DOROTA SOBCZUK
Institute of General Food Chemistry
Technical University of Łódź, Poland

REBECCA CRAIG
The School of Pharmacy
The Queen's University of Belfast
Medical Biology Centre, Belfast
BT9 7BL, Northern Ireland, U.K.

MARTA NOGUEROLES
Facultad de Ciencias
Universidad de Alicante, Alicante, Spain

SLAVICA RADIŠIĆ
Department of Pharmacy
Faculty of Medicine, Banja Luka, 78000 Banja Luka, Bosnia

BOHENG SHEN
School of Biosciences
University of Birmingham, Edgbaston, Birmingham, B15 2TT, U.K.

SUSANNE SIMON
Biozentrum of the University of Basel
CH-4056 Basel, Switzerland

THE STUDY OF THE INTERACTIONS BETWEEN NAPROXEN AND N-ACETYL-L-TYROSINE ETHYL ESTER

Review: **prof. dr hab. Stanisław Wysocki**

The interaction between naproxen and N-acetyl-L-tyrosine ethyl ester was investigated using UV-Vis absorption and fluorescence methods and the results indicate that an interaction is present in the

ground state. Hydrogen bond formation between these two molecules in complexation was illustrated by the computer optimised complex. This theory was supported by the increased fluorescence lifetime decay observed, the Gibbs free energy for complexation and also by the geometry of the complex.

1. Introduction

Naproxen, S(+)6-methoxy-β-methyl-2-napthalene acetyl acid (fig. 1), is a non-steroidal anti-inflammatory agent of the family of arylpropionic acids, with anti-inflammatory, analgesic, and anti-pyretic properties often used in the treatment of rheumatic and arthritic disease [1]. All of the drugs from this pharmacological group inhibit the cyclo-oxygenase enzyme by binding to the enzyme active site and presumably blocking access of the substrate to Tyr-385 [2].
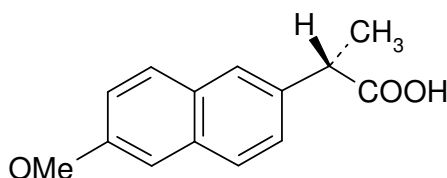

Fig. 1. The chemical structure of S-naproxen

N-acetyl-L-tyrosine ethyl ester (ATEE) (fig. 2) is used here to represent the amino acid tyrosine. In peptides, only the phenol group is available for interaction as the carboxyl and amino groups are involved in peptide bonds. Tyrosine is therefore esterified to ATEE, in which the only free group for interaction is also the phenol group.
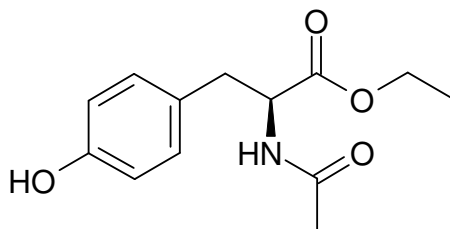

Fig. 2. The chemical structure of ATEE

The UV-Vis spectrum of naproxen in phosphate buffered saline (PBS), described by Moore et al, shows four absorption bands with maxima at 230nm, 270nm, 320nm, and 330nm. The emission spectra of naproxen in PBS at room

temperature shows maxima at 355nm, and the calculated fluorescence quantum yield (Φ_F) is 0.40 [3].

The UV absorption spectra of ATEE in water, measured by Ali et al, shows two peaks with maxima at 230nm and 275nm [4]. The fluorescence emission spectra of ATEE solution shows a maximum at 301nm, with excitation at 280nm, as described by Nevin et al [5].

