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NEW TRANSITION METAL COMPLEXES OF BIGUANIDE DERIVATIVES – BIOLOGICAL ACTIVITY ON MARINE ORGANISMS

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Abstract. The N-substituted biguanide derivatives have recieved a considerable attention for their antidiabetic and antimalarial activity and for the therapeutic treatment of pain, anxiety and memory disorders as 5-HT_3 serotonin receptors. The biological action of these complexes on the human body may be explained by the formation of these compounds. We have synthesised a new series of La(III) and Ce(III) complexes derived from N-substituted biguanide. These derivatives were characterised by elemental analysis, molar electrical conductivity, EPR, IR, UV-vis. and electronic spectroscopy. Their antibacterial activity was confirmed by using some pathogen bacteria cultures. In this paper we report the effects of some La(III) and Ce(III) compounds on the invertebrate species *Euxinia maeotica* Crustacean and bacterian *Halomonas* sp. isolated from the Romanian Black sea coast. Toxicity tests LD_{50} and LD_{100} were determined on the mentioned organisms. The physiological reactions were observed on the invertebrate crustacean species: the breathing rhythm modifications, the oxygen consumption and excretion.

Keywords: La(III) and Ce(III) complexes, N-substituted biguanide compounds, *Euxinia maeotica* Crustacean, *Halomonas* bacterial culture, biological activity,

AIMS AND BACKGROUND

The literature in the last years points out a large practical application spectrum of the lanthanides complexes of expanded porphyrins as efficient photosensitisers which produce aggressive singlet oxygen after controlled activation by light, as diagnostic tools in medicine for their fluorescence imagining, in therapeutic radiopharmaceutics, for body pH-monitoring and as biomarkers[1,2].

On the other hand, the N-substituted biguanide derivatives are known for their antidiabetic, antimalarial, antiseptic and disinfectant activity being used for therapeutic treatment of pain, anxiety and memory disorders[3] as 5-HT_3 agonist serotonin receptors[4-6].

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In other previous papers we described the synthesis and characterisation of the La(III) and Ce(III) complexes compounds with biguanide derivatives using elemental chemical analysis, molar electrical conductivity, electronic spectra, EPR, IR and UV-vis. spectra. The selection of the ligand and of the *f*-metal ions could be connected with the therapeutic activity on the human body. The ligand N,N-dimethylbiguanide (Fig. 1) and the inorganic salts $La(NO_3)_3.6H_2O$ and $Ce(NO_3)_3.6H_2O$ were obtained from Merck and Fluka and were used without further purification. The complex compounds were obtained by refluxing the methanol solution p.a. of $Ce(NO_3)_3.6H_2O$, $La(NO_3)_3.6H_2O$ and ligand L, in corresponding M:L molar ratio 1:2. The complexes were refluxed 2-3 h. The resulting precipitate was separated by filtration salt: ligand after the evaporation of the excess of solvent and washed in methanol-ether mixture and dried on $(P_2O_5)_2$ in vacuum dessicator. The compounds are cream coloured white powder soluble in alcohol, acetonitrile, N,N-dimethylformamide[7-10].

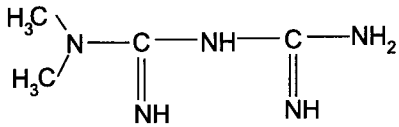


Fig. 1. N,N-dimethylbiguanide ligand

The antibacterial activity of the ligands, metallic salts and synthesised complexes was determinated using different pathogen bacteria and fungal strains such as β-haemolytical *Streptococcus* group A, *Pseudomonas aeruginosa* serotype VI, *Salmonella* group B, *Corynaebacterium* diphteriae-intermedius, *Corynebacterium* diphteriae-gravis toxigen, *Bacillus subtilis*, *Bacillus globigii*, *Bacillus sphericus*, *Bacillus vulgatus*, *Bacillus megatherium*, *Shigella flexneri*, *Candida albicans* and *Saccharomyces cerevisiae*[11].

