Sixth International Conference
South-West University
Faculty of Mathematics & Natural Sciences
Blagoevgrad, Bulgaria 10 - 14 June, 2015

LEU-ARG ANALOGUES: SYNTHESIS, IR CHARACTERIZATION AND DOCKING STUDIES

[1]**Tatyana Dzimbova,** [2]**Atanas Chapkanov,** [1]**Tamara Pajpanova**
[1]*Institute of Molecular Biology "Roumen Tsanev", Sofia, Bulgaria*
[2]*South-West University "Neofit Rilski", Blagoevgrad, Bulgaria*

Abstract. Leu-Arg is a kyotorphin receptor antagonist. It is very important to control antinociception and this could be achieved by modifications in agonist's or antagonist's molecules. Two analogues of Leu-Arg were synthesized, their IR- spectral characteristics were defined, and preliminary docking studies were performed. The results show that new analogues bind receptor thus they could be used for further analysis and their biological activity could be tested in vitro and in vivo.

Keywords: kyotorhin, IR, docking, Mu-opioid receptor, GOLD

Kyotorphin (L-tyrosyl-L-arginine) is a neuroactive dipeptide with morphine-like action, which plays an important role in pain regulation in the brain. The kyotorphin (KTP) is a unique neuropeptide which produces analgesia by releasing opioid pentapeptide. It is synthesized in a specific part of the brain. It is believed that kyotorphin binds to specific receptors, which binding site is not different from the binding site of the μ-opioid receptor (Machuqueiro & Baptista, 2007). When it binds Met-enkephalin is released, Kyotorphin rapidly degraded to Tyr and Arg by the action of peptidases *in vivo*. Therefore the main objective of all involved in kyotophin chemistry is to find ways to increase its stability, mainly by creating analogues containing modified and non-proteinogenic amino acid residues. The effect of many of them is investigated *in vivo* experiments and relationship structure – activity was explained with the help of computational methods (Dzimbova et al., 2014; Dzimbova, et al., 2006). The dipeptide, wherein Tyr-residue is replaced with Leu, exhibits strong antagonistic activity with respect to kyotorphin receptor. It is known that in order to be recognized by the opioid receptor, the ligand must have a free α-amino group, which reacted with Asp-residue in the transmembrane helix III receptor sequence and an aromatic residue Tyr (Trp, Phe) (Casy, 1993; Paterlini et al., 2000). The replacement of Tyr with Leu in the dipeptide structure leads to activity

changes. Frequent modifications in our works are the replacement of Arg-residue with Cav or Lys. Canavanine (Cav) have a remarkable structural similarity, where one of the methylene groups in the side chain of Arg is replaced by an oxygen atom. On the other hand the properties of the two amino acids are different. Arg is strongly basic amino acid due to the guanidino group (pKa 12.48), while the presence of oxyguanidino group of Cav leads to pKa value of 7.04. Lys possess a pKa value of 10.5 (Fig. 1).