Naproxen has been shown to photosensitize single stranded break in double stranded, supercoiled DNA *in vitro* , and was therefore chosen for this experiment due to its possible use as an inhibitory agent for some enzymes, including topoisomerases [6]. Topoisomerases form an intermediate between the phosphoryl group of the DNA backbone and the tyrosine of the enzyme active site, and are therefore important for DNA replication, which is augmented in cancer pathology [7]. It is hoped that this inhibitory process will occur on interaction with tyrosine, which is an amino acid important in many enzymes, including topoisomerase I. The anticipated complex formed between naproxen and tyrosine should result in inhibition. The suggested complex between these molecules involves the carboxyl group of naproxen and phenol group of ATEE, and if this occurs, it would facilitate the possible use of naproxen in photodynamic therapy in cancer treatment.

The aim of this investigation is to find if there is any interaction between naproxen and N-acetyl-L-tyrosine ethyl ester (ATEE), in the ground and excited states. We have applied a photophysical approach for studying the specific interaction between these two molecules. The methodology we used in our investigation is UV absorption spectroscopy, steady state fluorescence spectroscopy, and time-resolved fluorescence spectroscopy.

2. Experimental Details

2.1. Materials

Naproxen and N-acetyl-L-tyrosine ethyl ester (ATEE), produced by Sigma were used in the experiment, and were dissolved in 0.1M phosphate buffer (pH 7.0). All reagents were at least analytical grade and were used without further purification.

2.2. Methods

Absorption measurements were obtained using a Nicolet Evolution 300 UV-Vis spectrophotometer (resolution 1nm; A range 0-6) from Thermo Electron Corporation.

Steady-state fluorescence measurements were made using spectrofluorometer Fluoromax-2 (2000 signals/noise) from Jobin Yvon-Spex. Solutions were placed into a 10mm stoppered quartz cuvette and temperature was kept constant at 27°C. Titrations were made with a constant ATEE concentration while varying the concentration of naproxen.

Kinetics of the fluorescence decay were measured using a time-resolved fluorometer (Edinburgh Instruments OB-920) using a pulsed nanosecond nitrogen lamp as an excitation source and temperature was again constant at 27°C.The decay curves were analyzed using F 900 Spectrometer Software.

The complex structure between naproxen and ATEE was optimized with the program Gaussian 3.0, firstly utilizing the method Universal Force Field (UFF) [8] and then with AM1 [9]. S-naproxen was used in the complex optimization.

3. Results and Discussion

3.1. Experimental Results

3.1.1. UV

In fig. 3 the spectrum for ATEE shows a maximum at 275nm which corresponds with the literature value [4]. Naproxen shows four peaks with maxima 262nm, 271nm, 317nm and 330nm. The peak at 262nm differs from the literature values which do not include this maximum [3].
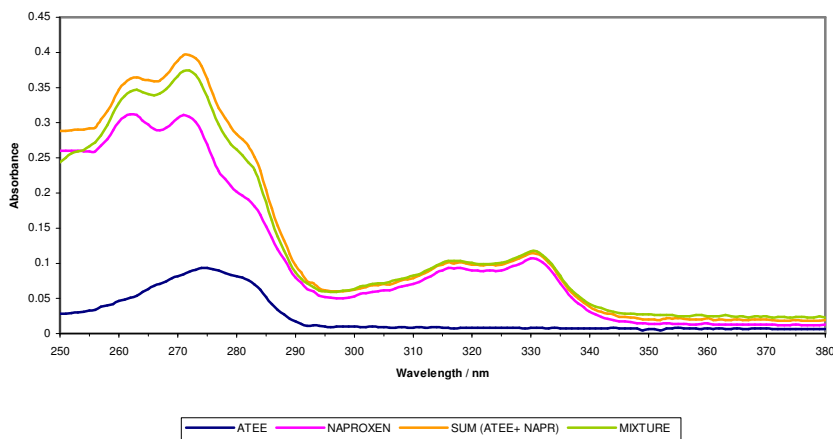

Fig. 3. The absorption spectra of ATEE (5×10^{-5}M), naproxen (5×10^{-5}M)and the mixture

The mixture and the theoretical sum of the absorbances of ATEE and naproxen show maxima at the same wavelengths but the absorbance of the mixture in the tyrosine region (260-285nm) is lower than that of the theoretical sum in comparison to the red end.

