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Nanoencapsulation of 3-chloropropylaminobenzoate Derivatives with Potential Insecticidal Activity †

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Abstract: Aminobenzoic acid derivatives have shown various pharmacological properties, one of which is pesticide activity, giving these compounds the ability to work as alternatives to current pesticides. Nanotechnology could efficiently support the use of these compounds by encapsulating them in stable nanoformulations, to improve their stability and effectiveness. In the present work, 3-chloropropylaminobenzoate derivatives were synthesised, evaluated against their effect upon the viability of the insect cell line *Sf9* (*Spodoptera frugiperda*) and nanoencapsulation studies of the most active compound were carried out. The most potent molecules reduced insect cell viability by around 40% at 100 μg/mL.

Keywords: aminobenzoic acid derivatives; aminobenzoates; nanoencapsulation; *Sf9* (*Spodoptera frugiperda)*; insecticides

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1. Introduction

According to FAO (Food and Agriculture Organization) by 2050, the world population will reach 9.1 billion, which represents an increase of 34% of the current population [1] and consequently, an increase of 70% in the food production will be required [1,2]. Agrochemicals have been fundamental to produce food, as well as for the control of disease vectors [3]. To control weed, insect various disease-carrying pests infestation in agricultural feeds, pesticides are widely used [3,4]. The intensive use of pesticides, promotes an insecticide resistance that affects the effectiveness and utility of pest protection compounds [5]. Thus, as alternative is necessary to develop pest control options, especially those with new mechanisms of action [3,5].

Aminobenzoic acid derivatives are of fundamental interest, as different relative positions of the functional groups on the aromatic ring (*para*, *meta* and *ortho*) can produce significant differences in chemical properties. *ortho*-Aminobenzoic acid, trivialy named as anthranilic acid and its analogues have a privileged profile as pharmacophores for the rational development of deliberate drugs for the management of pathophysiology and pathogenesis of various diseases. The structure substitution of the anthranilic acid pro-

vides a variety of compounds, which allow a comprehensive assessment of structure-activity relationship analysis for the identification of hits and leads in a typical drug development paradigm. Anthranilic acid derivatives exhibit interesting antimicrobial, antiviral and insecticidal properties [6].

Nano-structured materials have recently received increasing attention due to their unique properties and wide range of vital applications [7,8]. Nanotechnology could efficiently support the use of several biologically active compounds by encapsulating them in stable nanoformulations, such as nano-emulsions to improve their stability and effectiveness [9].

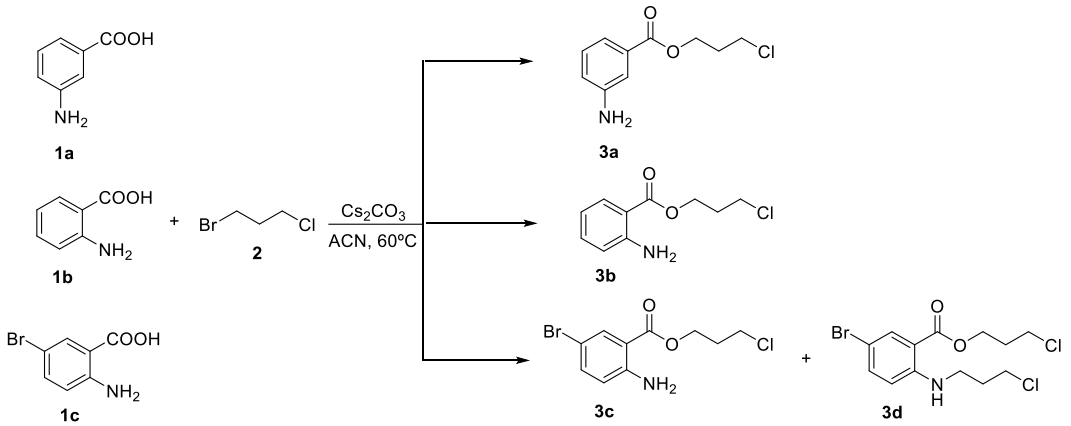
Given these facts into account, the synthesis of 2- and 3-aminobenzoic acid derivatives were carried out in order to further evaluate them as potential alternative insecticides and nanoencapsulation studies were performed with the most active compound.

