Inhibition of Acetylcholinesterase by Coumarin-Linked Amino Acids Synthesized via Triazole Associated with Molecule Partition Coefficient

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A previous study for the identification of acetylcholinesterase (AChE) inhibitors demonstrated that the hybrid between tyrosol, the 1,2,3-triazole nucleus, and the coumarin group, namely 7-({1-[2-(4-hydroxyphenyl)ethyl]-1H-1,2,3-triazol-4-yl}methoxy)-4-methyl-2H-chromen-2-one (10), has a high enzyme inhibitory activity. Here, we synthesized analogues of 10 via triazole with pharmacophoric groups represented by tyrosine, phenylalanine, tryptophan, and glycine in addition to evaluating the impact of coumarin-linked amino acids on AChE inhibition. We obtained eight triazoles, six of which are undescribed. In general, the presence of carboxylic acid decreased the inhibitory activity, while aromatic amino acids increased enzymatic inhibition compared to glycine. The derivative containing tyrosine, structurally most similar to 10, presented the lowest inhibition percentage, indicating that phenolic hydroxyl is not the preponderant factor for inhibition. Molecular docking was not enough to explain in vitro experiments. On the other hand, MlogP (logP calculated by the Moriguchi method) was related positively to enzymatic inhibition. To increase the hydrophobicity of the molecules, we tested the esterified triazole derivatives comparatively with the enzyme. The compound ethyl 2-(((4-methyl-2-oxo-2H-chromen-7-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)acetate (6) presented an increment of inhibitory activity of 46.97 ± 1.75% at 100 μmol L⁻¹. We also associated the best activity with the lowest van der Waals volume and molar mass values.

Keywords: amino acid, 1,2,3-triazoles, acetylcholinesterase, coumarin

Introduction

Coumarins are a large class of compounds naturally found in plants, bacteria, and fungi that display a wide range of interesting biological properties. These biological activities are antioxidant, anticancer, antimicrobial, anti-leishmanial, anti-tuberculosis, and therapeutic effects against Alzheimer’s disease. These compounds are known to make noncovalent interactions, such as hydrophobic, π-type, and hydrogen bonding. The capacity to bind to multiple targets, including amino acid residues, makes coumarin derivatives promising in the development of new drugs and agrochemicals.

In this context, molecular hybridization, a combination of biologically active molecules, has been shown to be an encouraging strategy to increase biological targets. Association between 2H-chromen-2-one core and the 1,2,3-triazole moiety has shown more pharmacologically active coumarins. Within the universe of click chemistry, the synthesis of 1,2,3-triazoles has been studied as a method for obtaining new compounds. This pharmacophoric
group is propitious because it presents several biological activities such as antimicrobial, antiprotozoal, antitumor, and enzyme inhibition, as is the case with elastase and acetylcholinesterase (AChE). For example, Moradi et al. synthesized a new series of coumarin and benzylamine hybrids, linked via the triazole ring, and evaluated the compounds as inhibitors of AChE and butyrylcholinesterase (BChE). Among the compounds, 7-((1-(3-methoxybenzyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one was the most active, presenting half maximal inhibitory concentration (IC$_{50}$) values of 3.4 and 1.1 μmol L$^{-1}$ for AChE and BChE, respectively.

In previous works, our research group was involved in the investigation of derivatives containing the 1,2,3-triazole nucleus. Tyrosol-derived triazoles, a tyrosine analogue biophenol, were evaluated as AChE inhibitors. The compound containing the coumarin group, namely 7-(1-[2-(4-hydroxyphenyl)ethyl]-1H-1,2,3-triazol-4-yl) methoxy)-4-methyl-2H-chromen-2-one (10) exhibited a high inhibitory effect, reducing 67.8 ± 5.8% of enzyme activity at 50 μmol L$^{-1}$. Amino acids perform different functions in the organism. Among these macromolecules stands out the tyrosine, which is a synthetic precursor of tyrosol. The association between amino acids and organic molecules is interesting because their association can qualitatively enhance some physicochemical properties, such as solubility, stability, and toxicity. Furthermore, amino acids have been reported in the development of prodrugs due to their propensity to be transported across biological membranes, increasing bioavailability. Hybrids between coumarins and amino acid have already been reported to have enzymatic inhibition capabilities and antimicrobial activity. However, the use of amino acids in organic chemistry is still scarce due to the difficulties of working with polar molecules while using typically nonpolar solvents.