The observed difference between the UV spectral lines is not large indicating that this technique has low sensitivity, therefore fluorescence emission was employed to improve sensitivity.

3.1.2. Fluorescence

The steady state fluorescence emission spectra (fig. 4) obtained for ATEE and naproxen show maxima at 303.5nm and 355nm respectively, which are comparable with the literature values [3,5]. The fluorescence quantum yield of the mixture (exc. 280nm) is lower than that of both ATEE and naproxen in the regions of the maximal intensity. This gives an indication of some interaction between naproxen and ATEE.
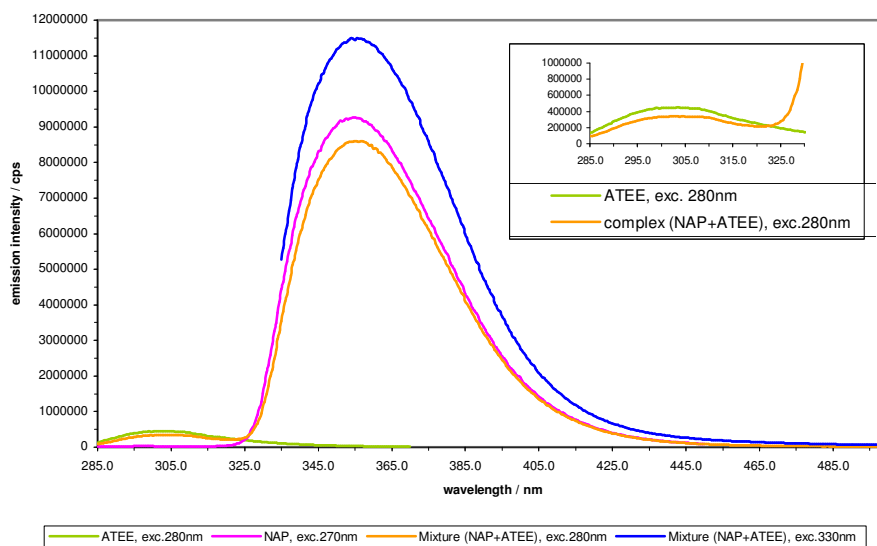

Fig. 4. Emission spectra of ATEE, naproxen and complex (NAP+ATEE); a magnification of the spectrum from 285-330nm can be seen in the insert
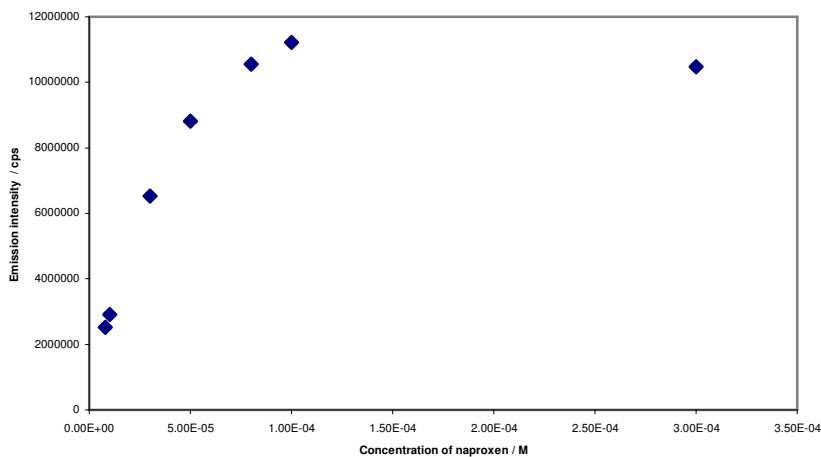
Fig. 5. A plot of the maximum emission intensity of the complex at 355nm against
the concentration of naproxen, excitation 270nm