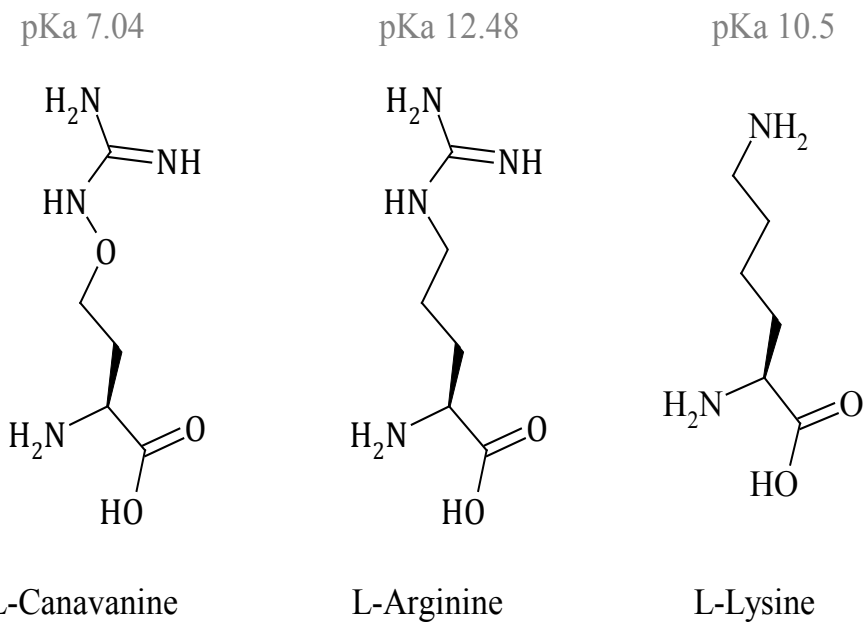


Fig. 1. Structures of Arg, Cav and Lys

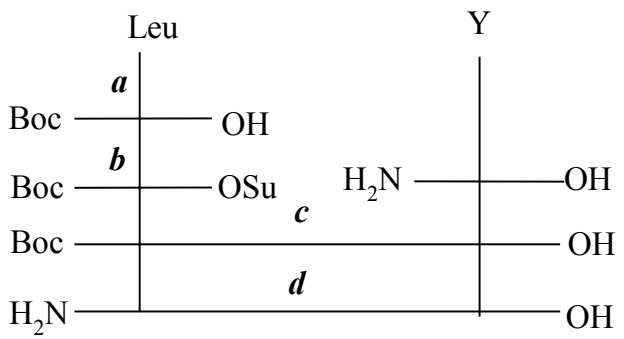
The aim of the presented work was to synthesize analogues of kyotorphin as well as to characterize them using FT-IR spectroscopy and to predict their activity to μ-opioid receptor (MOR) with a help of docking procedure.

Materials and methods

Chemistry

The synthesis of kyotorphin analogues (Fig. 2) was carried out in solution. All chemicals were of analytical grade and anhydrous solvents were obtained commercially (Fluka) and used directly. HPLC-grade acetonitrile and MeOH were purchased from Merck. Analytical TLC was performed on Merck silica gel (60F254) plates (0.25 mm)

using of the following solvent systems: A) *V* (C_6H_6) : *V* (CH_3COCH_3) : *V* (CH_3COOH) = 100 : 50 : 2; B) *V* ($CHCl_3$) : *V* (MeOH) : *V* (CH_3COOH) = 95 : 5 : 5; C) *V* (CH_3CN) : *V* (H_2O) = 4 : 1. Visualization was done with either UV, ninhydrin or a chlorine toluidine reagent. HPLC analyses were performed on Agilent Technologies HP 1100 and Waters 2695 LC instruments, using a Column: Lichrosphere® RP_8 (100 x 4,6 mm); mobile phase: acetonitrile/deionized water 40/60 (v/v).



a) Boc_2O, $NaHCO_3$, i-PrOH, H_2O;
b) HOSu, EDAC, EtOAc;
c) THF, Et_3N
d) TFA, CH_2Cl_2.

Y = Cav or Lys

Fig. 2. Synthesis of dipeptides

IR spectroscopy

The IR-spectra of analogues (Fig. 3 and Fig. 4) were recorded using a Thermo Scientific Nicolet iS10 FT-IR spectrometer (4000 – 400 cm^{-1}) with ATR accessory. A spectral resolution of ± 4 cm^{-1} was used and 64 scans were accumulated. The solid state IR spectra were recorded using ATR accessory and technique.

Computational tools

In order to perform computational studies the different software was used in the present work: - crystal structure of the μ-opioid receptor was obtained from RCSB (PDB id: 4dkl, http://www.rcsb.org/pdb/home/home.do); - ligand preparation was done with Avogadro (an open-source molecular builder and visualization tool – Version 1.0.3, http://avogadro.openmolecules.net/); - docking studies were performed by using GOLD

5.1 (Genetic Optimization for Ligand Docking, (Jones et al., 1997), run on Scientific LINUX 5.5 operating system; - for generation figures Molegro Molecular Viewer (http://molegro.com/index.php) was used.

Experimental part

Synthesis of Boc-Leu-Cav(Boc)$_2$ and Boc-Leu-Lys(Boc)

A solution of Cav(Boc)$_2$ (Lys(Boc)) (1.1 mM) and DIPEA (0.4 ml, 2.2 mM) in 5 ml DMF was added to a solution of Boc-Leu-OSu (0.42 g, 1.1mM) in 5 ml DMF. Reaction was carried out at room temperature for 24 hours. After complete the reaction 10 ml of water was added followed by extraction with $CHCl_3$ (3 x 10 ml). Combined organic layers were washed consequently with 5% $NaHCO_3$ (3 x 10 ml), 5% $NaHSO_4$ (3 x 10 ml), and brine, dried over anhydrous Na_2SO_4 and $CHCl_3$ was evaporated. The work-up procedure describe above yielded a 0.43 g (67 %) of Boc-Leu-Cav(Boc)$_2$ and 0.32 g (65 %) of Boc-Leu-Lys(Boc)$_2$, respectively.