In this present study, it was proposed the esterification of amino acids as a way of increasing the carbon chain and, consequently, the solubility in organic solvents. The last stage of the synthesis was the hydrolysis of the esters to obtain coumarin-linked amino acids. Thus, considering that the associated coumarin derivatives and triazole rings exhibit several biological properties, we reported on the synthesis of a new series hybrids of containing 4-methylumbelliferone, 1,2,3-triazole moiety, and amino acids, esterified or not (Figure 1). In vitro studies were also performed in the presence of AChE to determine their

![Figure 1](synthetic_steps_involved_in_the_preparation_of_triazole-derived_azide_amino_acids.png)

Figure 1. Synthetic steps involved in the preparation of triazole-derived azide amino acids. Reagents and conditions: (i) anhydrous dichloromethane (DCM), thionyl chloride (SOCl$_2$, 1.2 eq.), ethanol (4.8 eq.), rt, 30 min, 83% yield; (ii) sodium azide (2.0 eq.), dimethyl sulfoxide (DMSO), rt, 2 h; (iii) sodium azide (5.0 eq.), DCM/H$_2$O (2:1 v/v), rt, 2 h; (iv) 20% (v/v) ethanolic H$_2$SO$_4$ solution (60 °C, 96-98% yield range; (v) NaHCO$_3$ (10.0 eq.), CuSO$_4$·5H$_2$O (0.10 eq.), DCM/H$_2$O/methanol (2:1:1 v/v), rt, 24 h; (vi) HBr 48% (m/m), 70-80 °C, 70% yield; (vii) sodium azide (1.5 eq.), dimethylacetamide (DMAC), rt, 4-5 h; (viii) propargyl bromide (1.5 eq.), anhydrous K$_2$CO$_3$ (2.0 eq.), anhydrous acetonitrile, 50 °C, 24 h; (ix) CuSO$_4$·5H$_2$O (0.20 eq.), sodium ascorbate (0.40 eq.), ethyl ether (1), or ethyl acetate (2 and 4) or DCM (3)/H$_2$O (8:1 v/v), rt, 14 h, 58-93%; (x) 20% m/v NaOH solution (5 mL), ethanol, rt, 3 h, 57-98% yield range.
Results and Discussion

Amino acid-derived organic azides were chosen for molecular hybridization with propargylated 4-methylumbelliferone (5) to produce a series of derivatives containing a triazole ring as linker (6-9, Figure 1). The aromatic amino acid-derived azides, obtained from tyrosine, phenylalanine, tryptophan esterified (2b-2d) were used due to the similarity to tyrosol. Azides from these monomers were obtained for a diazo transfer reaction (v), with the configuration retention, using trifluoromethanesulfonyl azide (TfN₃). In addition, to verify the influence of the aromatic portion on the cholinesterase action of the compounds, we promote the synthesis of a derivative without this portion (glycine analogues, 6 and 11). For such, the ethyl 2-chloroacetate (2a) was utilized to produce the glycine analog-derived azide (3a). Concomitantly to azides production, the propargylation reaction of 4-methylumbelliferone (viii) was performed, producing the alkyne, 7-hydroxy-4-methyl-2H-chromen-2-one (5), in 77% yield. Finally, triazoles (6-10) were synthesized from azides (3a-3e) and alkylene 5 via copper(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC) in yields ranging from 58 to 93%. All triazolic derivatives were characterized through infrared (IR) and nuclear magnetic resonance (¹H and ¹³C NMR) spectroscopies as well as high-resolution mass spectrometry (HRMS), and specific rotation,  with Kumari et al. and 6 are in accordance with tyrosol. The characterization of 1 H NMR spectra, the signals of hydrogen atoms at the 1.18-1.29 and 1.29-4.26 range are absent, confirming hydrolysis. The carbon and hydrogen chemical shifts in ¹C and ¹H NMR are consistent with the compound structures.

