Spectral and Quantum - Mechanical Characterizations and Biological Activity of N-(P-nitrobenzoyl)-L-phenylalanine

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A systematic study on the acylation reaction of L-phenylalanine with the rest of p-nitrobenzoyl was performed. The yield of reaction shows dependence on the transient appearance of Δ^2-oxazolinone-5, on the working temperature, on the contact time and on the polarity of the solvent. The acylated derivative structure was confirmed by elemental analysis, spectral and computational methods of molecular mechanics. The value of DL_{50} and the antimicrobial action of acyl-derivative (IV) has been established.

Keywords: N-acyl-aminoacids, optimization reaction, oxazolone hydrolysis, biological activity

Finding that amino acids play an essential role in the structure and functions of some biomolecules, especially when they are associated with other elements substituted was a good sign for future development of researchers towards pharmaceutical applications.

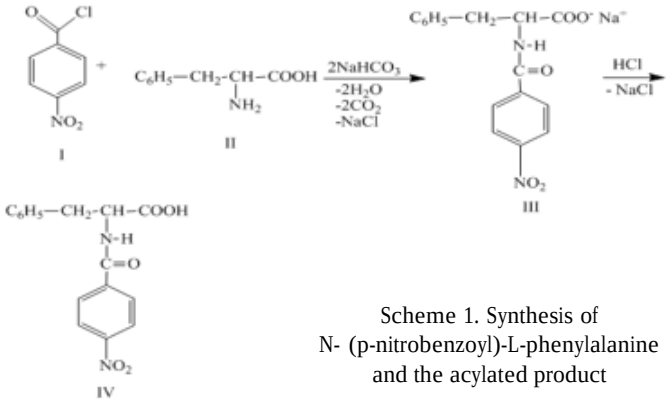
Investigations undertaken were aimed at further experimental study on the synthesis of a phenylalanine derivative, potentially biologically active.

We concentrated on phenylalanine, because it plays an important role in transamination reaction, there by establishing the connection between the metabolism of proteins and carbohydrates. On the other hand, phenylalanine is the supplier of the amine groups in the biosynthesis of the other amino acids, such as tyrosine. This amino acid is also involved in the urea cycle and also in the regulation of ammonia content in the body.

Literature indicates that the phenylalanine is found in the structure of peptides which are known as anti-tumor drugs [1-6].

Other studies have shown the structure and biological tests of some phenylalanine derivatives with obvious hypoglycemic action [7, 8]. Through a complex metabolic pathway, phenylalanine causes the release of colecistochinina, an hormone of particular importance in the maintenance of intestinal flora [9]. At the same time this amino acid and some its salts behave as stimulators in the synthesis of certain neurotransmitters, mainly L-dopa, dopamine, epinephrine and norepinephrine [9]. Some publications reported the effects of phenylalanine such as analgesic [10, 11], antitumor [12], with antidepressant properties [13, 14], with functions of Parkinson and Vitiligo inhibitors [15-17] and selective neuropeptide Y_1 receptor antagonists [18, 19].

Some researchers have synthesized biologically active substances containing o-, m- or p-nitrobenzoic acid residue in molecules [20-26]. The coupling of phenylalanine with p-nitrobenzoic acid was performed having in view these results (scheme 1).



Scheme 1. Synthesis of N- (p-nitrobenzoyl)-L-phenylalanine and the acylated product

Experimental part

Using the reaction between the p-nitrobenzoic acid chloride (I) and phenylalanine (II) in the presence of sodium bicarbonate solution (Schotten Baumann conditions), the intermediate product - the sodium salt of N-(p-nitrobenzoyl) - L-phenylalanine (III), in the reaction with hydrochloric acid (30%) gives the acylated product (IV) [40] (scheme 1).

When the reaction was done in water solution without cooling system, using sodium bicarbonate in excess and benzene as solvent for the acid chloride, the reaction yield of compound (IV) was 60%. The product had the melting point 139-141°C and specific optical rotation $[\alpha]_D^{20} = +18$. This fact shows that temperature is an important factor for acylation reaction.

The reaction takes place at the contact surface between the water and benzene layers, this is why a part of the acid chloride might be hydrolisated fact which determines a smaller yield of the reaction. This disadvantage had been removed using acid chloride in excess and decreasing the temperature of reaction (18-12°C) and assuring a vigorous and constant stirring.