Synthesis of HCl.Leu-Cav and HCl.Leu-Lys

The peptide Boc-Leu-Cav(Boc)$_2$ (Boc-Leu-Lys(Boc)) (0.1 mM) was dissolved 3M HCl/EtOAc (1 ml). The deprotection continued for one hour at room temperature. The solvent was evaporated, and the crude product was treated three times with MeOH (3 x 20 ml), which was also evaporated. Final peptides were obtained after column purification (Silicagel 60, CH_3CN : H_2O, 4:1).

Results and discussion

The synthesis of the analogues which structures are shown in Fig.1 was the main task in our work. The method of activated esters was used (Fig. 2.) which allows amino component to be added into the reaction without protecting the carboxyl group. This leads to a reduction of the reaction steps and racemization degree of the obtained products. First amino acid residue (Leu) was protected with Boc-group which is readily removed after complete reaction at mild acidic conditions. Coupling was carried out at room temperature as OSu-esters are very reactive and the reaction time is very short (2-4 hours).

To the best of our knowledge, there is no vibrational spectroscopic study on kyotorphin analogues, although there are a number of studies on Tyr (Grace et al., 2002) and Arg amino acid residues (Xie, et al., 2004). The FT-IR spectra the analogues are given in Figs. 3 and 4, respectively. The ring stretching mode of tyrosine which is observed at 1515 cm^{-1} in the IR spectrum in solid state of Tyr (Fig.3), is used as a marker band for protonation state of Tyr in proteins (Xie et al., 2004), since it is red shifted by 15 cm^{-1} in the deprotonated form. On the other hand, the C–O stretching, ν(C–O), and C–O-H

