NOVEL ACYL DERIVATIVES OF N-(p-AMINOBENZOYL)-L-GLUTAMINE ENCAPSULATED IN POLYMERIC NANOCAPSULES WITH POTENTIAL ANTITUMORAL ACTIVITY

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New compounds with antitumoral activity have been obtained, by synthesizing aminoacid derivatives with formyl or acetyl groups grafted on N-(p-aminobenzoyl)-L-glutamine. The suggested chemical structure of the new compounds was confirmed by elemental and spectral analysis (FT-IR and ^{1}H-NMR). The synthesized acyl derivatives have been encapsulated into polymeric nanocapsules, based on chitosan and sodium alginate. Similarly to a reference cytostatic drug (methotrexate), biological tests showed that all compounds and all loaded nanocapsules presented low toxicity and good antitumoral activity against Erlich ascetic tumour in mice.

Keywords: L-glutamine derivatives, polymers, nanocapsules, antitumoral activity, EAT, chitosan

INTRODUCTION

The synthesis of novel compounds with pharmacological action is a major desideratum, the present research representing an original contribution to the field of aminoacids chemistry.

Obtaining of L-glutamine derivatives with p-aminobenzoic acid as a pendant group is of great importance, especially due to the fact that this acid participates at the regulation of the general metabolism,[1] by entering the chemical composition of folic acid, which is indispensable for the metabolism of deoxyribonucleic acids.[2] Based on this property, numerous authors considered the p-aminobenzoic acid as a structural component in novel peptide derivatives;[3-6] moreover, the p-aminobenzoic acid presents remarkable antitumoral action[1,7,8] and some of its derivatives demonstrated favourable antiviral activity,[9] protection capacity against radiation,[10] antifibrinolytic[11,12] or vessel dilatation effect.[13]

Most organic antitumoral and antimicrobial drugs are harmful for both the healthy and affected human cells, especially when administered in repeated doses. Thus, their encapsulation in drug delivery systems is sometimes preferred, the encapsulated drug being released in a controlled, sustained manner to the targeted sites. Nanoparticulated systems are promising as vectors for drugs, enzymes and other biological or cosmetic active principles. Polymeric nanocapsules, in particular, present high drug encapsulation efficiency, the polymeric shell protecting the encapsulated drug against degradation factors.[14]

Nanocapsules based on natural polymers are usually prepared by an emulsion-coacervation process, using the emulsion as a template phase and stabilization by physical

intermolecular or covalent crosslinking.[14,15]

The present research was focused on the synthesis of N-benzoyl-L-glutamine derivatives substituted in p-position with -NO_2, -NH_2 or acyl-amine groups with potential biological properties, and on their encapsulation in polymeric nanocapsules based on sodium alginate and chitosan.

EXPERIMENTAL

Materials and method

All reagents were used as purchased (Aldrich, Fluka, Merk, S.C. Chemical Company S.A.). The FT-IR spectra were registered on a FT-IR spectrophotometer (ATR) Brucker Tensor-27; ^{1}H-NMR analysis was performed on a Brucker ARX 400 spectrometer (5 mm QNP probe; 1H/13C/31P/19F) and elemental analysis was made using an Exeter Analytical CE 440 elemental analyser. The melting points of the obtained compounds were determined with a Mel-Temp melting point module, provided with a digital thermometer.

N-(p-nitrobenzoyl)-L-glutamine (I)

In a reaction flask provided with a refluxing cooler and a mechanical stirrer, 250 mL distilled water, 0.05 mol L-glutamine and 0.25 mol sodium bicarbonate were introduced, under stirring. After solubilizing all components at 10-12 °C, 0.05 mol p-nitrobenzoic acid chloride dissolved in 125 mL anhydrous benzene was slowly added (using a dropping funnel), during 1 h, under continuous stirring. Stirring was maintained for another 90 min at 10-12 °C. Then, the reaction mixture was vacuum-filtered and the benzene layer removed, using a separating funnel. The remaining aqueous phase is then acidulated, under stirring, with a diluted hydrochloric acid solution (1:1; v/v), until reaching a pH of 1-1.5, when a solid product is precipitated. The final compound is purified by repeated recrystallization from boiling water.