Other observation that helped to explain the increase or decrease in reaction yield would be that a red-violet coloration appears during the reaction and it becomes more

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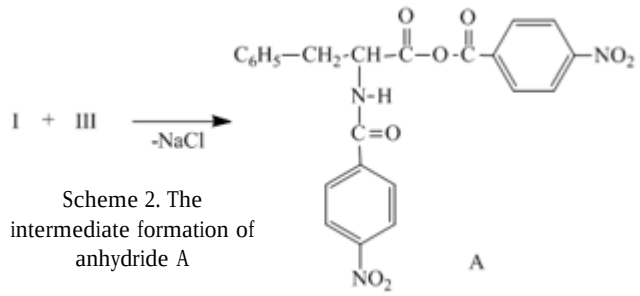
intense as the system temperature is higher. This coloration has a maximum of intensity and than in decreases.

The coloration can be due to some secondary reactions between the sodium salt of N- (p-nitro-benzoyl)-L-phenylalanine (III) and p-nitrobenzoic chlorure acid (IV), which would lead to a mixed anhydride with structure A (scheme 2).

An SN_2 type mechanism leads to anhydride (A) formation and it is influence mainly by the positive effect of the $-NO_2$ group.

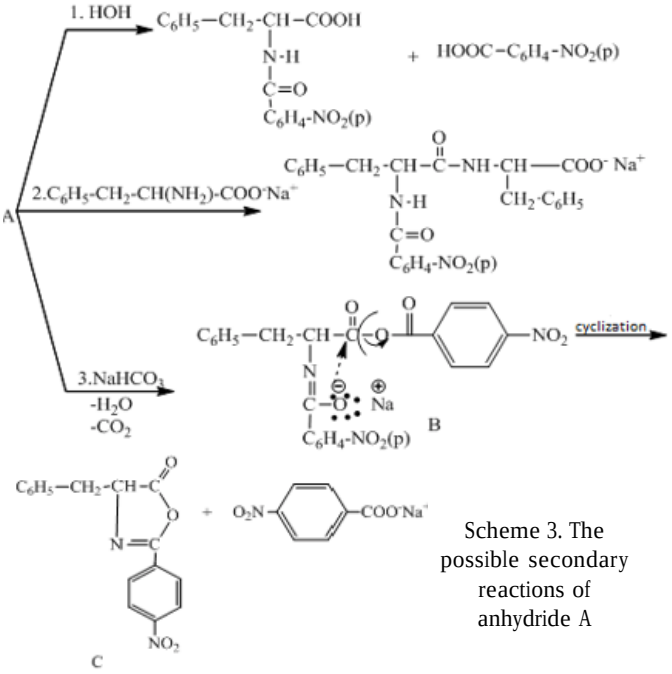


Scheme 2. The intermediate formation of anhydride A

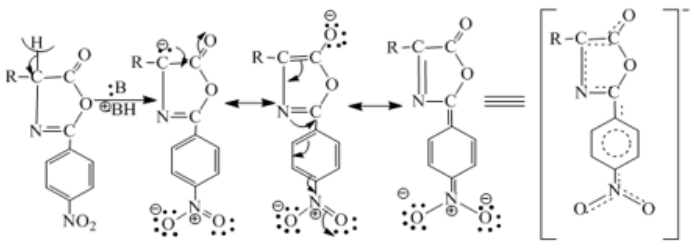
However only formation of mixed anhydride (A) cannot explain both the racemization and the red-violet coloration.

Following, other possible reactions of mixed anhydride were taken into consideration, as it results from scheme 3.

The reactions (1) and (2) could decrease the reaction yield. Only reaction (3) could explain the coloration of the system and it leads to the obtaining of 2-(p-nitrophenyl)-4-benzyl-Δ^2-oxazolinone-5, (C). This reaction takes place easy; this fact can be explained both by the activation of the α-carboxylic group in the present p-nitrobenzoyl and by the lability of the amidic hydrogen allowing the neutralization of the base before the hydrolysis of the anhydridic structure B.



Scheme 3. The possible secondary reactions of anhydride A



Scheme 4. Possible limit structure of the oxazolone carbanion

Molecular modeling

The structure of N-(p-nitrobenzoyl)-L-phenylalanine was estimated based on the theoretical and computational technics of molecular mechanics by using HyperChem 8.0.6 program, a semi-empirical method AM1 [41].

