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OPTIMIZATION REACTION FOR OBTAINING SOME N-[P-(R)-BENZOYL]-L-GLUTAMINE DERIVATIVES WITH PHARMACEUTICAL ACTION

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The conditions in which the obtaining reactions of some N-[p-(R)-Benzoyl]-L-Glutamine derivatives are made with the highest yield were established in this paper. Due to their antitumoral effects, the action of glutamine derivatives on the digestive enzymes has been evaluated.

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1. Introduction

The finding that amino acids play a key role in the structure and functions of biologically important molecules was a positive signal for the further development of research in the direction of pharmaceutical applications.

We set the glutamine because it plays an important role in transamination reactions, thus establishing the link between proteins and carbohydrates metabolism. On the other hand, it is a supplier of amino groups in the biosynthesis of other amino acids. This amino acid is also involved in the urea cycle and also in regulating the content of ammonia in the body. Glutamine is also used to improve glutathione level and to increase the muscle mass in HIV infection [1]. Through a complex metabolic pathway, glutamine causes a particularly important protective effect on the intestinal mucosa [2-4]. Also this amino acid and some of its derivatives are important in surgery, reducing the incidence of infections [5]. Recent researches established the structure of some glutamine derivatives for which the biological tests evidenced antitumoral effect [6,7].

Considering these results Grebinisan, et al. [7] obtained N-(p-aminobenzoyl)-L-glutamine (II) by reducing N-(p-nitrobenzoyl)-L-glutamine (I) sulfide sodium in ethanol medium and in the presence of sodium bicarbonate. The compounds N-[p-(formilamino)-benzoyl]-L-glutamine (III) and N-[p-(acetylamino)-benzoyl]-L-glutamine (IV) (scheme I) were synthesized by N-(p-aminobenzoyl)-L-glutamine (II) with formic acid or with mixture of acetic anhydride-acetic acid.

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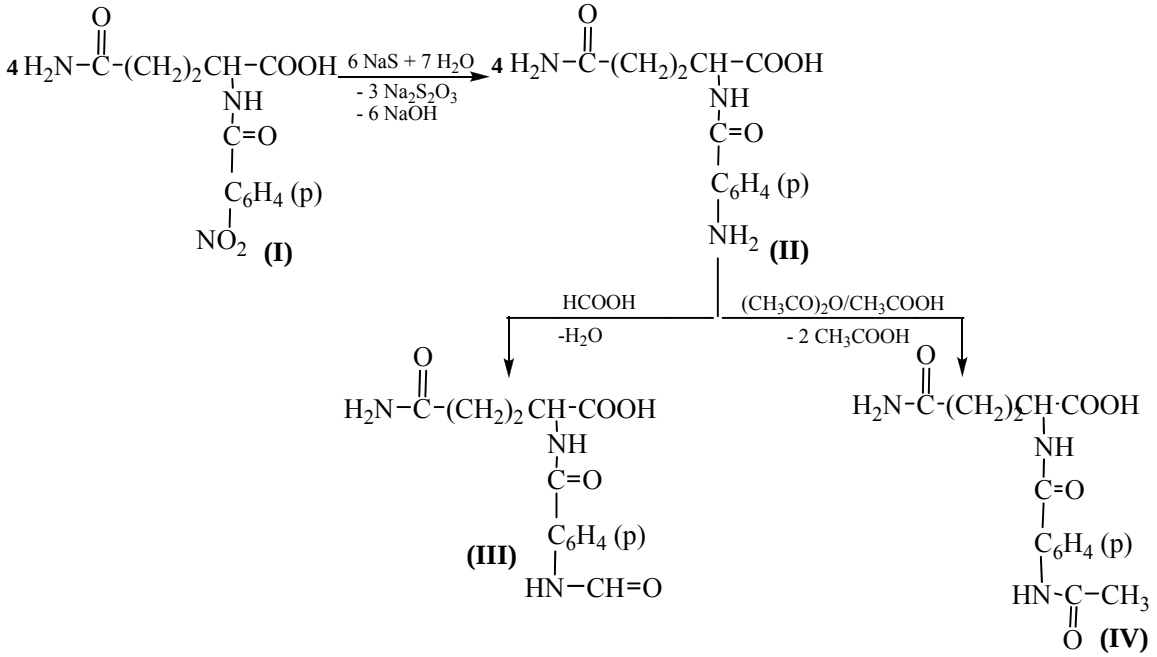
Scheme I