Molecular docking

Molecular docking was used to predict intermolecular interactions between the triazoles and acetylcholinesterase. The docking analysis was carried out with five compounds (10-14). We choose the enzyme Torpedo californica AChE to compare with data from Bousada et al. The energies for the best molecular orientation for the interactions between the ligands and the enzyme are presented in Table 1.

Table 1. Interaction affinity energy of the ligands for the molecular arrangements with lowest energies

| Compound | Interaction affinity energy / (kcal mol⁻¹) |
|----------|------------------------------------------|
| 10 (reference) | -12.0 |
| 11 (t-GlyCOOH) | -10.2 |
| 12 (t-TyrCOOH) | -11.5 |
| 13 (t-PheCOOH) | -11.1 |
| 14 (t-TrpCOOH) | -8.8 |

Molecular docking was also used to assess how many different positions each ligand can occupy at the catalytic site and which amino acids they interact with. The active site of the Torpedo californica acetylcholinesterase is composed of Ser200, His440, and Glu327 (catalytic triad); Trp84, Tyr130, Phe330, and Phe331 (anionic site); Phe288 and Phe290 (acyl pocket); Gly118, Gly119, and Ala201 (oxyanion hole); Asp72, Tyr70, Tyr121, Trp279, and Tyr334 (peripheral anionic site-PAS).

Ligand-receptor interactions for triazoles are demonstrated in Figure 2 as well as the pharmacophoric map of compound 10. All molecules interact with many amino acid active sites, including Phe330. Furthermore, amino acid-derived triazoles and 10 exhibited different interactions with the PAS portion, related to the binding of many inhibitors.

The molecular docking revealed that the compounds present affinities for the Torpedo californica acetylcholinesterase similarly to 10, and most often outnumber the amino acid interactions at the catalytic site when compared to 10. For example, (12) presented a conventional hydrogen bond with Asn85 and Ser122, which can hinder substrate-enzyme interaction (Figure 2). So, we decided to test the amino acid-derived triazoles against acetylcholinesterase in vitro.
Enzymatic inhibition assay

The acetylcholinesterase inhibition assay was initially performed with the triazole derivatives (11-14) as well as compound 10. The experiments were performed with compounds at the concentrations of 50, 100, and 200 μmol L\(^{-1}\). The percentages of inhibition of AChE by 10 are in accordance with the results obtained by Bousada et al.\(^{32}\)

Triazoles derived from aromatic amino acids were more active against acetylcholinesterase when compared to t-GlyCOOH (11), which inhibited only 15.84 ± 1.51%
at 200 μmol L⁻¹ (Table 2). Thus, the presence of aromatic amino acids associated with the coumarin nucleus via triazole tends to increase enzymatic inhibition in relation to aliphatic amino acids. This fact corroborates our initial hypothesis.

The presence of tryptophan and phenylalanine in triazoles formed inhibitors with higher potential than tyrosine. Compound 14 (t-TrypCOOH) inhibited 29.33 ± 1.45% of enzymatic activity, followed by 13 (t-PheCOOH), with 21.32 ± 1.90%, and 12 (t-TyrCOOH), with 17.78 ± 0.84% at 100 μmol L⁻¹. So, compound 12, the structurally closest to 10, presented the lowest inhibition percentage, indicating that the presence of phenolic hydroxyl is not the preponderant factor for inhibition. Derivative 10 reached 72.38 ± 1.11% inhibition, while t-TyrCOOH reached only 23.77 ± 3.10%. The carboxylic acid group present in tyrosine seems to decrease enzymatic inhibition when connected to the coumarin nucleus via triazole.