The molecule was geometrically optimized using the Polak – Ribiere algorithm as convergence criterion and the maximum gradient used to optimize chemical structures studied was 0.001 kcal / (Å.mol). A calculation type *single point* and a number of physical and structural properties have been obtained, after the molecular geometry optimization.

Results and discussions

The experimental study on this reaction was extended and it allowed us to draw some conclusions regarding yield and optical purity of the compound (IV), both landmarks influenced by the operating parameters and possible side effects.

The lability of the amidic hydrogen is supported by the presence of the $-NO_2$ group in para position, which has as effect the stabilization of B anion compared to A anhydride, favoring the cyclisation reaction and the formation of C heterocycle.

The results obtained proved that red-violet coloration is determined by the formation of the C compound which in basic media releases a proton from α-position and determine the formation of a carbanion with three chromophore groups >C=O, >C=N-, $-NO_2$ according to the structures from scheme 4.

From these results we could conclude that with the rise of the temperature, the secondary reactions occur having as result a reduction in yield and also the contamination of the optical part (racemization) of the final product. These data are consistent with the known data in the literature for the synthesis of N-acyl-amino acid or peptide, products more or less racemized through stage of Δ^2-oxazolidinone -5 [27-29].

The hydrolization rate of compound A

In order to establish the hydrolization rate of compound A, the absorbance in the maximum of the visible electronic absorption band (λ=485 nm) which gives violet-red color for a few minutes was recorded at intervals of 10 s and the graph of ln E_0/E vs. time was obtained in figure 1.

According Lambert-Beer law the extinction E in the maximum of the electronic absortion band is directly proportional with the concentration c of the spectrally active molecule:

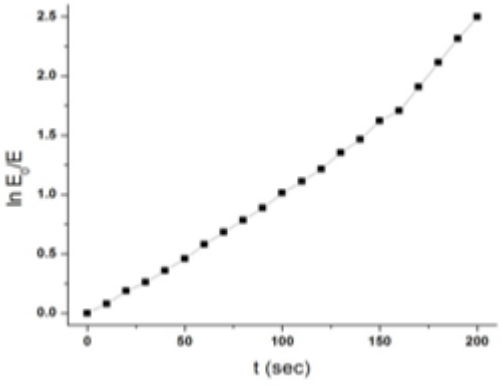


Fig. 1. ln E_0/E vs. time

$$E = \ln \frac{A_0}{A} = \varepsilon \cdot c \cdot L \quad (1)$$

In equation (1) A and A_0 are absorbances at times t and t=0, ε-extinction cofficient, L- thickness of the solution layer.

The extinction in the maximum of the absorbtion electronic band is a measure of the spectrally active substance concentration.

In chemical reaction, the variation ΔE of concentration in a time interval Δt, is described by a general formula:

$$dE = k \cdot E^n \cdot dt \quad (2)$$

in which k is the reaction rate and n is the reaction order.

For n=1, by integrating (2), one obtains:

$$\ln \frac{E_0}{E} = k \cdot t \quad (3)$$

In reaction (3), E_0 and E are the initial extinction and the extinction at the moment t.

Our measurements show the dependence illustrated in figure 1, from which it results that the hydrolization reaction is of the first order [30, 31].

The regression parameters (slope and the intercept; regression coefficient; standard deviation) of the line ploted from figure 1 are given in table 1.

The hydrolization rate of compound (A) is of about k=0.0107 s^{-1} for the first 150 s. For t>150 s, the reaction rate is higher, near 0.0198 s^{-1}, but the measurement precision is lower, due to color disappearance.

Factorial experiment for reaction optimization

Giving in view the connection between chemical structure and biological activity, the establishing optimal reaction conditions for obtaining acyl-phenylalanine (IV) is of a notable importance.

Statistical models [32, 33] based on experimental factorial design allow to establish the best conditions in which the reaction rate can be maximized. From the preliminary studies, it is clear that the yield is significantly influenced by two variables: reaction time (X_1) and the reaction temperature (X_2). The small variations of these parameters determine significant variations in the yield of the reaction (table 2).