The structure of compound (I) was confirmed by spectral and chemical analyzes. Its IR spectrum shows characteristic vibration band of NH bond at 3428 cm^{-1}, 3743 cm^{-1} and amide I band located at 1654 cm^{-1}. The characteristic bands of para-disubstituted benzene also appear at 715 cm^{-1} and 784 cm^{-1}. Intense bands at 1344 cm^{-1} and 1596 cm^{-1} attributed to symmetric and asymmetric vibration of nitro group were identified.

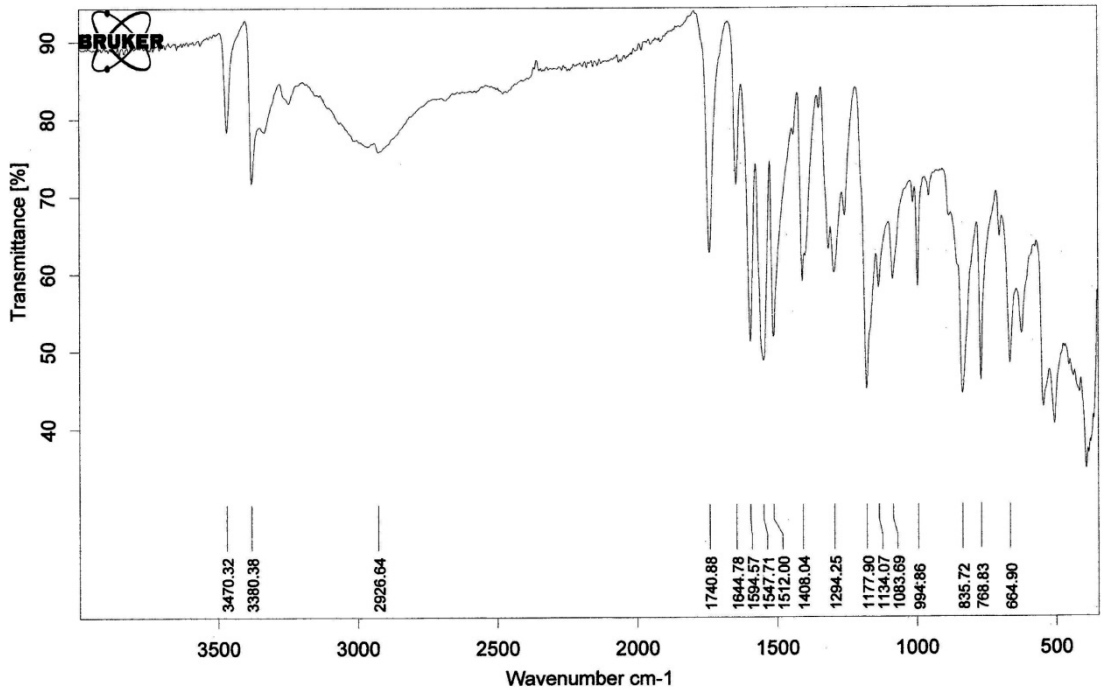


Fig. 1. FTIR spectrum of compound II

Nuclear magnetic resonance (NMR) spectra also confirm the structure (I). In the lower fields some signals corresponding to the aromatic protons appear at 8.11-8.35 ppm. The protons of