White solid (12.83 g; yield, %: 87), melting point: 180-181 °C. FT-IR; ν_{max}cm^{-1}: 3428-3743 (NH); 1654, 1719 (C=O); 1344 (NO_2 symmetrical); 1596 (NO_2 asymmetrical); 715-784 (CH Ar). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.95-1.99 (m, 1H, CH_2); 2.01-2.16 (m, 1H, CH_2); 2.23-2.27 (m, 2 CH, CH_2); 4.36-4.42 (m, 1H, CH); 6.84 (d, 1H, NH_2); 7.38 (s, 1H, NH_2); 8.11-8.18 (d, 2H, Ar); 8.31-8.35 (d, 2H, Ar); 9.08-9.1 (d, 1H, NH); 12.79 (s, 1H, COOH).

Anal. calcd. for $C_{12}H_{13}N_3O_6$ (%): C, 48.81; H, 4.40; N, 14.23; found: C, 49.00; H, 4.77; N, 14.52.

N-(p-aminobenzoyl)-L-glutamine (II)

In a reaction flask provided with a reflux condenser, 0.016 mol N-(p-nitrobenzoyl)-L-glutamine (I) and 100 mL ethanol (96%) were added to 0.016 mol sodium bicarbonate in 50 mL distilled water. The components were allowed to react for 45 min, under stirring at 60 °C, until complete dissolution of acyl-aminoacid as a sodium salt, then the reaction mixture was treated with 50 mL preheated (50-60 °C) aqueous solution of 0.04 mol Na_2S x $9H_2O$ and 0.04 mol sodium bicarbonate, and allowed to react for 4 h, under reflux. The hot reaction mixture was then filtered and left to cool down to room temperature before being acidulated with a diluted hydrochloric acid solution (1:1; v/v), until reaching a pH of 1.5-2. A crystalline compound was formed and then purified by repeated recrystallization from boiling water.

Light-yellow solid (2.71 g; yield, %: 64), melting point: 200-201 °C. FT-IR; ν_{max}cm^{-1}: 2500-3700 (NH); 1710 (C=O); 1530 (amide II); 740 (CH Ar). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 2.50-2.59 (m, 4H, CH_2); 3.37 (s, 2H, NH_2); 3.84-3.87 (m, 1H, CH); 5.65-5.67 (d, 2H, NH_2); 6.53-6.65 (d, 2H, Ar); 7.57-7.59 (d, 2H, Ar); 8.32-8.35 (d, 1H, NH); 12.40 (s, 1H, COOH).

Anal.calcd. for $C_{12}H_{15}N_3O_4$ (%): C, 54.33; H, 5.66; N, 15.84; found: C, 54.74; H, 5.90; N, 16.17.

N-[p-(formylamino)-benzoyl]-L-glutamine (III)

A mixture of 0.018 mol N-(p-aminobenzoyl)-L-glutamine (II) and 10 mL formic acid (80%) introduced into a reaction flask provided with a reflux condenser was heated (80-85 °C) on a water bath for 30 min and then transferred into a crystallizing dish to cool for 6 h at room temperature, when a viscous product, precipitating upon addition of 20 mL of distilled water, was separated. The final compound was purified by recrystallization from boiling methanol.

White solid (3.32 g; yield, %: 63), melting point: 184-185 °C. FT-IR; ν_{max}cm^{-1}: 3296 (NH); 1676-1703 (C=O); 1531 (amide II); 767 (CH Ar). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 3.35-3.40 (m, 4H, CH_2); 3.90-3.91 (m, 1H, CH); 7.27-7.29 (d, 1H, CH); 7.66-7.68 (d, 2H, Ar); 7.84-7.85 (d, 2H, Ar); 8.33-8.35 (d, 1H, NH); 8.73-8.75 (d, 1H, NH); 10.34-10.43 (m, 2H, NH_2); 12.30 (s, 1H, COOH).