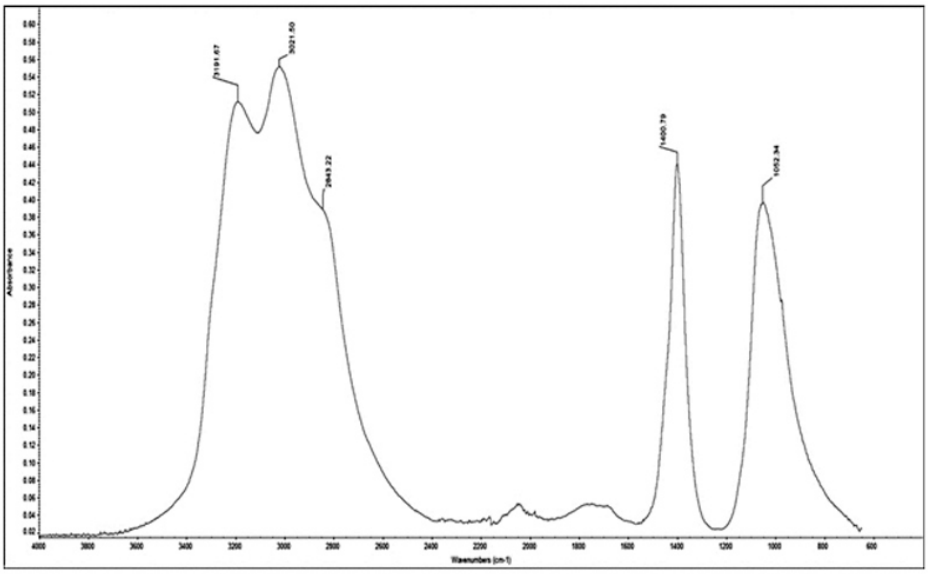


Fig. 3. FT-IR spectrum (solid state) of L-Leu-Cav-OH.HCl

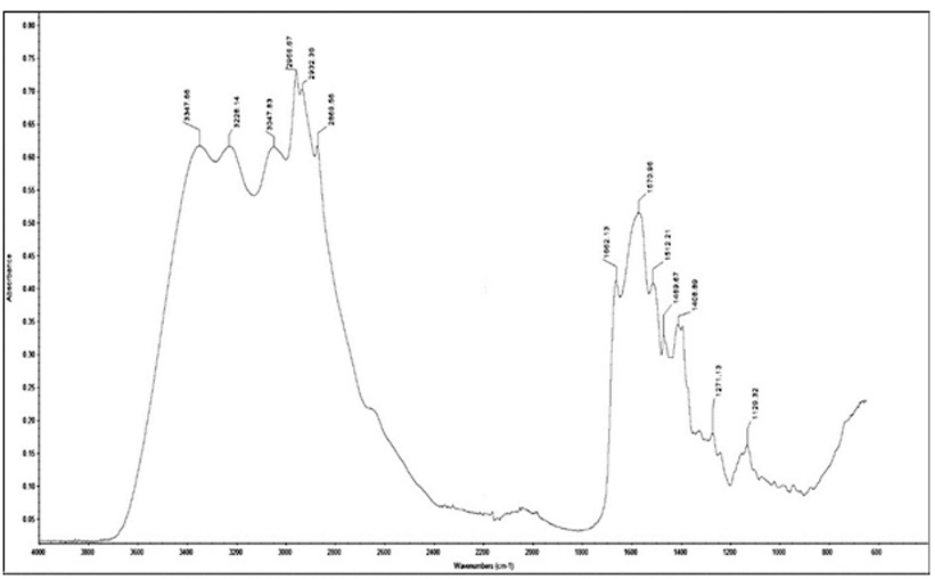


Fig. 4. FT-IR – spectrum (solid state) of L-Leu-Lys-OH.2HCl

deformation, δ (COH), modes of tyrosine are known to be sensitive to H-bonding (Bart 2000) and merge to one broad band near 1250 cm^{-1} in water.

In the spectra of investigated compounds missing the bands -OH-Tyr group over 3500 cm^{-1}. A multiple character of the IR-bands within 3300 – 2500 cm^{-1} region corresponds to asymmetric and symmetric stretches $\nu^{as}NH_3^+$ and $\nu^{s}NH_3^+$ vibrations of protonated NH_3^+-groups in the structure compounds (Figs. 4 and 5). The bands at 1662 cm^{-1} and 1570 cm^{-1} in the L-Leu-Lys-OH spectrum (Fig.4) correspond to Amide-I ($C{=}O_{str}$ + $N{-}H_{def.}$ + $C{-}N_{str}$) and Amide II ($C{-}N_{str}$ + $N{-}H_{def}$) bands respectively. The peaks at 1400 cm^{-1} and 1052 cm^{-1} correspond to NH_2 (wag. Arg) and νCN chain (Fig.3).

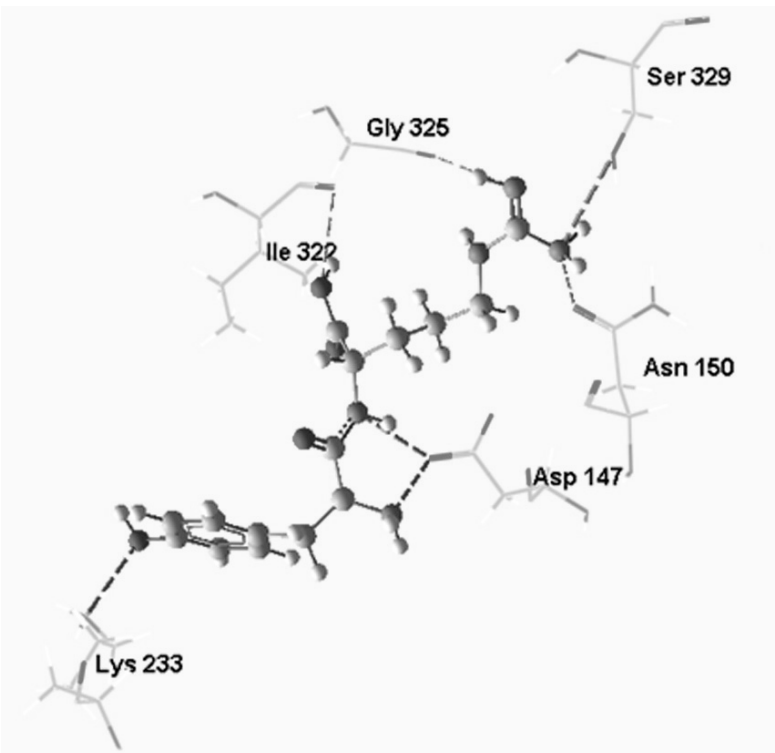


Fig. 5. Interactions of kyotorphin with the active site of MOR
(Picture was generated with MMV)

Further, we tried to find relationship between structure and activity, and to predict the biological effect of the newly synthesized Leu-Arg analogues, using a docking software.