It can be seen from fig. 5 that the fluorescence quantum yield of the complex increases with increasing naproxen concentration until a constant intensity is reached, where all the ATEE (constant concentration, 5×10^{-5}M) is involved in complex formation so that no further complexes can be produced.
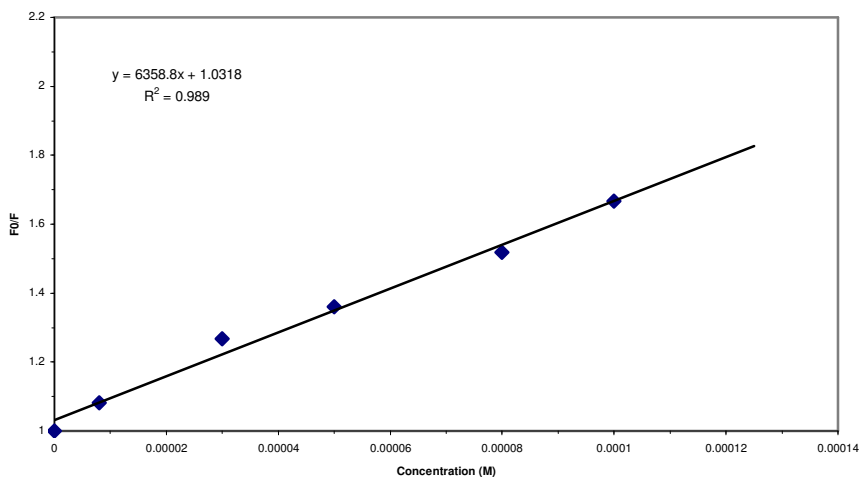
3.1.3. Stern-Volmer

Fig. 6. Stern-Volmer plot for ATEE fluorescence quenching
by naproxen ([ATEE] = 5×10^{-5} M)

The quenching of ATEE fluorescence by naproxen is described by the Stern-Volmer plot (fig. 6), and the relationship:

$$\frac{F_0}{F_i} = \left(1 + k_q \cdot \tau_0 \cdot [Q]_i\right) \cdot \left(1 + K \cdot [Q]_o\right) \tag{1}$$

where: F_0 – fluorescence intensity in the absence of quencher (naproxen)

F_i – fluorescence intensity for changing concentration of quencher

k_q – rate constant for quenching

τ_0 – lifetime of fluorescence decay of chromophore in the absence of quencher

$[Q]_i$ – concentration of free quencher

K – equilibrium constant for complex formation in ground state, in the presence of quencher

$[Q]_o$ – analytical concentration of quencher

The first bracket describes dynamic quenching and the second describes static quenching. The straight line obtained in the Stern-Volmer plot is therefore indicative of the presence of either purely static or dynamic quenching but it is not possible to determine which occurred. Time resolved fluorescence spectroscopy was therefore used to determine the decay times, thus enabling the differentiation between the presence of static and dynamic quenching.