Our paper presents two aspects: the effects of the La(III) and Ce(III) complexes with N,N-dimethylbiguanide on the invertebrate *Euxinia maeotica crustacean* species adapted at a saline aquatic medium and on the marine saprophitic bacterium *Halomonas* sp. laboratory cultures compared with the effects of Ce(III) and La(III) salts, ligand N,N-dimethylbiguanide and synthesised complexes, solvents acetonitrile and N,N-dimethylformamide on the same organism. The experiments had as aim to establish some significant doses of these compounds for estimating their toxicity[12].

We chose *Pontagammarus* (*Euxinia*) *maeoticus* S o w i n s k i, 1984 (Crustacea Amphipoda) as biological material for its qualities: one of the most psalmicole species, very easy to access towards the year, optimal size for experimental, forms specific crowds with a density of thousands of samples on meter square. *Euxinia maeotica* has also a great tolerability at salinity variations and it is very easy to

adapt in laboratory conditions no matter the age of the individuals in the sample used. This species is always used as biotester organism in experiments *in vitro*[11,13-17]. It's easy to observe the physiological modifications at this invertebrate, respiratory rhythm, oxygen consumption.

The saprophytic bacterian *Halomonas* sp. isolated from the Romanian Black aea coastal sediments is extremely salt-tolerant aerobic, heterotrophic gram-negative, easy to adapt at experimental conditions and can be used in ecotoxicology tests[12].

The experiment consisted in observations of the effects of the contamination with the new synthesised compounds, Ce(III) and La(III) salts, ligand and solvents on the marine organisms. Physiological reactions observed at the invertebrate crustacean species were the breathing rhythm modifications, mobility, the oxygen consumption, nutrition and excretions, first signs of stress. At the marine bacteria strain we observed growth and inhibition actions, depending of the contaminants.

EXPERIMENTAL

Marine invertebrate organism data. For this study we used *Euxinia maeotica* adults (10-12 mm length and 0.0319 g weight/individual) which had been maintained in laboratory conditions for 10 days, then we have taken samples of 20 individuals each, examinated in contamination conditions in sea water vessels of 1000 ml, comparative to the control sample. Increased doses were used for each tested compounds. The stock solutions for contamination are presented in Table 1. The physico-chemical parameters of the water (temperature (°C), salinity (‰), oxygen consumption (mg/l), pH, density (mg/l) and electrical conductivity) were measured daily in experimental vessels (Table 2).

Table 1. Stock solutions for the contaminants

$Ce(NO_3)_3$	$La(NO_3)_3$	N,N-dimethyl biguanide	Ce:N,N-dimethyl biguanide complex (1:2)	N,N-dimethyl formamide	Acetonitrile	La:N,N-dimethyl-biguanide complex (1:2)
0.4340 g/ 100 ml distillated water	0.4330 g/ 100 ml distilated water	0.1655 g/ 100 ml distilated water	0.1800 g/ 100 ml solvent	saturation conc./20°C 12 g/m^3	saturation conc./20°C 163 g/m^3	0.1790 g/ 100 ml solvent

Table 2. Experimental series and physicochemical parameters

Experimental series	Contamination concentration (µl/l)	Number of individuals in experimental vessels	Oxygen consumption in water (mg/l)	pH	Water temperature (°C)	Salinity (%)	Electrical conductivity (mV)
			$Ce(NO_3)_3$				
Sample I	20	20	4.46	8.46	26	16	–73
Sample II	50	20	4.90	8.47	25.5	16	–74
Sample III	100	20	4.45	8.46	26	16	–73
Control	–	20	4.59	8.46	26	16	–73
			$La(NO_3)_3$				
Sample I	20	20	2.66	8.49	23.25	18	–75
Sample II	50	20	2.77	8.48	23.2	18	–74
Sample III	100	20	2.79	8.48	24.3	18	–74
Control	–	20	2.77	8.49	25.1	18	–75
			N,N-dimethylbiguanide				
Sample I	50	20	3.34	8.46	17.7	18	–73
Sample II	100	20	3.27	8.47	16.4	17	–74
Control	–	20	3.12	8.46	16.3	17	–73
			Acetonitrile				
Sample I	50	20	2.9	8.45	18.8	17	–73
Sample II	100	20	2.92	8.46	18.7	17	–73
Control	–	20	3.12	8.47	16.3	17	–74
			N,N-dimethylformamide				
Sample I	20	20	5.47	8.48	22.3	18	–75
Sample II	50	20	5.56	8.49	22.7	18	–76
Sample III	100	20	5.22	8.48	23.4	18	–75
Sample IV	200	20	5.31	8.49	23.5	18	–74
Sample V	250	20	5.03	8.48	23.6	18	–74
Sample VI	300	20	4.37	8.48	22.5	18	–73
Sample VII	350	20	3.96	8.48	24.1	18	–73
Sample VIII	400	20	3.36	8.48	23.8	18	–73
Control	–	20	5.03	8.49	23.8	18	–75
			Ce:N,N-dimethylbiguanide complex 1:2				
Sample I	20	20	4.72	8.47	26.1	16	–74
Sample II	50	20	4.40	8.46	26	16	–73
Sample III	100	20	4.29	8.46	26.1	15	–73
Control	–	20	5.27	8.48	24.4	16	–74
			La: N,N-dimethylbiguanide complex 1:2				
Sample I							
Sample II							
Sample III							