Table 2. Influence of amino acids bound to the coumarin nucleus via triazole against the enzymatic activity of acetylcholinesterase (Electrophorus electricus, type VI). Enzyme inhibition percentage at 50, 100, and 200 μmol L⁻¹, concentrations of each compound with their respective standard deviations. Different letters were used to compare the significance between compounds at the same concentration (p < 0.05 according to analysis of variance (ANOVA) followed by Tukey’s post-test)

| Compound     | Inhibition / % |
|--------------|---------------|
|              | Concentration / (μmol L⁻¹) |
|              | 50  | 100  | 200  |
| 10 (reference) | 63.94 ± 3.08  | 65.90 ± 1.13  | 72.38 ± 1.11  |
| 14 (t-TrypCOOH) | 17.25 ± 1.56  | 29.33 ± 1.45  | 43.26 ± 1.43  |
| 13 (t-PheCOOH) | 12.66 ± 2.19  | 21.32 ± 1.90  | 34.13 ± 3.54  |
| 12 (t-TyrCOOH) | 10.20 ± 1.00  | 17.78 ± 0.84  | 23.77 ± 3.10  |
| 11 (t-GlyCOOH) | 4.63 ± 0.87   | 8.06 ± 1.11   | 15.84 ± 1.51  |
| Galantamine   | 89.2 ± 1.0    | 89.2 ± 1.0    | 89.2 ± 1.0    |

*Galantamine was used as a positive control.

From these results, a question was raised: why amino acid-derived triazoles do not show inhibition against acetylcholinesterase comparable to 10 even while interacting in high affinity with the enzyme? This occurs because molecular docking is a computational simulation which assumes that there will be an interaction between a ligand and receptor, therefore, the simulation attempts to make it happen. In that regard, some parameters are not taken into account in bioinformatics analysis, like solubility. The in vitro activities are realized in methanol and water, influencing the migration of the inhibitors to the active center of the enzyme. Besides this, the docking assay does not consider the different conformations that the enzyme may have, regarding it as a rigid structure.

In short, differences between biological assays and docking results can be associated with the physicochemical characteristics of the compounds. In that regard, the compounds were evaluated for the possible structure-activity relationship. We evaluate three characteristics: (i) MlogP (logP calculated by the Moriguchi method), (ii) van der Waals volume, and (iii) molar mass (Figure 3).

The MlogP may be related to inhibitor interactions within hydrophobic amino acids in the enzyme. This idea is supported by the fact that the anionic site and the PAS site, related to the binding of many inhibitors, are formed by 100 and 80% of aromatic amino acids, respectively. The AChE anionic site is of paramount importance because the proper orientation of acetylcholine in the gorge. The aromatic character of the gorge might contribute to the high catalytic activity. The aromatic lining can be associated with initial absorption of ligand to low-affinity sites, followed by rapid diffusion to the active site. Besides, the anionic and hydrophobic subsites confer specificity for alkyl (R) groups and stabilize the methyl group of the acetylcholine.
The high activity of compound 10 against acetylcholinesterase can be associated with the greatest hydrophobicity. To confirm this, we decided to verify whether increasing MlogP values would increase the inhibitory activity against acetylcholinesterase. With that, we also tested the hypothesis that the presence of carboxylic acid in the derivatives decreases this activity. We tested the esterified triazole derivatives against the enzyme. Due to solubility in methanol compounds, concentrations were 50 and 100 μmol L⁻¹. MlogP, van der Waals volume, and molar mass values are presented in Table 3.

Only half of the compounds increased their activities compared to their analogues. The compounds t-PheCOOEt (8) and t-GlyCOOEt (6) were 60 and 480% more active than t-PheCOOH (13) and t-GlyCOOH (11) at 100 μmol L⁻¹, respectively (Figure 4). The other triazoles decreased their inhibitory activities against acetylcholinesterase when acetylated.