3.1.4. Time-resolved Fluorescence

The kinetics of the fluorescence decay of ATEE and the complex (em. 302nm) are di-exponential, whereas the decay of naproxen and the complex (em. 355nm) is mono-exponential. The τ_2 value for ATEE and the complex are comparable. The τ_1 value of the complex (em. 302nm) is larger than that for ATEE. This result is opposite to that expected if quenching occurs in the excited state, therefore indicating that complex forms in the ground state. As dynamic quenching can only occur in the excited state, it can be concluded from this result that only static quenching is present so analysis hereafter will be performed neglecting the former.
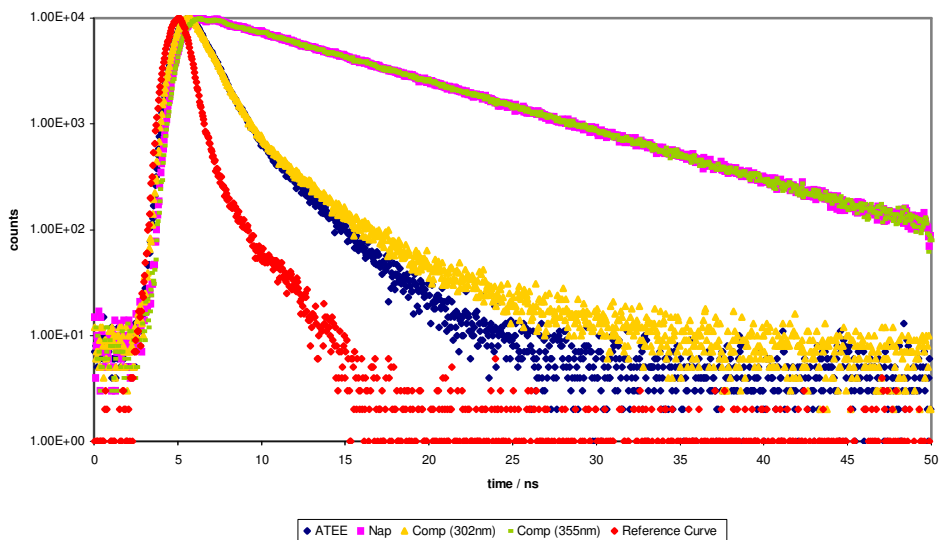
Fig. 7. Fluorescence time decay for ATEE (5E-5M), naproxen (5E-5M)
and complex (ATEE+NAP)

Table 1

Fluorescence lifetime decays for ATEE, naproxen and complex em. 302nm
and 355nm, obtained from time resolved fluorescence spectroscopy

	τ_1		τ_2		Average τ
	(ns)	B_1	(ns)	B_2	(ns)
ATEE	3.377	0.004	1.013	0.068	1.400
Complex, em. 302nm	4.683	0.003	0.997	0.071	1.607
Naproxen	9.283	0.039	-	-	-
Complex, em. 355nm	9.306	0.040	-	-	-

The first bracket of equation 1 can be omitted:

$$\frac{F_0}{F_i} = \left(1 + K \cdot [Q]_o\right) \qquad (2)$$

and the equilibrium constant, K attained from the slope of the Stern-Volmer plot.
This value is $6.36 \times 10^3 \, M^{-1}$, and will be denoted as K_1.

To compare the results from practical and theoretical, the K value from the practical was recalculated using equation 3. The Stern-Volmer equation describing K_1 assumes that the naproxen (quencher) concentration is much greater than that of ATEE but this assumption does not hold for this experiment as the concentrations are comparable so it was necessary to use equation 3. The ratio F_o/F_i can be calculated from:

$$\frac{F_0}{F_i} = \frac{C_T^o}{\left(C_T^o - C_K\right)} \tag{3}$$

where: C_T^o – analytical concentration of ATEE

$\quad\quad C_K$ – concentration of complex

Because:

$$F_i = \alpha \cdot \sum_i \cdot \Phi_i \cdot \varepsilon_i \cdot C_i \tag{4}$$

where: α – apparatus factors

$\quad\quad \Phi_i$: – fluorescence quantum yield of i

$\quad\quad \varepsilon_i$ – molar extinction coefficient of i

$\quad\quad C_i$ – concentration of i

with the consideration that $A \leq 0.1$

$$F_i = \alpha\left(\Phi_T \cdot \varepsilon_T \cdot C_T + \Phi_N \cdot \varepsilon_N \cdot C_N + \Phi_K \cdot \varepsilon_K \cdot C_K\right) \tag{5}$$

From the experimental conditions ($\lambda_{exc}=280nm$, $\lambda_{em}=302nm$), it can be seen that:

$$\Phi_N = 0 \quad\quad \Phi_K = 0$$

In this case the equation for F_i has the form:

$$F_i = \alpha \cdot \Phi_T \cdot \varepsilon_T \cdot C_T \tag{6}$$

Under the equilibrium conditions, the concentration of free ATEE molecules is less than the analytical value of the concentration of the complex so that equation 6 has the form:

$$F_i = \alpha \cdot \Phi_T \cdot \varepsilon_T \cdot \left(C_T^o - C_K\right) \tag{7}$$