The real significant variables X_1 and X_2 are transformed in a-dimensional variables x_1 and x_2.

The polynomial describing the reaction yield of compound IV depends on the a-dimensional significant parameters x_1 and x_2 as it is shown in equation (4):

$$\eta_I = 70.445 + 1.50x_1 + 0.833x_2 - 1.50x_1x_2 - 3.167x_1^2 - 3.167x_2^2 \quad (4)$$

The student test was made by organizing other three experiments in the center of the variation domain of the a-dimensional significant variables.

The obtained data are listed below:

$$\eta_1 = 70;\ \eta_2 = 72;\ \eta_3 = 69;\ \bar{\eta} = 70.333;\ P = 0.509 \quad (5)$$

The formula (6) is used to compute the precision P:

$$P = \sqrt{\frac{S\eta}{N}} \quad (6)$$

The t-student test for the coefficients of polynomial from equation (4) has the following values:

$$t_0 = 138.40;\ t_1 = 2.947;\ t_2 = 1.637;\ t_{12} = 2.497;$$

$$t_{11} = 6.222;\ t_{22} = 6.222 \quad (7)$$

These values attest the equation (4) as a good choice to express the reaction yield for obtaining the compound IV.

The conditions for the extreme values of the yield were obtained by deriving equation (4) in rapport with the two a-dimensional variables. The obtained equations are given in (8):

$$\begin{aligned} -6.334x_1 - 1.50x_2 + 1{,}50 &= 0 \\ -1.50x_1 - 6.334x_2 + 0{,}833 &= 0 \end{aligned} \quad (8)$$

and the solutions for the extreme values of the a-dimensional variables and for the reaction yield are listed in (9):

$$x_{1e} = 0.218;\ x_{2e} = 0.080;\ \eta_e = 70.67 \quad (9)$$

The obtained values are in accord with the established polynomial eq.(4).

Spectral analyses

The structure of compound IV was confirmed by elemental and spectral analysis [34 – 36].

The IR spectra of this compound has a characteristic signal for the amidic -NH bond at 3108 cm^{-1}, 3345 cm^{-1} and

Time (sec.)	Intercept	Slope	No points	Adjusted regression square	Standard deviation
0-150	-0.04901	0.0107	16	0.99682	0.02878
160-200	-1.4636	0.01984	5	0.99939	0.00774

Table 1
HYDROLIZATION RATE OF COMPOUND A; INTERCEPT AND SLOPE OF LINE (3)

Nr.	$x_1[X_1(^0C)]$	$x_2[X_2(\min)]$	x_1x_2	$x_1^2 - 2/3$	$x_2^2 - 2/3$	η_I
1	-1[10]	-1[85]	1	1/3	1/3	60
2	-1[10]	0[90]	0	1/3	−2/3	67
3	-1[10]	1[95]	-1	1/3	1/3	64
4	0[11]	-1[85]	0	−2/3	1/3	66
5	0[11]	0[90]	0	−2/3	−2/3	70
6	0[11]	1[95]	0	−2/3	1/3	69
7	1[12]	-1[85]	-1	1/3	1/3	67
8	1[12]	0[90]	0	1/3	−2/3	68
9	1[12]	1[95]	1	1/3	1/3	65

Table 2
SIGNIFICANT PARAMETERS AND THE REACTION YIELD FOR COMPOUND IV

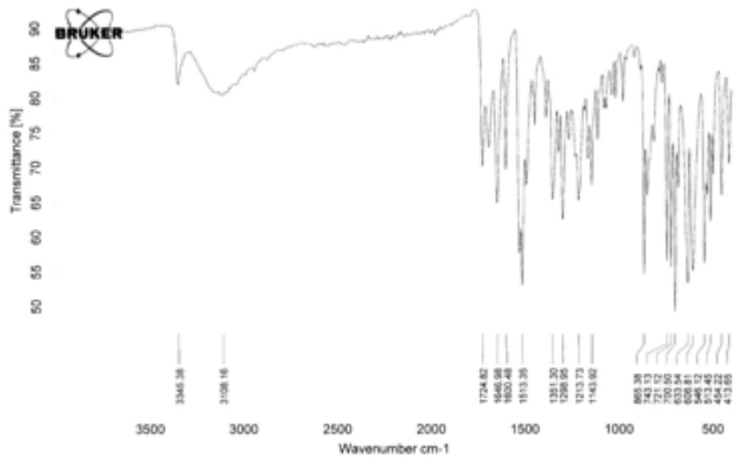


Fig.2. The IR spectrum of N- (p-nitrobenzoyl) -L-phenylalanine (IV)