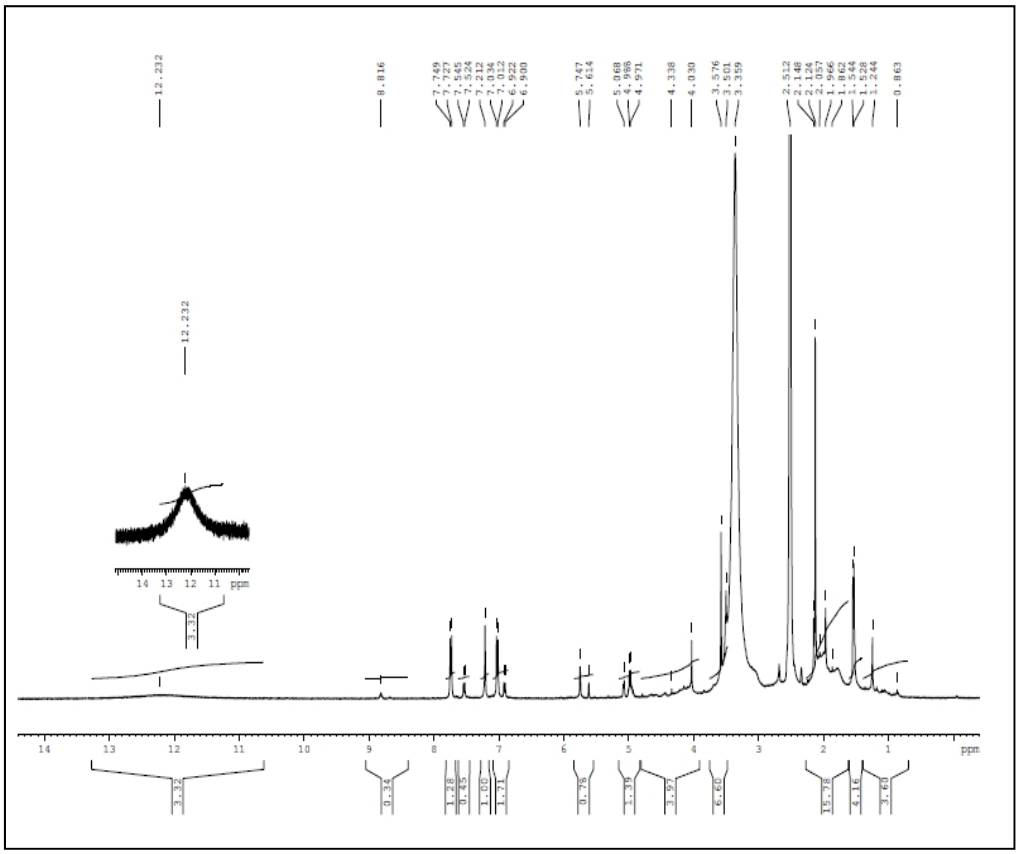
aliphatic chains are identified by signals occurring at 1.95-1.99 ppm, 2.01-2.16 ppm, 2.23-2.27 ppm and 4.36-4.42 ppm. The protons of group NH_2 appear as a singlet at 6.84 ppm and 7.38 ppm, while the proton characteristic of NH amide group is indicated by a doublet at 9.08-9.10 ppm. Hydrogen carboxylic group is assigned to a signal that appears at δ = 12.79 ppm.

The structure of compound (II) was also investigated by elemental analysis and by spectroscopic measurements. In the IR spectrum the valence vibrations of NH produce an intense band at 3470 cm^{-1} and OH group appear at 2926 cm^{-1} respectively.

The CO amidic group is identified by the vibration valence band at 1740 cm^{-1}. Para-disubstituted benzene ring is highlighted by the pick from 768 cm^{-1}. The two bands assigned to symmetric and asymmetric vibrations of NO_2 group do not appear in IR spectrum.

^{1}H-NMR spectrum confirmed the proposed structure. The signals corresponding to the two protons of the NH_2 groups newly formed appear at 5.74 ppm, thereby supporting successful reduction reaction.

The nature of the product III and IV was also confirmed by elemental and spectral analyzes.



Fig, 2. ^{1}H NMR Spectrum of compound II

For products III and IV, the amide bands appear in the IR spectra located between 2565-3319 cm^{-1} and the amide II bands in the 1635-1748 cm^{-1}. Both compounds show a characteristic absorption band similar to para - disubstituted benzene in the 785-852 cm^{-1} range.