The intensity of fluorescence emission without naproxen is equal to:

$$F_0 = \alpha \cdot \Phi_T^o \cdot \varepsilon_T \cdot C_T^o \tag{8}$$

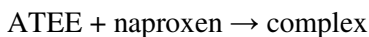Therefore the Stern-Volmer equation can be described with the formula:

$$\frac{F_o}{F_i} = \frac{\alpha \cdot \Phi_T^o \cdot \varepsilon_T \cdot C_T^o}{\alpha \cdot \Phi_T \cdot \varepsilon_T \cdot \left(C_T^o - C_K\right)} = \frac{C_T^o}{\left(C_T^o - C_K\right)} \tag{9}$$

Because $\langle \tau_T \rangle = \langle \tau_k \rangle$ it can be concluded that

$$\Phi_T^o = \Phi_K$$

Therefore equation 3 has the form as it is written above.

From the definition of the equilibrium constant for the reaction:

ATEE + naproxen $\rightarrow$ complex

and the mass law, the following equation is obtained:

$$K = \frac{C_K}{\left(C_T^o - C_K\right) \cdot \left(C_N^o - C_K\right)} \tag{10}$$

The acceptable physical root of this equation gives C_K:

$$C_K = \frac{1}{2}\left\{ \left(C_N^o + C_T^o + \frac{1}{K}\right) - \sqrt{\left(C_N^o + C_T^o + \frac{1}{K}\right)^2 - 4C_N^o C_T^o} \right\} \tag{11}$$

C_T^o and C_N^o are the initial concentrations of ATEE and naproxen respectively, calculated from the computer data, C_K is the concentration of the complex.

C_K from equation 11 can be substituted into equation 3 in order to calculate K.

The graph obtained by fitting the experimental data to equation 3 using the Slidewrite program has a better fit than the Stern-Volmer plot (fig. 6).
The K value (K_3) obtained is $6.05 \times 10^3 \, M^{-1}$ (Fig. 8).
From this K value, it is possible to calculate the Gibbs free energy:

$$K = \exp\left(-\frac{\Delta G}{RT}\right) \tag{12}$$

where R is the universal gas constant and T is the absolute temperature (298K).

The calculation of ΔG with K_3 using a rearrangement of equation 12, gives a value of –21.56KJ/mol.
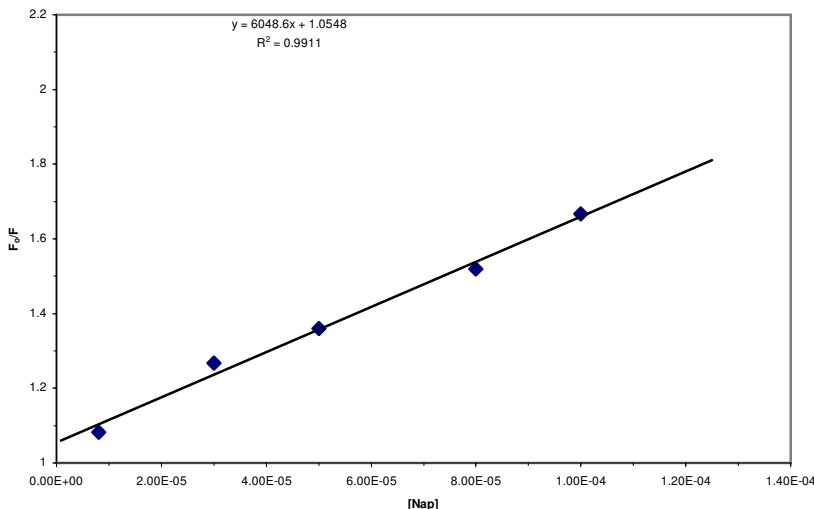
Fig. 8. Fitting graph for Stern-Volmer plot

3.2. Theoretical results

To calculate the theoretical ΔG, the computer data values were substituted into equation 13.

$$\Delta G = G_k - \left(G_N + G_T\right) \tag{13}$$

where ΔG is the Gibbs free energy, G_K is the free energy of the complex, G_N is the free energy of naproxen, G_T is the free energy of ATEE.