Apparatus. Multichannel WTW (oxygenometre, salinometre, pH-metre, potentiometre).

Saprophytic strain data. The saprophytic strain used in this experiment was isolated from the Romanian Black sea coastal sediments (50 m depth). On the basis of their 16S rRNA sequences it was identified as *Halomonas* sp. (members of the gamma subdivision of Proteobacteria).

Halomonas sp. is colourless, extremely salt-tolerant aerobic, heterotrophic gram-negative, motile, rod-shaped bacteria with 6.6-0.8 μm wide and 1.6-1.9 μm long and does not produce endospores (Fig. 2).

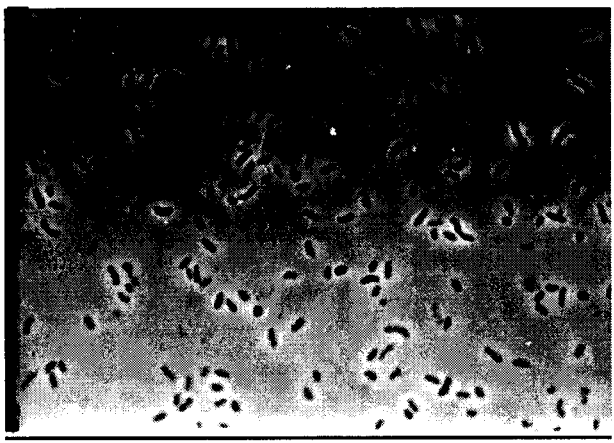


Fig. 2. Phase contrast photograph of *Halomonas* sp.; bar = 5 μm

Pure culture of *Halomonas* sp. was tested for the ability to growth on the marine solid medium (ZoBell) suplemented with the following compounds (Table 1):

- $Ce(NO_3)_3$ and $La(NO_3)_3$;
- N,N-dimethylbiguanide;
- new synthesised complex compounds (Ce(III):N,N-dimethylbiguanide and La(III): N,N-dimethylbiguanide in molar ratio 1:2);
- solvents for the mentioned complexes (acetonitrile and N,N-dimethylformamide).

The Petri dishes (in duplicate) were inoculated with 0.2 ml diluted culture and 0.2 ml sterile solution of each mentioned compounds (sample). Two controls consisted of inoculated medium (medium + bacterium is control I) and uninoculated medium suplemented with 0.2 ml compounds is control II were used.

All solid cultures were incubated for 7 days at room temperature (20 to 25°C) under aerobic conditions.

RESULTS AND DISCUSSION

Observations of the marine crustacean invertebrate. Our study consisted in testing the toxicity of these complex compounds using one species of crustacean already used as bio-marker organism.

The tests regarding the action of the Ln(III) complexes with biguanide derivatives on the *Euxinia maeotica* Crustacea Amphipoda revealed the fact that the $La(NO_3)_3$ has toxic effects more rapidly in 72 h from the contamination, 50% of the organism died (DL_{50}), the rest of the individuals having lethal reactions in the next 5 days (Fig. 3).