^{1}H-NMR spectra recorded for compounds III and IV show specific signals to the proton group -C = O at 3.90-3.93 ppm, and for the group – CO – CH_3 at 2.05 ppm. The characteristic signals for the protons of carboxylic groups appear at 12.58 ppm and 12.56 ppm. The optimization of the chemical reactions for obtaining of the I-IV glutamine derivatives is of notable importance in biochemistry field and its applications.

2. Optimization of the obtaining reactions for compounds I-IV

Our purpose is to establish the conditions in which the reactions for obtaining the compounds I - IV can be realized with the highest yield. Some preliminary studies revealed the influence of temperature and reaction time on the obtaining reaction yield for compounds N-(p-nitrobenzoyl)-L-glutamine (I), N-(p-aminobenzoyl)-L-glutamine (II), N-[p-(formilamino)-benzoyl]-L-glutamine (III) and N-[p-(acetylamino)-benzoyl]-L-glutamine (IV).

Let us consider the reaction yield (η) to be the indicator of the reaction optimization. The reaction temperature (X_1 expressed in Celsius degrees) and the reaction time (X_2 expressed in minutes) were considered as being significant variables for the studied reactions.

In order to obtain the conditions in which the reactions are made with the highest yield, a factorial experiment of the 3^2 type [8-10] was organized for each reaction. This experiment permits to obtain the reaction yield as a function of the second degree in the significant independent variables and to have a three-dimensional, spatial representation of the obtained function.

The relevant variables $X_i \ (i = 1,2)$ were transformed in a-dimensional variables $x_i \ (i = 1,2)$, by the procedure previously developed [8, 9].

Table 1. Extreme values of the significant variables for the obtaining reactions of compounds I - IV.

Compound	Temperature X_1 *(°C)*		Time X_2 *(minutes)*	
	Minim	Maxim	Minim	Maxim
I	80 ($x_1 = -1$)	90 ($x_1 = 1$)	15 ($x_2 = -1$)	55 ($x_2 = 1$)
II	10 ($x_1 = -1$)	14($x_1 = 1$)	100 ($x_2 = -1$)	140 ($x_2 = 1$)
III	19 ($x_1 = -1$)	21($x_1 = 1$)	300 ($x_2 = -1$)	420 ($x_2 = 1$)
IV	55 ($x_1 = -1$)	65($x_1 = 1$)	210 ($x_2 = -1$)	270 ($x_2 = 1$)

The reaction yield is expressed as a function (1) on the a-dimensional relevant orthogonal variables:

$$\eta = a_0 + a_1 x_1 + a_2 x_2 + a_{12} x_1 x_2 + a_{11} x_1^2 + a_{22} x_2^2 \tag{1}$$

In order to obtain orthogonal quadratic terms, reaction yield (1) was averaged. Let be $\overline{\eta}-$ the average value of the reaction yield in $N = 9$ experiments.

$$\overline{\eta} = \frac{\sum_{i=1}^{N} \eta_i}{N} \tag{2}$$

By using relation (1), the condition of orthogonality and the constant values of the regression coefficients a_0, $a_{i,i}; \ i,i = 1,2$ and $a_{i,j}; \ \ i,j = 1,2$, the average reaction yield can be expressed as [11, 13]:

$$\overline{\eta} = a_0 + \frac{2}{3}(a_{11} + a_{22}) \tag{3}$$

From (1) and (3), one obtains:

$$\overline{\eta} = \eta + a_1 x_1 + a_2 x_2 + a_{12} x_1 x_2 + a_{11}\left(x_1^{\,2} - \frac{2}{3}\right) + a_{22}\left(x_2^{\,2} - \frac{2}{3}\right) \tag{4}$$

The values of the a-dimensional variables and the reaction yield for the studied compounds I-IV are given in the Table 2.

Table 2. Relevant a-dimensional variables and optimizing indicator (reaction yield) for compounds I-IV.