Anal.calcd. for $C_{13}H_{15}N_3O_5$ (%): C, 53.24; H, 5.11; N, 14.33; found: C, 53.35; H, 5.32; N, 14.59.

N-[p-(acetylamino)-benzoyl]-L-glutamine (IV)

A mixture of 0.018 mol N-(p-aminobenzoyl)-L-glutamine (II), 1 mL acetic anhydride and 2.5 mL acetic acid (96%) was heated at 85-90 °C for 4 h in a reaction flask provided with a reflux condenser. Then, the reaction mixture is transferred to a crystallizing dish to cool (3-4 h) at room temperature, when a crystalline product, finally purified by recrystallization from boiling water, was separated.

Light-yellow solid (3.64 g; yield, %: 66), melting point: 214-215 °C. FT-IR; ν_{max}cm^{-1}: 3304

(NH); 1732 (C=O); 1530 (amide II); 766 (CH Ar). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 2.07 (s, 3H, CH_3); 2.59-2.63 (m, 4H, CH_2); 3.80-3.82 (m, 1H, CH); 5.68 (s, 2H, NH_2); 6.50-6.52 (d, 2H, Ar); 7.50-7.53 (d, 2H, Ar); 8.0 (d, 1H, NH); 10.16 (s, 1H, NH); 12.56 (s, 1H, COOH).

Anal. calcd. for $C_{14}H_{17}N_3O_5$ (%): C, 54.72; H, 5.53; N, 13.68; found: C, 55.11; H, 5.93; N, 14.06.

Preparation of alginate-chitosan nanocapsules

Alginate-chitosan nanocapsules were prepared using a modified version of the methods described elsewhere.[16-18] Briefly, an o/w emulsion was first prepared by dropping a mixture of 1.3 mL ethanol, 12 mg benzyl alcohol and 0.7 mL drug solution in dimethylsulphoxide (30 mg/mL) into 60 mL of aqueous sodium alginate solution (0.6 mg/mL) containing 2% (w/v) Tween80. After sonication (Bandelin ultrasonic homogenizer Sonopuls HD 2070) for 15 min, 9 mL of a $CaCl_2$ solution (0.7 mg/mL) were added dropwise and the emulsion was left under magnetic stirring for 45 min. Then, 12 mL of a chitosan solution (0.6 mg/ml in 2% (v/v) acetic acid) were added dropwise into the crosslinked nanocapsule suspension and magnetic stirring was continued for another 45 min. Finally, the nanocapsules aqueous suspension was allowed to equilibrate overnight prior to ethanol and water removal by rotary evaporation at 40-60 °C for 30 min. The nanocapsules suspensions were kept either refrigerated or at room temperature.

RESULTS AND DISCUSSION

Synthesis and characterization of L-glutamine derivatives

The investigation was focused on the synthesis of L-glutamine derivatives by its reaction with p-nitrobenzoic acid chloride (Fig. 1). The reaction took place at low temperatures (10-12 °C), in a sodium bicarbonate aqueous solution, the chloride being dissolved first in anhydrous benzene and then added to the cold reaction mixture. The reaction took place for 90 min, under vigorous mechanical stirring. By treating the sodium salt obtained from the condensation reaction with hydrochloric acid, N-(p-nitrobenzoyl)-L-glutamine (I) was formed.

Compound (I) was obtained by recrystallization from hot water (yield of 87% and melting point – 180-181 °C).

The anticipated chemical structure of (I) was confirmed by elemental and spectral analyses (FT-IR and ^{1}H-NMR). The FT-IR spectra showed absorption bands characteristic of the symmetrical/ asymmetrical vibrations of the NO_2 group found in p-position of the benzene ring. In the ^{1}H-NMR spectra, characteristic displacements occurred at 8.11-8.35 ppm for the aromatic protons, while the secondary amide proton group was deshielded, due to the conjugation effect of the >C=O group from glutamine.