The calculated theoretical ΔG is -30.456 KJ/mol. This value is similar to literature values for hydrogen bonding (20 kJ/mol) [10]. The theoretical structure was optimized in vacuum, neglecting the effect of solvation which occurred in the experiment, and therefore resulting in the difference in values ΔG.

From this theoretical value of ΔG, K_2 was calculated using equation 12 giving a value of $2.19 \times 10^5 \, M^{-1}$, which is higher than K_3 ($6.05 \times 10^3 \, M^{-1}$) due to the solvation effects previously discussed.

The distance in O-H···O in the optimized complex is 2.92 Å, which is comparable to the literature value of 2.8Å [10]. The angle 1-4-2 is 149˚, and not linear due to disruption of the bond angle by oxygen 3. The angle of the hydroxyl group in free ATEE is 107.9˚, compared with 109.4˚ when in the complex. A red shift in the frequency of the stretch vibration of the C-O bond in the hydroxyl group of ATEE is observed from $1686.15 cm^{-1}$ in free ATEE to $1677.73 cm^{-1}$ when in the complex. This is characteristic for the formation of a hydrogen bond.
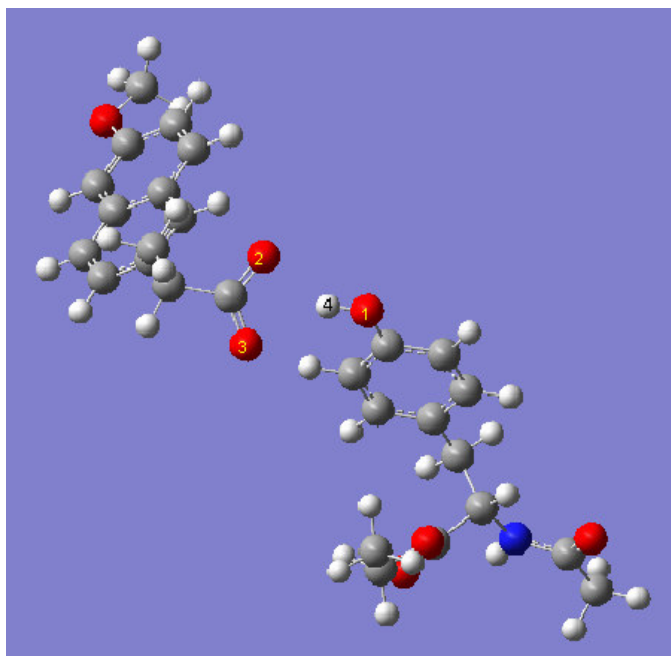
Fig. 9. The optimized structure of the complex between naproxen and ATEE

4. Conclusions

Both the theoretical (-30.456 KJ/mol) and experimental (–21.56KJ/mol) ΔG values agree with the energy of formation of hydrogen bonds [10].
It has been shown that there is an interaction between naproxen and ATEE in the ground state due to the small difference in absorbance values between the complex and the sum of the absorbances of ATEE and naproxen, and to the increase in fluorescence lifetime decay. The suggested complex structure obtained from optimization is stabilized by hydrogen bonds and this theory is supported by the increased τ_1 value, as it is known that hydrogen bond formation can increase the fluorescence lifetime of a chromophore group [11].

The geometry of the optimized complex structure and the red shift in hydrogen bond vibrations supports the proposed formation of hydrogen bonds in complex formation between naproxen and ATEE.
It can therefore be concluded that naproxen and ATEE interact by hydrogen bond formation.

knowledge of the Gaussian and HyperChem programs. Furthermore, thanks to IAESTE for the opportunity to gain invaluable work experience.