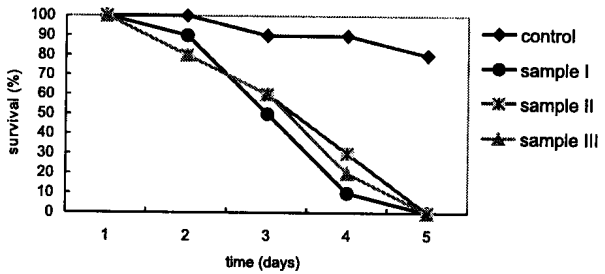


Fig. 3. Survival rate of *E. maeotica* contaminated with $La(NO_3)_3$

The $Ce(NO_3)_3$ has a reduced toxic effect, only in the eight day the DL_{50} is registered and the DL_{100} is registered after 10 days for all concentrations used (Fig. 4).

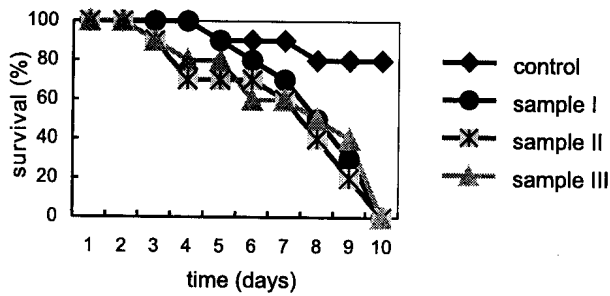


Fig. 4. Survival rate of *E. maeotica* contaminated with $Ce(NO_3)_3$

The acetonitrile solution used as solvent for the analysed complexes has such an acute toxicity that in 3 days 50% of the organisms manifest lethal effects for both concentration used (Fig. 5).

In the next 3-4 days a constant value with no fatalities can be observed. This aspect could be explained by the organisms' reaction of stocking the toxic substances in excretory cells (nefrocites) situated at the bases of the bronchi, reason

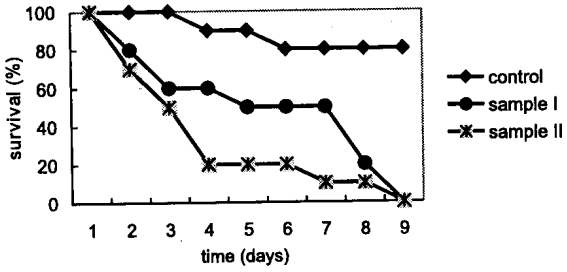


Fig. 5. Survival of *E. maeotica* contaminated with acetonitrile sat. sol.

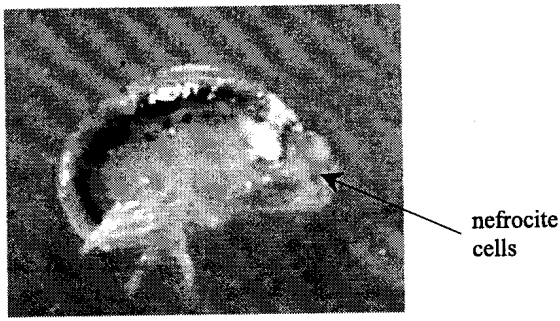


Fig. 6. *Euxinia maeotica* S o w. after acetonitrile contamination

for which this excretory bags become dark and prominent (Fig. 6). After this period of resistance, the organisms die, reaching for the DL_{100} after 9 days of experiment.

In case of N,N-dimethylbiguanide contamination, the DL_{50}, with a more reduced toxicity than the acetonitrile and the $La(NO_3)_3$, but with a more increased toxicity than the $Ce(NO_3)_3$ appears after 6 days (Fig. 7).