2. Results and Discussion

2.1. Synthesis of Aminobenzoic Acid Derivatives 3a–d

Esterification of the aminobenzoic acids **1a**-**c** with the intended alkyl bromide was carried out with cesium carbonate since, as it is known, compared to other alkali metal carboxylate salts, cesium salts have been shown to be especially efficient in esterification, under non-aqueous conditions. Acetonitrile is a convenient reaction solvent due to its appropriate boiling point, as well as high dielectric constant and polar nature, which provide good solubility for the cesium carboxylate salts favorable for the esterification [10]. Thus, the reaction of 3-aminobenzoic acid **1a**, 2-aminobenzoic acid **1b**, and 2-amino-5-bromobenzoic acid **1c**, with 1-bromo-3-chloropropane **2** was carried out in presence of cesium carbonate, in acetonitrile, at 60 ºC. After silica gel column chromatography purification, the corresponding esters derivatives, namely 3-chloropropyl 3-aminobenzoate **3a**, 3-chloropropyl 2-aminobenzoate **3b** and 3-chloropropyl 2-amino-5-bromobenzoate **3c,** accompanied with 3-chloropropyl 5-bromo-2-((3-chloropropyl)amino)benzoate **3d**, respectively, were obtained in yields up to 41% (Scheme 1), and characterized by NMR (^{1}H and ^{13}C) spectroscopies.

In the ^{1}H NMR spectra, signals related to the methylene protons of the new substituent in all derivatives are showed as a quintet (δ 2.21-2.24 ppm) and triplets (δ 3.67-4.44 ppm). For compound **3d** it was also visible the presence of methylene protons linked to amine group also as a quintet (δ 2.24 ppm) and triplets (δ 3.41-3.70 ppm). The presence of aromatic protons was detected by the presence of three signals for all derivatives (δ 6.32-7.86 ppm). The ^{13}C NMR spectra showed the carbons of the methylene groups, for all derivatives (δ 31.65-61.51 ppm), as well as the aromatic carbons (δ 107.28-161.43 ppm). The confirmation of the presence of the newly formed ester linkage in all compounds was also supported by ^{13}C NMR spectra, which displayed signals of the carbonyl group (δ 166.54 - 167.39 ppm).



Scheme 1. Synthesis of esters derived from amino benzoic acids **3a-d.**

2.2. Toxicity of Aminobenzoic Acid Derivatives 3a-d

The impact of aminobenzoic acid derivatives **3a**-**d** in the viability of *Sf9* cells was evaluated at 100 μg/mL, following 24h of exposure. As shown in Figure 1, compound **3c** containing, simultaneously, a bromine and an amine group in the benzen ring was completely devoid of toxicity. On the other hand, the derivatives **3a**, **3b** and **3d**, elicited a significant reduction in viability, compound **3b** being the most potent causing ca. 40% cell death. For this reason, **3b** was chosen for further nanoencapsulation assays, keeping in mind a future application as insecticides.

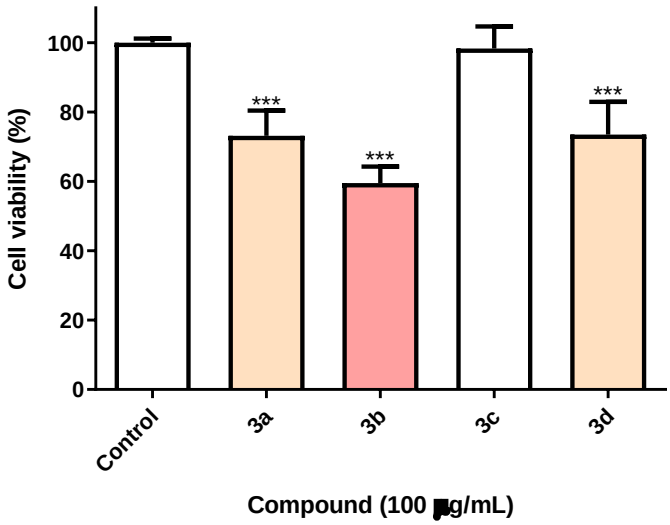


Figure 1. Viability of *Sf9* cells after incubation with the indicated molecules (100 μg/mL) for 24 h.

2.3. Nanoencapsulation Studies

Before encapsulation, a preliminary study of the photophysical properties of compound **3b** was carried out, by measuring its absorption and emission spectra in solution. This study was needed for determination of the encapsulation efficiency and release kinetics, and the results are displayed in Figure 2. The absorption spectrum shows two peaks, the first at 250 nm and the other at 340 nm. The fluorescence spectra revealed a band between 360 nm and 500 nm, with a maximum around 410 nm.