We believe that the increase in the physicochemical parameters could have influenced the inhibitory activity, both positively and negatively. In fact, the increase in MlogP resulted in an enhancement of the inhibitory activity of t-GlyCOOEt (6) and t-PheCOOEt (8), but this positive correlation was not observed for molecules derived from tryptophan (9) and tyrosine (7). Between esterified amino acids, triazoles derived from phenylalanine (8) and glycine (6) presented the lowest van der Waals volume and molar mass values compared to the other esterified compounds. t-GlyCOOEt (6) exhibited van der Waals volume and molar mass approximately 28 and 26% less than t-PheCOOEt (8), respectively. The low values for these parameters represent greater degrees of conformational freedom for structures within the enzymatic active site. Therefore, the substrate enzyme interaction is more likely to be optimized since more conformational possibilities are allowed. On the other hand, although the esterification of t-TyrCOOH (12) and t-TrypCOOH (14) also resulted in an increase of MlogP, they were accompanied by the increase of the others parameters as well, such as Waals volume and molar mass. These parameters may have breached a limiting value which permits optimal interaction with the enzyme, thus reducing their activities.

**Table 3.** Comparison between MlogP (logP calculated by the Moriguchi method), van der Waals volume, and molar mass values of triazoles derived from esterified (6-9) and non-esterified (11-14) amino acids

| Compound   | MlogP | van der Waals volume / Å³ | Molar mass / (g mol⁻¹) |
|------------|-------|---------------------------|-----------------------|
| 9 (t-TrypCOOEt) | 2.721 | 649.51 | 472.49 |
| 8 (t-PheCOOEt) | 2.176 | 609.43 | 433.46 |
| 7 (t-TyrCOOEt) | 1.705 | 620.79 | 449.46 |
| 6 (t-GlyCOOEt) | 0.825 | 474.97 | 343.33 |
| 14 (t-TrypCOOH) | 2.315 | 579.86 | 405.40 |
| 13 (t-PheCOOH) | 1.750 | 539.77 | 444.44 |
| 12 (t-TyrCOOH) | 1.279 | 550.90 | 421.40 |
| 11 (t-GlyCOOH) | 0.332 | 405.65 | 315.28 |

MlogP, van der Waals volume and molar mass were calculated by the MedChem Designer® 3.1.0.30, Marvin Sketch® 17.28.0 and ChemDraw Ultra® 12.0.2.1076, respectively.

**Conclusions**

Once the biological activities of the coumarin nucleus were reported, and once our research group discovered the inhibitory activity of AChE by triazoles...
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Inhibition between the esterified (6) and non-esterified (11) forms, 46.97 ± 1.75%, and 8.06 ± 1.11%, at 100 μmol L⁻¹, respectively. The physicochemical parameters related to molecular spatial dimension were the ones that best explained the relationship between structure and activity. These findings indicate the bioconjugation between the amino acid, the coumarin, and the triazole can be considered an important foundation for further studies on AChE inhibition.

Experimental

General procedure

All reagents and solvents were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) and Vetec (Duque de Caxias, Rio de Janeiro, Brazil) and used without prior purification. Column chromatography was performed over silica gel 60 (70-230 mesh, Macherey-Nagel, Düren, Germany) as a stationary phase using a mixture of hexane/ethyl acetate as eluents. Analytical thin-layer chromatography (TLC) was performed on silica gel plates (Macherey-Nagel DC-Fertigfolien ALUGRAM® Xtra SIL G/UV254, Düren, Germany). ¹H NMR (300 MHz) and ¹³C NMR (75 MHz) experiments were performed on a Bruker 300 MHz equipment (Billerica, Massachusetts, USA) using CDCl₃, dimethyl sulfoxide (DMSO-d₆), and (CD₃)₂CO as solvents. The spectra were referenced using the solvent residual signals. ¹H NMR data are presented as follows: chemical shift (δ) in ppm, multiplicity, the number of hydrogens, and coupling constant (J) values in hertz (Hz). Multiplicities are shown as the following abbreviations: s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet).

Melting temperature (mp) ranges were determined in a GladiATR apparatus (Varian, Palo Alto, California, USA). HRMS were carried out on a high-resolution Q-TOF (quadrupole-time of flight) mass spectrometer Impact II geometry (Bruker Daltonics Corporation, Bremen, Germany) equipped with an electrospray ionization source (ESI). Samples were directly injected into the ESI source using a syringe pump (KDS Legato 100, KD Scientific, Holliston, Massachusetts, USA) at a flow rate of 10 μL min⁻¹. The capillary voltage was operated in positive ion mode, set at 4000 V with an end plate offset potential of −500 V. The dry gas parameters were set to 8 μL min⁻¹ at 180 °C with a nebulization gas pressure of 0.4 bar. Data were collected in the range of 50 to 800 m/z in continuous acquisition mode. The mass spectra were obtained in the Data Analysis program 4.3 (Bruker Daltonics Corporation, Bremen, Germany).