The N,N-dimethylformamide, at low concentrations (20, 50, 100, 200 µl/l) did not present toxic effects for the first 5 days. In the sixth day, the doses were

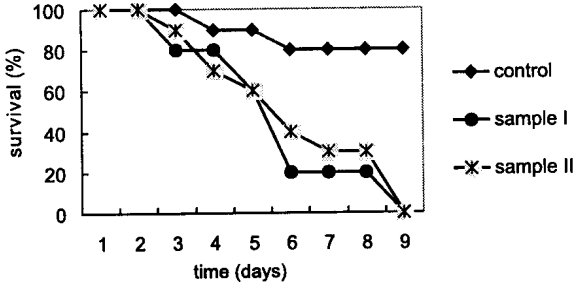


Fig. 7. Survival of *E. maeotica* contaminated with N,N-dimethylbiguanide

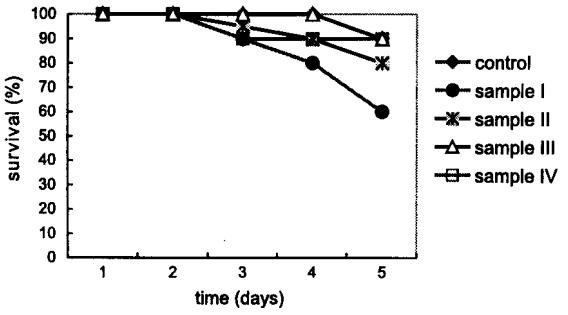


Fig. 8. Survival of *E. maeotica* contaminated with DMF (low concentrations)

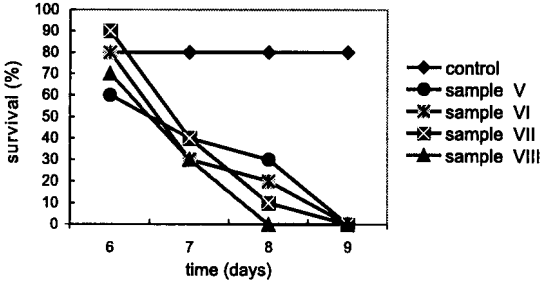


Fig. 9. Survival of *E. maeotica* contaminated with DMF (high concentrations)

increased to 250, 300, 350, 400 µl/l. The growth of the concentration had determined the death of the organisms in the next 5 days, but the effects came out gradually depending on the individual sensibility of the organisms (Figs 8 and 9).

The Ce:N,N-dimethylbiguanide complex solved in DMF presents high toxicity at the 50 and 100 µl/l concentrations; a high rate of fatalities in the next four days can be observed, while at the 20µl/l concentration the organisms have survived in the same ratio as the ones from control; fatalities can be observed after 7 days (Fig. 10).

Physiological responses. The organisms reactions at the stress conditions caused by the induced intoxication consists in biochemical, physiological and behaviour

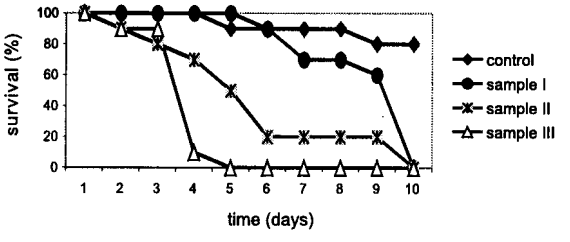


Fig. 10. Survival of *E. maeotica* contaminated with Ce:N,N-dimethylbiguanide

parameters modification. At the marine crustacean invertebrate, the physiological reactions are the most specific, easy to follow and consist in the increase or decrease of respiration rhythm, depending on metabolism, depositing and excretion pathway of the toxic. In the period of contamination the organisms did not fed as much as the control organisms. This showed an alteration of the metabolism induced by contamination and depending on the physiological needs.

The oxygen consumption (Table 2) indicates that the La(III) and Ce(III) salts, no matter the concentration used, did not have a significant modification immediately after the contamination. It must be noticed that in the first 2 or 3 days after the exposure at different La(III) and Ce(III) concentrations, the organism survival is high (80-100%). Immediately after the acetonitrile and N,N-dimethylformamide contamination an increasing respiratory oxygen consumption was observed. In the first minutes after exposure to the substances mentioned, the mobility of the organism has increased, they started to swim easily and rose to the surface of the vessel. Also the oxygen consumption has increased at 2.405 mg/g/h (at the concentration of 50 μl/l) and doubled (compared with control 3.392 mg/g/h at 100 μl/l). The ligand N,N-dimethylbiguanide induced a significant oxygen consumption (5.5472 mg/g/h), increasing especially at small concentrations (50 μl/l) and a lower oxygen consumption (2.8485 mg/g/h) at high concentration (100 μl/l). In similar conditions of testing at the same species but with another Ce(III) complex (Ce:α-ketoglutaric acid 1:2) and N,N-dimethylformamide as solvent, the respiratory rhythm is amplified 2 or 3 times. The N,N-dimethylformamide solvent for both complexes maintained this physiological parameter at proximate values of control.