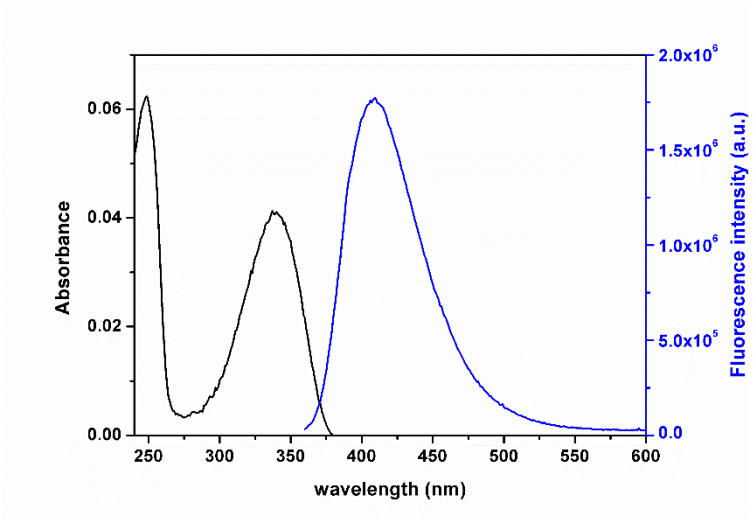


Figure 2. Absorption and fluorescence emission (excitation at 340 nm) spectra of compound **3b** in ethanol (1×10^{-5} M for absorption and 1×10^{-6} M for emission).

Compound **3b** was encapsulated into nanoliposomes of egg lecithin/cholesterol (Egg-PC:Ch 7:3), aiming at allowing an effective release of the loaded compound. A high encapsulation efficiency of 97.1% was obtained, showing that derivative **3b** can be efficiently encapsulated into the nanoliposomes. The structural characterization of the prepared loaded nanoliposomes was performed by DLS, by measuring their hydrodynamic diameter, polydispersity index and zeta potential. As expected, liposomes with size of 82.4 ± 1.3 nm, PDI of 0.16 ± 0.02, and zeta potential of −4.75 ± 1.28 mV were obtained, similarly to previous results obtained for the same formulation loaded with other compounds [11].

The release kinetic profile of compound **3b** from the nanoliposomes was determined during 24 h and the obtained experimental data is displayed in Figure 3. The data of the release profile was fitted to two kinetic models, the Weibull model and the first-order model, and the obtained parameters are summarized in Table 1.

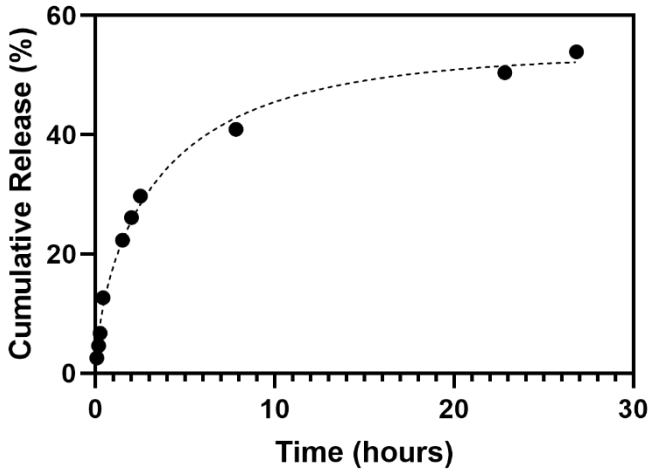


Figure 3. Cumulative release of compound **3b** from nanoliposomes of Egg-PC:Ch, fitted to the Weibull model.

Table 1. Parameters obtained by fitting the release profile to the first-order kinetic model and Weibull model, and the respective coefficients of determination (R^2).

First-order		Weibull		
K *(s⁻¹)*	R^2	*b*	*a*	R^2
0.38	0.97	0.41	0.67	0.99

A high release percentage of 54% was achieved, after 26 h, indicating that the Egg-PC:Ch formulation is suitable for the efficient release of compound **3b.** The Weibull model fitted better the experimental results, showing a higher coefficient of determination (Table 1). From the Weibull model fit, a Fickian diffusion is expected, as the *b* value is below 0.75. Despite not so good, the first-order model also fits well the data, allowing the determination of a rate constant of 0.38 s^{-1}.

3. Material and Methods

3.1. Typical Procedure for the Preparation of Compounds 3a-d (illustrated for 3b).

To a solution of 2-aminobenzoic acid **1b** (0.200 g, 1.46 mmol) in acetonitrile (3 mL), cesium carbonate (2.38 g, 7.30 mmol) and 1-bromo-3-chloropropane **2** (0.173 mL, 2.56

mmol) were added. The reaction mixture was stirred for 25 h at 60 ºC, and was monitored by TLC (silica: dichloromethane/light petroleum ether 9:1). 3-Chloropropyl 2-aminobenzoate **3b** was obtained as a brown oil (0.098 g, 32%). Rf = 0.74 (dichloromethane). ^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 2.22 (quint, *J* = 6.0 Hz, 2H, $OCH_2CH_2CH_2Cl$), 3.70 (t, *J* = 6.4 Hz, 2H, $OCH_2CH_2CH_2Cl$), 4.35 (t, *J* = 6.4 Hz, 2H, $OCH_2CH_2CH_2Cl$), 5.76 (s broad, 2H, NH_2), 6.63 – 6.68 (m, 2H, H-3 and H-5), 7.28 (dt, *J* = 8.8 and 1.6 Hz, 1H, H-4), 7.85 (d, *J* = 8.0 and 1.6 Hz, 1H, H-6) ppm. ^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 31.65 ($OCH_2CH_2CH_2Cl$), 41.31 ($OCH_2CH_2CH_2Cl$), 60.86 ($OCH_2CH_2CH_2Cl$), 110.36 (C-1), 116.10 (C-3), 116.61 (C-5), 130.95 (C-6), 134.11 (C-4), 150.52 (C-2), 167.75 (C=O) ppm.