Docking was carried out with GOLD 5.2 software. It uses genetic algorithm and considers full ligand conformational flexibility and partial protein flexibility. For docking studies the crystal structure of μ-opioid receptor, published in RCSB was used. It was published (Befort et al., 1996) that the binding site for opioid receptors was defined as residues within 10 Å radius of aspartic acid of third trans membrane domain, which is involved in the most crucial interaction. In the case of μ-opioid receptor this is Asp147. GoldScore algorithm was used and Fitness scoring function was calculated for each ligand. All Fitness function's values are listed in the Table 1.

Table 1. Fitness function values of the kyotorphin and Leu-Arg analogues and total energy of the complexes which they form with μ-opioid receptor

Peptide	Fitness function value	Total energy, kJ/mol
Tyr-Arg	58.43	-96.655
Leu-Arg	58.56	-92.186
Leu-Cav	55.34	-69.133
Leu-Lys	54.06	-87.578

As can be seen from the table, the complex of kyotorphin and the receptor has the lowest total energy. This means that it is the most stable of the study series. The complex of Leu-Cav and receptor has the highest energy. From an energetic point of view, the stability of the complexes increased in the order Leu-Cav / MOR, Leu-Lys / MOR, Leu-Arg / MOR, Tyr-Arg / MOR.

Analyzing the interactions of peptides with binding site of the receptor is seen following. Kyotorphin with its free amino group is bound to the carboxyl group of the side chain of residue Asp147 (Fig. 5). This interaction is particularly important for the activation of the receptor.

In the complex Leu-Arg / MOR interaction between the final free amino group and the carboxyl group of the side chain of Asp147 residue is again observed (Fig. 6), which explains the low enough total energy of the complex.

In the other two complexes, however, residue Asp147 interacts with various groups of the molecules of the peptides (Fig. 7). Oxyguanidino group of Cav residue of Leu-Cav is forming a hydrogen bond with the carboxyl group of the side chain of residue Asp147. This bond is weaker energy, which leads to the formation of unstable complex (Fig. 7A). In the other case, Leu-Lys, carboxyl group of the side chain of residue Asp147 form a hydrogen bond with the terminal carboxyl group of the dipeptide (Fig. 7B).

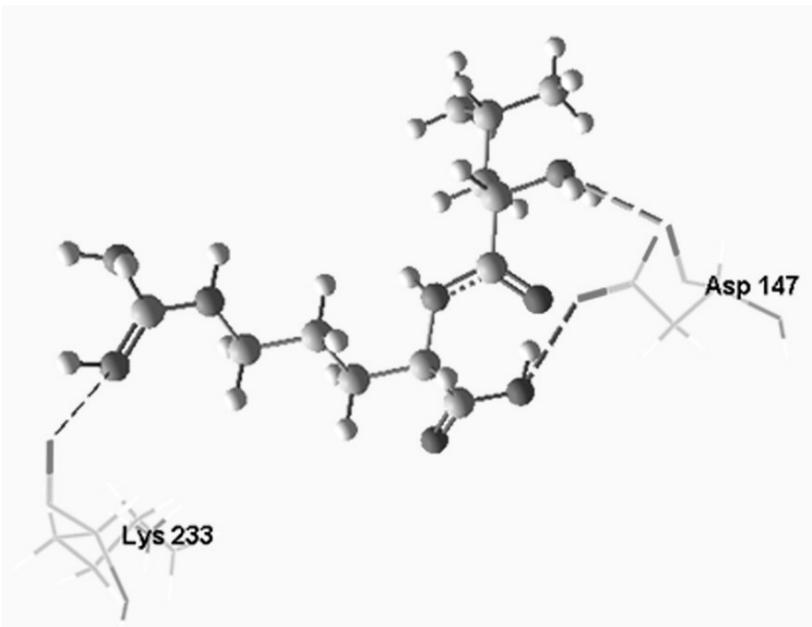


Fig. 6. Interactions of Leu-Arg with the active site of MOR
(Picture was generated with MMV)