N-(p-aminobenzoyl)-L-glutamine, as well as its N-acylated derivatives could present an antagonist effect against the p-aminobenzoyl-L-glutamic acid. A series of reactions (Fig. 2) were performed to obtain the derivatives. Thus, N-(p-aminobenzoyl)-L-glutamine (II) was synthesized by reducing the N-(p-nitrobenzoyl)-L-glutamine sodium salt with sodium sulphide, in the presence of sodium bicarbonate. The reaction mixture was refluxed for 4-5 h, then cooled and acidulated with hydrochloric acid until reaching a pH of 1.5-2. The final product (II) was obtained by recrystallization from boiling water with a yield of 64% and a melting point of 201 °C.

The spectroscopic data (FT-IR and ^{1}H-NMR) summarised below prove the anticipated chemical structure of this dipeptide. Thus, the two bands characteristic of the symmetrical/asymmetrical vibrations of NO_2 no longer appeared in the FT-IR spectrum. The ^{1}H-NMR spectra showed chemical shifts of the newly formed NH_2 protons at 5.65-5.67 ppm, thus confirming the reduction reaction.

Some p-substituted phenyl derivatives of aminoacids present remarkable biological effects.[3,4,6,19-23] Considering this, our research focused on obtaining new acyl derivatives (III and IV) with potential pharmacological activity by grafting various substitutes (Fig. 3) to the amino group of compound (II). N-[p-(formylamino)-benzoyl]-L-glutamine (III) was synthesized by the formulation of compound (II) with high yield, when using an excess (5/1 molar ratio) of formic acid (80%) at 80-85 °C. By reacting compound (II) with an acetic anhydride/acetic acid mixture under reflux, N-[p-(acetylamino)-benzoyl]-L-glutamine (IV) was obtained (66% yield).

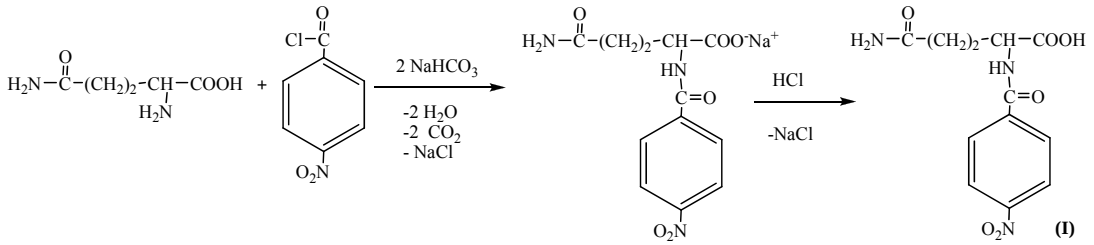

Figure 1: Synthesis of N-(p-nitrobenzoyl)-L-glutamine (I)

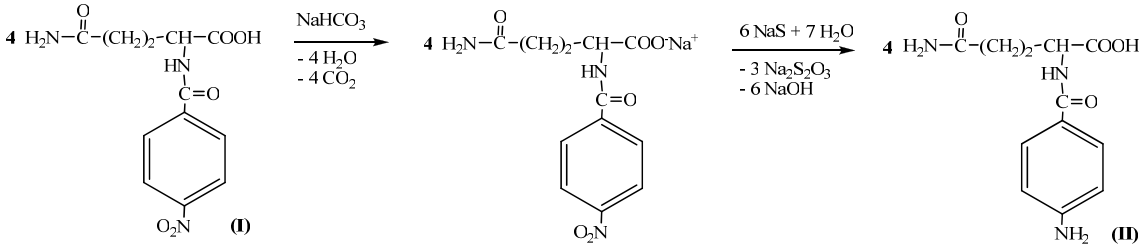

Figure 2: Synthesis of N-(p-aminobenzoyl)-L-glutamine (II)