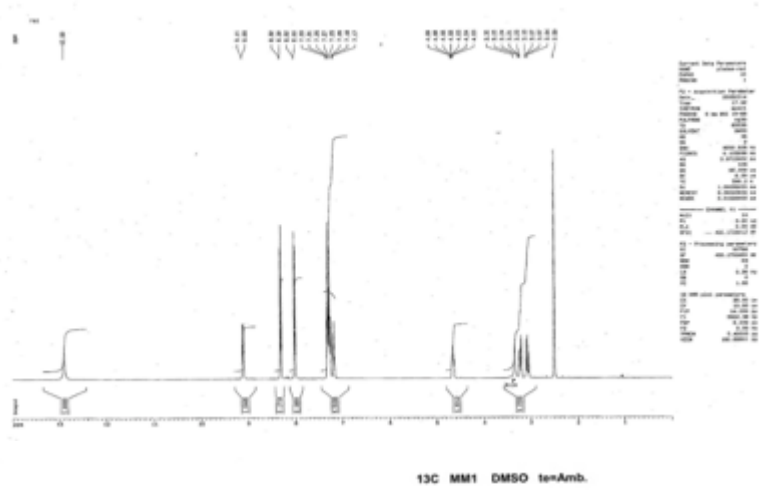


Fig.3. The ^{1}H-NMR spectrum of N- (p-nitrobenzoyl)-L --phenylalanine (IV)

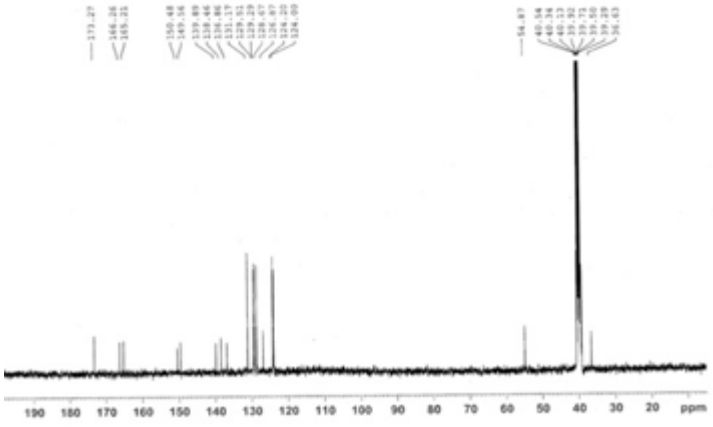


Fig.4. The ^{13}C-NMR spectrum of N- (p-nitrobenzoyl) -L-phenylalanine (IV)

an amidic bond signal at 1646 cm^{-1}. Characteristic bands at 743 cm^{-1} for para disubstituted nucleus and also at 865 cm^{-1} appear in IR spectrum. The symmetrical and asymmetrical vibrations for -NO_2 (fig. 2) are founded at 1351 cm^{-1} and 1513 cm^{-1} in IR spectrum.

The ^{1}H-NMR spectrum brings a confirmation for the structure of the IV compound. At 7.25-8.3 ppm there are specific signals for protons in the aromatic rings, the protons in the aliphatic chain can be identified by signals at 3.2 ppm and 4.7 ppm. The characteristic proton of NH group

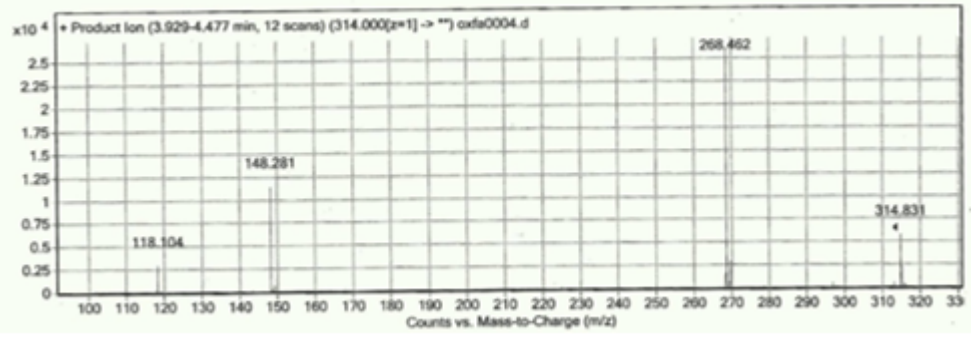


Fig.5. Mass spectrum of N-(p-nitrobenzoyl)-L-phenylalanine (IV)