3.2. Biological Assays of Aminobenzoic Acid Derivatives 3a-d

The potential of compounds **3a**-**d** was evaluated as biopesticides in assays using the *Sf9* (*Spodoptera frugiperda*) insect cell line. Cells were maintained at 28 °C and cultivated in Grace's medium with 10% FBS. For the evaluation of viability, cells were plated at 3.0×10^4 cells/well and exposed to the molecules, after which resazurin was added, resulting being read at 560/590 nm after 60 min. of incubation.

3.3. Nanoencapsulation and Release Studies of Compound 3b

The ethanolic injection method was used for the preparation of nanoliposomes loaded with compound **3b** [12]. Liposomes of 1,2-diacyl-sn-glycero-3-phosphocholine from egg yolk (egg phosphatidylcholine, Egg-PC) and cholesterol (Ch) (70% Egg-PC and 30% Ch) were used [10]. Briefly, an ethanolic solution of Egg-PC:Ch (7:3) and compound **3b** was injected, drop-by-drop, into an aqueous solution, under vortexing. For determination of the encapsulation efficiency, EE (%), equation 1 was used,

$$EE(\%) = \frac{C_{total} - C_{non-encapsulated}}{C_{total}} \times 100 \quad (1)$$

where c_{total} is the compound concentration used for the preparation of liposomes and $c_{non\text{-}encapsulated}$ is the compound concentration that was not encapsulated into the nanoliposomes. The separation of the compound-loaded liposomes from the non-encapsulated compound was performed using Amicon® Ultra centrifugal filter units of 100 kDa, by centrifugation at 3000 rpm, during 10 min. The emission of the non-encapsulated compound (the filtrate part) was measured for the determination of its concentration, using a previously obtained calibration curve of fluorescence intensity vs. concentration.

The release kinetic profiles of compound **3b** from the nanoliposomes were obtained using Amicon® centrifugal filters, in which the upper compartment was filled with the **3b**-loaded nanoliposomes and the bottom with water. For determination of the cumulative release, aliquots of 200 μL were collected from the bottom part of the Amicon®, and replaced with an equal volume of water, during 24 h. The concentration of released compound was determined by measuring the emission of the aliquots, and the experimental data was fitted to the Weibull model [13] and first-order model [14]. The Weibull model expresses the compound fraction accumulated (m) in solution at time t, following equation 2,

$$m = 1 - e^{\left[-(t-T_i)^b/a\right]} \quad (2)$$

where a defines the timescale of the process, Ti is a location parameter representing the latency time of the release mechanism, and b parameter denotes the curve type shape. For $b > 1$, the transport follows a complex release mechanism; $b \leq 0.75$ indicates Fickian diffusion (in either fractal or Euclidian spaces), and $0.75 < b < 1$ indicates a combined mechanism (Fickian diffusion and Case II transport).

The first-order model follows equation 3, in which F(%) and M_0 are the percentage and the total amount of compound released, respectively, k represents the first-order rate constant, and t is time.

$$F(\%) = M_0 \times (1 - e^{-kt}) \quad (3)$$

The emission spectra were collected in a Fluorolog 3 spectrofluorometer (HORIBA Jobin Yvon IBH Ltd., Glasgow, UK), and the UV-Vis absorption spectrum was obtained in a Shimadzu UV-3600 Plus UV-Vis-NIR spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The structural characterization of the nanoliposomes was performed by Dynamic Light Scattering (DLS), using a Litesizer 500 equipment from Anton Paar (Anton Paar GmbH, Graz, Austria) with a solid-state laser of 648 nm and 40 mW. For the hydrodynamic diameter, polydispersity index, and zeta potential, three independent measurements were performed.

4. **Conclusions**

In the present work, four 3-chloropropylaminobenzoate derivatives were synthesized and used in biological studies against the *Sf9* cell line, with the aim of evaluating their potential as insecticides.

The encapsulation in liposomes of Egg-PC:Ch allowed a high encapsulation efficiency and an effective release of the most active compound **3b**, being a suitable formulation for this potential insecticide.

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