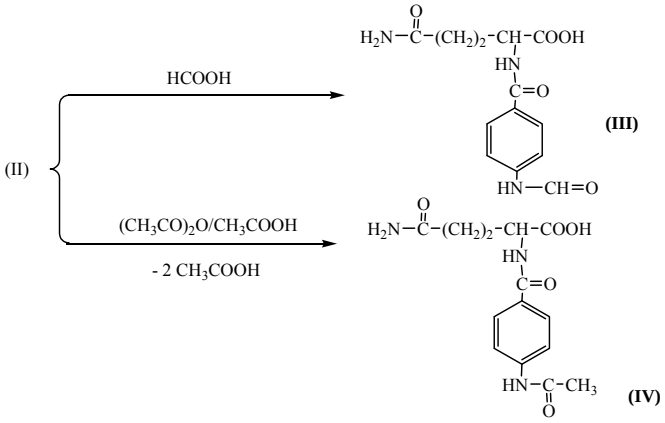

Figure 3: Synthesis of N-[p-(formylamino)-benzoyl]-L-glutamine (III) and N-[p-(acetylamino)-benzoyl]-L-glutamine (IV)

The anticipated chemical structures of compounds (III) and (IV) were confirmed by elemental and spectral analyses (FT-IR and ^{1}H-NMR). FT-IR spectra revealed the presence of amide I and amide II and of an absorption band characteristic of the p-disubstituted benzene ring. In the ^{1}H-NMR spectra, the signal at 5.67 ppm for compound (II) appeared shifted at 8.33 ppm, and at 8.75 ppm, respectively, for compounds (III) and (IV), due to the conjugation with the >C=O group.

Nanocapsule preparation and characterization

Alginate-chitosan nanocapsules were prepared using a multiple-step process, involving coacervation and O/W emulsification. Nanomicelles instantly formed when dispersing the oily phase containing the active principles (N-[p-(formylamino)-benzoyl]-L-glutamine or N-[p-(acetylamino)-benzoyl]-L-glutamine) into the aqueous alginate solution. The polymeric shell was further solidified by crosslinking with calcium chloride and chitosan by electrostatic interactions, followed by solvent removal.

The FT-IR data showed an absorption band at 3100-3500 cm^{-1} specific to the hydrogen bonds, and an N-H bending vibration specific to deacetylated chitosan at 1570 cm^{-1}. Moreover, no absorption peaks appeared in the spectrum for asymmetrical/asymmetrical –C-O stretching (~1210-1320 cm^{-1}) of sodium alginate or for the -NH_3^+ of chitosan, indicating crosslinking of alginate with chitosan and calcium chloride.

Nanocapsule morphology was evaluated by scanning electron microscopy, which showed almost spherical nanocapsules with sizes of approximately 400 nm (Fig. 4).

The physical stability of nanocapsules was evaluated by determining the effect of storage temperature (5 °C and room temperature) on the mean diameter of nanocapsules (Table 1). The nanocapsules presented only slight modifications of the mean diameter at 5 °C, during storage. However, storage at room temperature determined an increase in nanocapsule size.

Biological activity

The toxicity of the synthesized compounds and loaded nanocapsules was evaluated following their intraperitoneal administration as suspensions in Tween80 to groups of 14 mice each (20±5 g), according to Karber method.[24] Mice were monitored, their mortality after 7 days being noted and the value of LD_{50} being calculated (Table 2).

All synthesized compounds and nanocapsules encapsulating compounds (III) and (IV) are virtually non-toxic, compared to methotrexate (the reference cytostatic drug), which recommends them for further laboratory screening.

The antitumoral activity was evaluated on experimental Ehrlich ascetic tumours (EAT) inoculated in groups of A_2G mice (20±2 g) of 20 or 10 animals (control group), provided by the Oncology Institute of Cluj-Napoca. The tumours were transplanted by intraperitoneal injection from donor mice bearing EAT for 14 days. As acyl-dipeptides (III and IV) and the polymeric nanocapsules are difficult to solubilize, they were administered as suspensions in 1% methyl cellulose (chemically and biologically inert on animal tissues). The compounds were injected as single doses (40, 200 and 400 mg/kg body), 7 days after tumour transplantation.