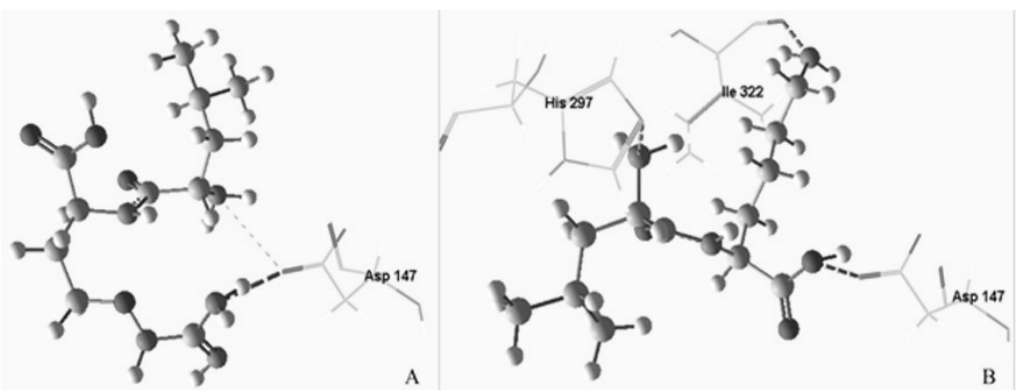


Fig. 7. Interactions of Leu-Cav (A) and Leu-Lys (B) with the active site of MOR
(Picture was generated with MMV)

As a result of docking the following conclusions could be drawn: a) both newly synthesized analogues will exhibit a low activity with respect to the MOR; b) substitution of the tyrosine residue of kyotorphin with leucine results in a peptide with an altered MOR activity; c) replacement of the arginine residue with amino acid residue with a weaker basicity leads to differences in binding of the analogues to the receptor. All tested peptides bind to the active site of the receptor, but due to differences in the type of interactions only kyotorphin will exhibit the desired effect. The other three peptides will occupy the active site without being able to produce an effect. They will act as reversible antagonists of MOR, as higher concentrations of kyotorphin can successfully removed them from the active site of the receptor.

Conclusion

As a result of the presented work two new analogues of Leu-Arg, antagonist kyotorphin were synthesized. They were characterized by infrared spectroscopy, and their effect to MOR was tested using the docking experiments. It was found that they will act as reversible antagonists, such that their effect is to be examined by means of biological studies.

Acknowledgment. Authors' thanks for the financial support of the SWU''N. Rilski''-Blagoevgrad, Bulgaria. This work is partially supported by the project of the Bulgarian National Science Fund, entitled: "Bioinformatics research: protein folding, docking and prediction of biological activity", code NSF I02/16, 12.12.14.

REFERENCES

Bart, A. (2000). The infrared absorption of amino acid side chains. *Prog. Biophys. Mol. Biol.,74,* 141-173.

Befort, K., Tabbara, L., Bausch, S., Chavkin, C., Evans, C. & Kieffer, B. (1996). The conserved aspartate residue in the third putative transmembrane domain of the delta-opioid receptor is not the anionic counterpart for cationic opiate binding but is a constituent of the receptor binding site. *Mol. Pharmacol.*, *49*, 216-223.

Casy, A.F. (1993). *Steric factor in medicinal chemistry: dissymetric probes of pharmacological receptors.* New York: Plenum Press.

Dzimbova, T., Pajpanova, T. & Golovinsky, E. (2006). Synthesis of novel kyotorphins containing non-proteinogenic amino acid norsylfoarginine. *Bul. Chem. Comm.*, *38*, 45-48.

Dzimbova, T., Bocheva, A. & Pajpanova, T. (2014). Kyotorphin analogues containing unnatural amino acids: synthesis, analgesic activity and computer modeling of their interactions with μ-receptor. *Med. Chem. Res.*, *23*, 3694-3704.

Grace, L.I., Cohen, R., Dunn, T.M., Lubman D.M., de Vries, M.S. (2002). The R2PI spectroscopy of tyrosine: a vibronic analysis. *J. Mol. Spectrosc. 215*, 204-219.

Jones, G., Wilett, P., Glen, R.C., Leach, A.R. & Taylor, R. (1997). Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.*, *267*, 727-748.

Machuqueiro, M. & Baptista, A.M. (2007). The pH-dependent conformational states of kyotorphin: a constant-ph molecular dynamics study. *Biophys. J*, *92,* 1836–1845.

Paterlini, M.G., Avitable, F., Ostrowski, B.G., Ferguson, D.M. & Portoghese, P.S. (2000). Stereochemical requirements for receptor recognition of the m-opioid peptide endomorphin-1. *Biophys. J.*, *78*, 590–599.

Xie, Y., Zhang, D., Jarori, G.K., Davisson, V.J. & Ben-Amotz, D. (2004). The Raman detection of peptide tyrosine phosphorylation. *Anal. Biochem., 332*, 116-121.

✉ **Dr. Atanas Chapkanov** (corresponding author)
Departrment of Chemistry
South-West University Neofit Rilski
66 Ivan Michailov Str.
2700 Blagoevgrad, Bulgaria
E-mail: chapkanov@swu.bg