of the amide is indicated by a doublet at 9.1 ppm. The carboxyl hydrogen appears at 12.9 ppm (fig. 3).

In the ^{13}C-NMR spectrum of the compound IV (fig. 4), the carbon atoms from benzene rings give signals at 124.0-139.8 ppm, while the carbons belonging to the aliphatic chain the signals appear at 36.6 and 54.5 ppm. For the carboxylic groups the signal appears at 173.2 ppm and the CO-NH group is present at the 165.2 ppm.

A signal for the molecular ion M^+ (fig. 5), attesting the presence of compound IV appears in the mass spectrum at m/z 314.

HyperChem calculations

Using HyperChem 8.0.6 program, semi-empirical method AM1 [41], minimum energy state of system was estimated and it involves refining structure until optimum conformation of molecule (fig. 6).

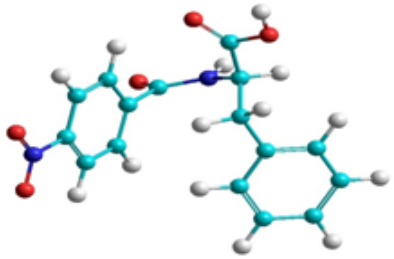


Fig.6. Optimized geometry of N- (p-nitrobenzoyl) -L-phenylalanine (IV)

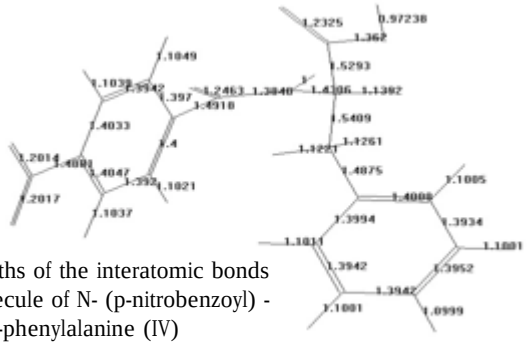


Fig.7. Lengths of the interatomic bonds in the molecule of N- (p-nitrobenzoyl) -L-phenylalanine (IV)

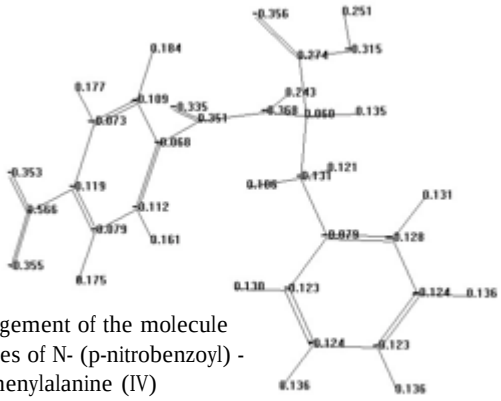


Fig.8. Arrangement of the molecule atomic charges of N- (p-nitrobenzoyl) -L-phenylalanine (IV)

Table 3

THE QSAR PARAMETERS OF N- (p-NITROBENZOYL) -L-PHENYLALANINE (IV)

Log *P*	-3.31
Hydration energy (kcal/mol)	-15.39
Refractivity (Å^3)	88.22
Polarizability (Å^3)	31.43
Mass (u.a.m.)	314.30
Volume (Å^3)	841.59
Surface area (Å^2)	435.69
Dipole moment (D)	6.31

Other parameters that characterize the molecule: interatomic bond lengths (fig. 7), and charges for each of the component atoms (fig. 8), were determined using HyperChem 8.0.6 program.

Application QSAR (Quantitative Structure - Activity Relationship) attempts to correlate the molecular structure with a certain type of chemical or biochemical activity.

The mass, the volume, the surface area, the hydrophobicity (log P), the molar refractivity, the polarizability, the energy of hydration and the dipole moment (table 3) were determined by using QSAR method [37].