References

[1] **Junquere, E., Aicart, E.:** A fluorimetric, potentiometric and conductimetric study of the aqueous solutions of naproxen and its association with hydroxypropyl-β-cyclodextrin. International Journal of Pharmaceutics, 176: 169-178, (1999).

[2] **Loll, P. J., Picot, D., Ekabo, O., Garavito, R.M.:** Synthesis and use of iodinated nonsteroidal anti-inflammatory drug analogues as crystallographic probes of the prostaglandin H2 synthesase cyclooxygenase active site.   Biochemistry, 35: 7330-7340, (1996).

[3] **Moore, D. E., Chappuis, P.P.:** A comparative study of the photochemistry of the non-steroidal anti-inflammatory drugs naproxen, benoxaprofen and indomethacin. Photochem. Photobiol., 65: 828-832, (1988).

[4] **Ali, F. E., Barnham, K. J., Barrow, C. J., Sparovic, F.:** Metal catalysed oxidation of tyrosine residues by different oxidation systems of copper/hydrogen peroxide. J. Inorganic Biochemistry, 98: 173-184, (2004).

[5] **Nevin, A., Cather, S., Anglos, D., Fotakis, C.:** Analysis of protein-based binding media found in paintings using laser induced fluorescence spectroscopy. Analytica Chimica Acta, 573-574: 341-346, (2006).

[6] **Bosca, F., Marin, M. L., Miranda, M. A.:** Photoreactivity of the nonsteroidal anti-inflammatory 2-arylpropionic acids with photosensitizing side effects. Photochemistry and Photobiology, 74 (5): 637-655, (2001).

[7] **Pommier, Y., Pourquier, P., Fan, Y., Strumberg, D.:** Mechanism of action of eukaryotic DNA topoisomerase I and drug targeted to the emzyme. Biochimica et Biophysica Acta, 1400: 83-106, (1998).

[8] [8]**Rappé, A. K., et al.:** UFF, a full periodic table force field for molecular mechanics and molecular dynamics simulations. J. Am. Chem. Soc, 114: 10024-10035, (1992).

[9] **Dewar, M. J. S., Zoebisch, E. G., Healy, E. F., Stewart, J. J. P.:** AM1: A new general purpose quantum mechanical molecular model. J. Am. Chem. Soc., 107: 3902-3909, (1984).

[10] **Atkins, P., De Paula, J.:** Atkin's Physical Chemistry 8th ed. Oxford; New York: Oxford University Press, 634, (2006).

[11] **Armbruster C., Knapp M., Rechthaler K., Schamschule R., Parusel A. B. J., Kohler G., Wehrmann W.:** Fluorescence properties of 1-heptanoylpyrene: a probe for hydrogen bonding in microaggregates and biological membranes. Journal of Photochemistry and Photobiology A: Chemistry, 125: 29-38, (1999).

BADANIA ODDZIAŁYWAŃ POMIĘDZY NAPROXENEM I N-ACETYLOWYM ESTREM ETYLOWYM L-TYROZYNY

Streszczenie

W pracy zbadano oddziaływania pomiędzy naproxenem i N-acetylowym estrem etylowym L-tyrozyny metodami spektroskopii absorpcyjnej UV-Vis i fluorescencyjnymi. Wyniki wskazują na istnienie interakcji między cząsteczkami w stanie podstawowym. Powstawanie wiązania wodorowego pomiędzy tymi dwoma molekułami zobrazowane zostało poprzez komputerową optymalizację kompleksu. Teorię tą potwierdza wydłużenie czasu życia fluorescencji kompleksu, energia kompleksowania Gibbsa oraz geometria cząsteczki kompleksu.

Wydział Biotechnologii i Nauk o Żywności
Politechnika Łódzka