Tumour growth inhibition determined by compounds (III) and (IV) and by the loaded nanocapsules was calculated following a method cited in literature,[25] 7 days after tumour inoculation (Table 3).

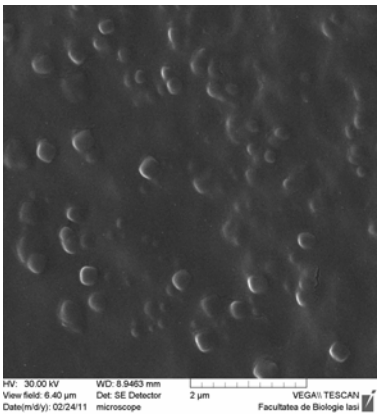

Figure 4: Morphology and size of polymeric nanocapsules loaded with compound (III)

Table 1

Physical stability of alginate-chitosan nanocapsules

Time (days)	Mean diameter (nm)			
	Nanocapsules loaded with compound (III)		Nanocapsules loaded with compound (IV)	
	5° C	RT*	5° C	RT*
0	422	422	412	412
30	449	495	410	473

* room temperature

Table 2
LD_{50} for compounds (I-IV) and loaded nanocapsules

Compound	Administration route	Tested animals	LD_{50} (mg/kg body)
I	intraperitoneal	mice	8240
II	intraperitoneal	mice	9055
III	intraperitoneal	mice	8540
IV	intraperitoneal	mice	9200
Nanocapsules loaded with III	intraperitoneal	mice	8750
Nanocapsules loaded with IV	intraperitoneal	mice	9315
Methotrexate	intraperitoneal	mice	146

Table 3
Tumour growth inhibition determined by acyl-peptides (III and IV) and acyl-peptides loaded nanocapsules

Compound	Administration route	Inhibition of EAT growth, %		
		Compound concentration (mg/kg body)		
		400	200	40
III	Intraperitoneal	45	40	35
IV	Intraperitoneal	66	61	53
III loaded nanocapsules	Intraperitoneal	47	43	37
IV loaded nanocapsules	Intraperitoneal	69	61	54
Methotrexate	Intraperitoneal	70	62	55

Table 3 shows that compounds (III) and (IV), as well as the loaded nanocapsules had a good tumour inhibition capacity, compared to methotrexate (reference antimetabolite drug). It is evident that compound (IV) and the nanocapsules loaded with compound (IV) presented the highest antitumoral activity, due to enhancement of the cytostatic effect determined by the acetyl group and, probably, due to a controlled and sustained release of the enclosed derivative from the core of nanocapsules to the tumour site.

CONCLUSIONS

The optimum reaction conditions for the synthesis of N-(p-nitrobenzoyl)-L-glutamine (I) by condensation of L-glutamine with p-nitrobenzoic acid chloride (Schotten-Bauman reaction) have been established. Compound (I) was reduced to N-(p-aminobenzoyl)-L-glutamine (II), from which formyl (III) and acetyl (IV) derivatives were synthesized. Their anticipated chemical structure was demonstrated by elemental and spectral analyses (FT-IR and ^{1}H-NMR).

The new acyl derivatives were further encapsulated in polymeric nanocapsules, prepared by an emulsion-coacervation process.

Compounds (I-IV), as well as the nanocapsules loaded with (III) and (IV), evidenced very low toxicity, which recommends them for other biological experiments.

The antitumoral effect of acyl-dipeptides (III, IV) and of the loaded nanocapsules was evaluated against the development of Ehrlich ascetic tumours in mice. The tested compounds showed an inhibition of EAT growth efficiency similar to that of methotrexate (reference cytostatic drug). However, N-[p-(acetylamino)-benzoyl]-L-glutamine (IV), as a free drug or encapsulated in polymeric nanocapsules, demonstrated the highest antitumoral activity, as influenced by the presence of the p-acetylamino group in the chemical structure of N-[p-(acetylamino)-benzoyl]-L-glutamine (IV), which, by gradual release from nanocapsules, can determine a retarded action.

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