No.	x_1	x_2	$\eta_I(\%)$	$\eta_{II}(\%)$	$\eta_{III}(\%)$	$\eta_{IV}(\%)$
1	-1	-1	53	40	46	61
2	-1	0	55	65	52	70
3	-1	1	52	42	47	61
4	0	-1	54	55	54	67
5	0	0	58	80	59	76
6	0	1	54	57	55	68
7	1	-1	53	43	47	62
8	1	0	56	66	53	71
9	1	1	53	45	50	63

The regression coefficients from (1) were determined by using relations established in [9]. They are listed in Table 3.

Table 3. Regression coefficients in relation (1) for compounds I-IV.

Compound	a_0	a_1	a_2	a_{12}	a_{11}	a_{22}
I	57.445	0.333	-0.167	0.250	-1.667	-3.167
II	79.555	1.167	1.000	0.000	-13.833	-23.333
III	59.222	0.833	0.833	0.500	-6.833	-4.833
IV	76.112	0.667	0.333	0.250	-5.667	-8.667

The values and the signs of the coefficients listed in Table 3 indicate the intensity and the sense of the effect corresponding to the a-dimensional variables and to their cumulative actions. So, if the coefficients a_{11} and a_{22} are negative, the reaction yield will have a maximum in the variation domain of the variables x_1, x_2 respectively. When a_{11} and a_{22} are positive, the extreme of the reaction yield will be a minimum.

Table 4. Reaction yield in the center of the variation domain, square average deviation S_η *and precision* P.

Compound	$\eta_1(\%)$	$\eta_2(\%)$	$\eta_3(\%)$	$\overline{\eta}_c(\%)$	S_η	P
I	53.0	51	50	51.33	7.17	2.39
II	81.0	85.0	83.0	83.00	65.79	21.94
III	58	59	58	58.33	17.01	5.67
IV	75	76	75	75.33	21.35	7.12

In order to obtain t-Student tests, a supplemental experiment in the center of the considered domain of variables has been organized. The values obtained for the reaction yield and their average values $\overline{\eta}_c$ in the center of the variation domain are listed in Table 4 for each of the studied compounds.

The t_j-Student tests [11-13] are expressed by:

$$t_j = \frac{|a_j|}{P}; t_{ij} = \frac{|a_{ij}|}{P}, i, j = 1,2 \quad (5)$$

The t - Student tests, given in Table 5, were computed by considering the same precision, P, for all coefficients.

Table 5. t - Student tests for obtaining reactions of compounds I-IV.

Compound	t_0	t_1	t_2	t_{12}	t_{11}	t_{22}
I	24.06	0.14	0.07	0.11	0.7	1.33
II	3.63	0.05	0.05	0.00	0.63	1.06
III	10. 45	0.15	0.15	0.09	1.21	0.86
IV	10.7	0.09	0.05	0.04	0.8	1.22

Let suppose that the equations expressing the reaction yield for the compounds I-IV contain only the variables with t - Student tests higher than 0.050. The values of t-Student tests from Table 5, permit us to write the equations for the reaction yield dependent on x_1 and x_2 a-dimensional parameters:

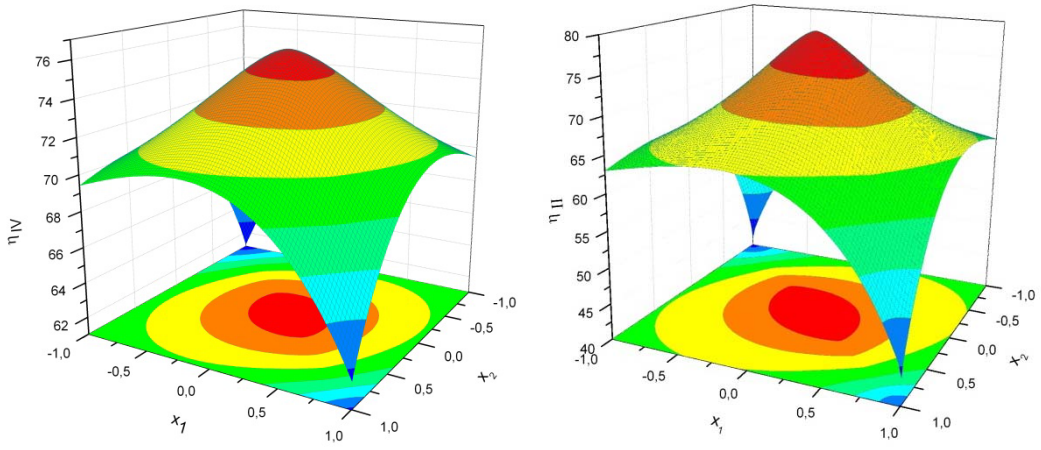
$$\begin{aligned}
\eta_I &= 57.445 + 0.333x_1 - 0.167x_2 + 0.25x_1x_2 - 1.667x_1^2 - 3.167x_2^2 \\
\eta_{II} &= 79.555 + 1.167x_1 - 13.833x_1^2 - 23.333x_2^2 \\
\eta_{III} &= 59.222 + 0.833x_1 + 0.833x_2 + 0.500x_1x_2 - 6.833x_1^2 - 4.833x_2^2 \\
\eta_{IV} &= 76.112 + 0.667x_1 - 5.667x_1^2 - 8.667x_2^2
\end{aligned} \tag{6}$$