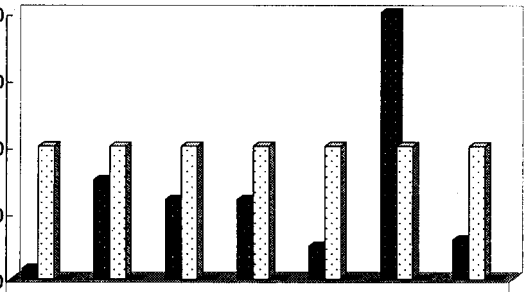
Saprophytic strain observations. After 7 days of incubation the solid medium from the probe and controls was analysed for bacterium growth.

Our results indicated that all tested compounds do not induce the inhibition of saprophytic strain growth, in all cases the bacterium was proliferated as following (Fig. 11):

– the $Ce(NO_3)_3$ produced a lower growth than organic ligand, but in the Ce: N,N-dimethylbiguanide 1:2 complex, Ce (III) did not influence the growth (the same values between ligand and complex were shown);

– the effect of $La(NO_3)_3$ on the bacterium growth was higher than ligand, but in the La : N,N-dimethylbiguanide 1:2 complex this effect is decreased;

– N,N-dimethylbiguanide has the same effect on the bacterium growth as the Ce : N,N-dimethylbiguanide 1:2 complex and higher than La:N,N-dimethylbiguanide 1:2 complex; this effect could be explained knowing their antidiabetic activity and also the fact that some microorganisms can use another metabolic pathways for hexoses oxidation, such as pentose phosphate pathway or the Entner–Doudoroff patway, than classical monosaccharides degradation pathway (the citric acid cycle) (Fig. 12);



	$Ce(NO_3)_3$	$La(NO_3)_3$	ligand N,N-dimethyl	complex Ce: N,N-dimethyl	complex La: N,N-dimethyl	solvent acethony-trile	solvent N,N-dimethyl
■ substance sample	8	75	60	60	25	200	30
☐ control I	100	100	100	100	100	100	100
■ control II	0	0	0	0	0	0	0

Fig. 11. Effect of synthesised compounds, ligand, inorganic salts and solvents on the *Halomonas* sp. density

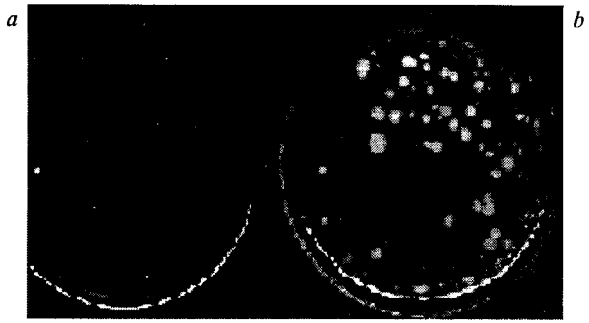


Fig. 12. *Halomonas* sp. bacterial culture with the ligand N,N-dimethylbiguanide: *a* – control (medium + ligand); *b* – sample (medium + bacterial inoculum + ligand)

– the newly synthesised complexes had different effect on the bacterium growth in our experimental conditions, such as the Ce : N, N-dimethylbiguanide complex is higher than La:N, N-dimethylbiguanide complex;

– in Petri dishes suplemented each of them with the solvents used for complexes dissolution, the highest bacterium density for acetonitrile and, respectively, a middling growth activity for N,N-dimethylformamide (knowing its mutagenic effects) was observed.

CONCLUSIONS

The action of all the substances we have used for the contamination are different in time and depend on the concentrations of the solutions.

First answers as sublethal effects for the *Euxinia maeotica* Crustacea Amphipoda involved the mobility and the ventilation of the abdominal appendix (pleopodes).

