCTHY) was studied by affinity mass spectrometry. It was observed that only the ligand binds to the synthetic *RacEa* peptide.

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