By deriving the relations (6) and using the conditions for the extreme, one can determine the corresponding a-dimensional coordinates of the extreme, $(x_{1,e}; x_{2,e})$ and also the extreme value for the reaction yield $\eta(x_{1,e}; x_{2,e}) = \eta_e$. They are given in Table 6.

Table 6. Extreme values of the reaction yield, $\eta(x_{1,e}; x_{2,e})$ and the corresponding coordinates $(x_{1,e}; x_{2,e})$.

Compound	$x_{1,e}$	$x_{2,e}$	$\eta(x_{1,e}; x_{2,e})$
I	0.1	-0.02	57.46
II	0.04	0	79.58
III	0.06	0.09	59.29
IV	0.06	0	76.13

The dependences of the reaction yield vs. the a-dimensional variables are illustrated in Figures 3a) and b) for compounds II and IV for which one obtains the highest values of the reaction yield.



a) b)

Fig. 3a),b). Reaction yield vs. a-dimensional coordinates for compounds II and IV

The results obtained in this section can help us in choosing the more appropriate conditions for obtaining the compounds I-IV with the highest reaction yield.

By using optimization procedures, one can limit the number of trials in obtaining the compounds I-IV with a superior yield.

3. Biological action of N - acyl - derivatives of L-Glutamines on digestive enzymes

The significant antitumoral activity resulting from biological test on Jensen carcinoma [7] justified the present study regarding the influence of glutamine derivatives I-IV on the digestive enzymes trypsin, lipase and amylase in the case of their oral administration.

The studied N-acyl-derivatives synthesized from the L-glutamine were tested from their action on the digestive enzyme. The known methods for testing the enzyme activity [14,15,16,17] allow to estimate the activity of the synthesized compounds I-IV on the digestive enzymes. A small amount from each of the compounds I to IV was used for testing. The "Triferment" pills were utilized as a source of enzymes: trypsin, 0.6 uW, lipase, 0.2 uW, and 0.416 g amylase. The pill content, without the outer shell, was ground and mixed with distilled water. This mixture was left at room temperature for one hour, after which it was centrifuged. The clear solution obtained, with an 3% average concentration, served as the enzyme source for determining the activity of trypsin, lipase and amylase, important factors in the digestion process.

3. a. The action on trypsin

The trypsin activity, with optimum pH between 7-9, was determined by the Sörensen method described in [18] and consisting in measuring the amino-acids released from gelatin, as a result of the enzyme action, for one hour, at 37 °C. The free amino groups of peptides and amino-acids are blocked by formaldehyde, while the free carboxyl groups are titrated with NaOH n/50 in the presence of phenolphthalein as indicator. A neutralized gelatin solution 5% was used for the preparation of each sample at 37 °C, each sample containing a small amount of finely pulverized investigated substance. 1 mL of this mixture was added to the enzyme solution and kept at 37 °C for one hour. Two samples were prepared: active and inactive (where the enzyme is destroyed by boiling) and also two controls (substrate-enzyme and substrate-substance). The tripsin hydrolysis was followed by titration. The solution was titrated with NaOH n/50 solution until pink color, in the presence of phenolphthalein. Formalin was added, followed by titration to intense pink color.