The complexes used have a high toxicity leading to lethal effects on the marine organisms in this order: DMF < N,N-dimethylbiguanide < Ce(III) salt < La(III) salt < Ce:N,N-dimethylbiguanide < acetonitrile.

The stress induced by intoxication has determined changes in the physiological parameters: respiratory rate, excretion rate depending on the contaminant and the concentrations used.

Taking into account our preliminary experimental results regarding the clear response of the growing or inhibition bacterial strain at the analysed La(III) and Ce(III) compounds groups, *Halomonas* sp. as well as *Euxinia maeotica* Crustacean could be used as 'test organism' for new synthesised compounds for biological activity evaluation.

REFERENCES

1. S. L. SHAPIRO, V. A. PARRINO, E. ROGOW, L. FREEDMAN: J. Am. Chem. Soc., **81**, 2220 (1959).
2. S. L. SHAPIRO, V. A. PARRINO, E. ROGOW, L. FREEDMAN: J. Am. Chem. Soc., **81**, 3725 (1959).
3. C. R. SIRTORI, C. PASIK: Pharmacol. Res., **30**, 187 (1994).
4. W. M. WATKINS, J. D. CHULAY, D. G. SIXSMITH, H. G. SPENCER, R. E. HOWELLS: J. Pharm. Pharmacol., **39**, 261 (1987).
5. P. MORAIN, C. ABRAHAM, B. PORTEVIN, G. de NANTENIL: Mol. Pharmacol., **46**, 732 (1994).
6. W. M. WATKINS, J. D. CHULAY, D. G. SIXSMITH, H. G. SPENCER, R. E. HOWELLS: J. Pharm. Pharmacol., **39**, 261 (1987).
7. C. R. SAHA: J. Inorg. Nucl. Chem., **38**, 1635 (1976).
8. L. THUNUS, R. LEJEUNE: Coord. Chem Rev., **184**, 125 (1999).
9. V. WING-WAH Y., K. KAM-WING LO: Coord. Chem. Rev., **184**, 157 (1999).
10. D. E. REICHERT, J. S. LEWIS, C. J. ANDERSON: Coord. Chem. Rev., **184** (1999)
11. T. NEGREANU-PIRJOL, C. CARPUS, L. CARPUS, R. SIRBU, C. GURAN: Biological Activity Studies of Some Ln(III) Complex Compounds with Difunctional Organic Ligands. In: The 12th Romanian International Conference on Chemistry and Chemical Engineering RICCCE, 12 Bucharest, 13th –15th Sept. 2001.
12. T. NEGREANU-PIRJOL, V. SERBAN, A. STROE, M. CRASMARU: Physiological Response of Aquatic Invertebrates Intoxicated with Ln(III) Complex Compounds Potential Use in Ecotoxicology-Preliminary Data. In: The 12th Romanian International Conference on Chemistry and Chemical Engineering RICCCE, 12 Bucuresti, 13th –15th Sept., 2001.
13. P. G. MOORE, P. S. RAINBOW: Copper and Zinc in an Ecological Series of Talitroidean Amphipoda (Crustacea). Oecologia, **73**, 120 (1987).
14. D. J. H. PHILLIPS, P. S. RAINBOW: Barnacles and Mussels as Biomonitors of Trace Elements: A Comparative Study. Marine Ecology (Progress Series), **49**, 83 (1988).
15. D. J. H. PHILLIPS, P. S. RAINBOW: Barnacles and Mussels as Biomonitors of Trace Elements: A Comparative Study. Marine Ecology (Progress Series), **49**, 83 (1988).
16. C. PLATAS, F. AVECILLA, A. de BLAS, T. RODRIGUEZ-BLAS, C. F. G. GERALDES, E. TOTH, A. MERBACH, J. C. G. BUNZLI: J. Chem. Soc., Dalton Trans., 611 (2000).
17. P. S. RAINBOW, P. G. MOORE, D. WATSON: Talitrid Amphipods (Crustacea) as Biomonitors for Copper and Zinc. Estuarine, Coastal and Shelf Science, **28**, 567 (1989).

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