Negative value of log P indicates that the product (IV) has hydrophilic character, which favors a better absorption and distribution in the body.

Toxicity study of the N- (p-nitrobenzoyl) -L-phenylalanine (IV)

To determine the acute toxicity we worked in lots of 6 white mice, male. The substances tested (II, IV) were administered intraperitoneally as a suspension in Tween 80 and the mortality was recorded 24, 48 h and 7 days.

The obtained results are presented in table 4.

The obtained results reveal that N-(p-nitrobenzoyl) -L-phenylalanine (IV) has a higher toxicity compared with L-phenylalanine (II), but it remains within acceptable limits, which recommending a laboratory screening.

The antimicrobial activity of N- (p-nitrobenzoyl) -L-phenylalanine (IV)

The evaluation of antimicrobial activity of acyl-phenylalanine (IV) was performed in the Laboratory of Microbiology of the Institute of Public Health Iasi.

Bacterial strains of the following species: *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis* P1131 were used to assess the activity of acyl-amino acid (IV). Sulfonamide, sulfaphenazole,

Compound	DL$_{50}$ (mg/kilo body)			
	24 hours	48 hours	7 days	The average
II	9700	9700	9340	9580
IV	6300	6300	5442	6014

Table 4

DL$_{50}$ VALUES OF THE TESTED COMPOUNDS

Compound	DL$_{50}$ (mg/kg corp)			
	Staphyl. aureus	*E. coli*	*Pseud. aeruginosa*	*Salmonella enteritidis*
IV	22 – 23	21 – 23	–	25
sulphonamide	29	27 – 29	–	25
sulphofenazole	24	20 – 21	–	20
sulfomethoxypyridazine	28 – 29	28	–	28
sulphamethoxazole	28 – 29	28	–	28

Tabel 5

ANTIMICROBIAL ACTIVITY FOR THE COMPOUND IV AND THE REFERENCE SULPHONAMIDES WITH A CONCENTRATION OF 50µg /DISC

- no inhibitory action.

sulfamethoxypyridazine and sulfamethoxazole, pharmaceutical products for commercial use, were used as reference substances

The seeds were grown on agar Mueller - Hinton and incubated at 37° C for 8 h. To assess the sensitivity of the microbes to the action of compound (IV), it was used as powder, in a concentration of 50 mg / disc. The results of the antimicrobial effect of the compound (IV) and of the reference sulphonamides were expressed by inhibition zone diameter, as determined by diffusion method Kirby - Bauer [41] (table 5).

Conclusions

It was established that the occurrence of coloration is due primarily to a secondary reaction that allows the formation of mixed anhydride (A) between N-(p-nitrobenzoyl)-L-phenylalanine sodium salt (III) and p-nitrobenzoic acid chloride (I), anhydride which leads through a cyclization process to Δ^2-oxazolinone-5 (C) due to the steric effects.

In contact with the base, the compound (C) remove the proton from á position forming a carbanion extented over three cromophore groups (> C = O, > C = N-, -NO_2) also determining the occurrence of red-violet coloration, which disappears with oxazolone hydrolysis.

The mathematical modelling describing the optimal process for obtaining N-(p-nitrobenzoyl)-L-phenylalanine (IV) was achieved.

The structure of compound (IV) was established by chemical and spectral (FT-IR, ^{1}H-NMR, ^{13}C-NMR, MS) analyses.

The molecular geometry, the bond lengths between atoms, the task of each atom in compound (IV) molecule, were determined using HyperChem 8.0.6.

The mass, the volume, the surface area, the hydrophobicity, the molar refactivity, the polarizability, the dipole moment and the hydration energy af acyl-derivative were established by QSAR method.

The DL_{50} value was determined and antimicrobial activity as studied for the compound (IV) compared with sulph-drug reference. The product (C) shows evident inhibitory effect on microbial strains of Gram-positive and Gram-negative bacteria.

Acknowledgments: This work was supported by the strategic grant POSDRU/159/1.5/S/137750, Project Doctoral and Postdoctoral programs support for increased competitiveness in Exact Sciences research cofinanced by the European Social Found within the Sectorial Operational Program Human Resources Development 2007 – 2013.

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Manuscript received: 21.05.2015