The amount of free amino acids in the two controls and that in the inactive sample was also measured, recording the used volume of NaOH n/50 (see Table 7).

From this table it result the moderate action of the studied compounds on the trypsin activity. The stimulating action of N-(p-acyl-amino)-benzoyl-derivatives III and IV, as well as the slightly inhibiting action of N-(p-nitrobenzoyl)-L-glutamine I and respectively N-(p-aminobenzoyl)-L-glutamine II - can be noted (Table 7). An increased activity is seen for the compound IV.

3. b The action on lipase

Assessing lipase activity was done by the Bondi, Chiroy and Lebon method described in [19]. The principle of method consists in determining the amount of fatty acids released by the action of lipase. The fatty acids are titrated with NaOH n/50 in the presence of phenolphthalein as indicator.

Neutralized olive oil was used as a substrate. The enzyme source was the same as for the determination of trypsin activity. Two samples were prepared, active and inactive, and also two controls (substrate-enzyme and substrate-substance) for each determination. Two measurements were made for samples and controls.

The active sample was prepared by mixing neutralized olive oil with a small amount of the analyzed substance. Enzyme was added to this mixture by stirring. The resulting emulsion was kept in a thermostat for an hour at 37°C, then 96% ethylic alcohol and 1-2 drops of phenolphthalein were added. The whole mixture was titrated with NaOH n/50, till pink in color.

The inactive sample was prepared in a similar manner, excepting that the enzyme was boiled (to be inactivated); after which it was added to the substrate and kept at constant temperature at 37°C. The control-substance was prepared by stirring in olive oil, a small amount of the analyzed substance, 96% ethylic alcohol and 1-2 drops of phenolphthalein.

The control-enzyme contains olive oil, 2 mL enzyme solution, 96% ethylic alcohol and 1-2 drops of phenolphthalein. Both controls are titrated with NaOH n/50 till intense pink color is obtained. The results, expressed in mL NaOH n/50, are shown in Table 7. As it can be noted from our research, the synthesized compounds I-IV, practically, do not affect the lipase activity.

3. c The action on amylase

This action was investigated using Benedikt method mentioned in [20, 21]. The active sample was prepared as it follows: 10% starch solution as substrate, a small amount of the finely pulverized substance and 1 mL enzyme solution. This mixture was stirred and the sample was kept for one hour at 37°C. 1 mL of Benedikt solution was added to the mixture and heated for 5 minutes.

The influence of the analyzed substance on amylase activity was assessed by comparing with controls, enzyme with substrate, enzyme with substance and substrate with substance. The displayed results show that compounds I-IV do not affect amylase activity.

Table 7. The results regarding the influence of compounds I-IV on digestive enzymes

20 mg Substance	Enzyme concentration (g/mL) in Triferment solution 3%		Enzyme activity expressed in mL NaOH n/50/mL in Triferment solution 3%	
	Trypsin	***Lipase***	***Trypsin***	***Lipase***
I	3.3	1.81	1.7	1.2
II	3.3	1.81	3.2	1.8
III	3.3	1.81	4.1	2.9
IV	3.3	1.81	5.9	3.4

Compounds I-IV do not present negative influence on the tested enzyme's activities. Consequently, it makes possible their orally administration. The pharmacological importance of the studied compounds imposed to establish the optimal conditions for their synthesis in microproduction process.

4. Conclusions

Some preliminary studies revealed the influence of temperature and reaction time on the reaction yield for obtaining compounds I-IV. By using optimization procedures, one can minimize the trials to obtain the studied compounds with a convenient yield. This study can help the chemists in obtaining biologic active N-[p-(R)-Benzoyl]-L-Glutamine derivatives in the best conditions.

The biological study has been shown the compounds I-IV do not influence the lipase and amylase activity. Trypsin is slightly influenced in its activity.

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