Values of rotation angle (α) were measured on a polarimeter ADP220, serial no. PF05050 (Bellingham+Stanley Ltd., Tunbridge Wells, Kent, UK) using sodium as a monochromatic light source (λ = 598.3 nm) at 25 °C and an optical path of 0.5 decimeters. The final concentration (c) of the solutions was calculated in g 100 mL⁻¹.

Synthesis of azides

Ethyl 2-azideacetate (Gly-N₃, 3a) preparation

Azide 3a was obtained from ethyl 2-chloroacetate (2a) which, in turn, was produced from esterification of chloroacetic acid (1a). To produce 2a, 5.32 mmol of chloroacetic acid and 10 mL of anhydrous dichloromethane (DCM) were transferred to a round-bottom flask on ice bath. Then, 6.38 mmol (1.2 equiv.) of thionyl chloride were added dropwise and the mixture was kept under magnetic stirring for 5 min. After this period, 1.5 mL of ethanol were added and the solution was stirred for 30 min. At the end (as observed by TLC), the reaction was quenched by adding 5 mL of distilled water, and the obtained solution was washed with 3 times with 5 mL of diethyl ether. The organic phases were combined, dried over anhydrous magnesium sulfate, filtered, and concentrated on a rotary evaporator. Compound 2a was obtained in 83% yield with no need for additional purification.

For the synthesis of 3a, 2a (2.04 mmol), sodium azide (4.08 mmol, 2 equiv.) and 2 mL of dimethyl sulfoxide (DMSO) were transferred to a round-bottomed flask and the mixture was kept under stirring for 12 h. After this time, 5 mL of saturated NaHCO₃ solution were added and the obtained solution was extracted with diethyl ether (3 × 5 mL). The organic phases were combined, dried over
anhydrous magnesium sulfate, filtered, and concentrated on a rotary evaporator.\textsuperscript{53} Compound 3a was obtained in 74\% yield as a transparent oil without further purification.

General procedure for the preparation of (S)-ethyl 2-azido-3-(4-hydroxyphenyl)propanoate (3b), (S)-ethyl 2-azido-3-phenylpropanoate (3c) and (S)-ethyl 2-azido-3-(1H-indol-3-y1)propanoate (3d)

Tyr-N\textsubscript{3} (3b), Phe-N\textsubscript{3} (3c), and Tryp-N\textsubscript{3} (3d) azides were synthesized by reacting TfN\textsubscript{3} (2) with tyrosine, tryptophan, and esterified phenylalanine, respectively. Firstly, TfN\textsubscript{3} was prepared \textit{in situ} from trifluoromethanesulfonic anhydride (Tf\textsubscript{2}O, 1) according to a reaction procedure described by Robillard \textit{et al.}\textsuperscript{54} and used in solution only.\textsuperscript{55} For TfN\textsubscript{3} synthesis, Tf\textsubscript{2}O (0.48 mmol) and 2 mL DCM were combined, dried, filtered, and concentrated under reduced pressure with ethyl acetate (3 × 5 mL). The organic phases were worked-up using ethyl acetate (3 × 5 mL), dried, filtered and concentrated to a volume of 3 mL under reduced pressure to obtain the triflylamino-transfer solution.

The amino acid esterifications were performed according to the methodology proposed by Laulloo\textsuperscript{56} with modification. Briefly, sulfuric acid (200 μL) was added dropwise to a solution of each amino acid (0.28 mmol) in ethanol (1.8 mL), the reaction was heated at 60 °C for 24 h, neutralized with saturated Na\textsubscript{2}CO\textsubscript{3} solution and extracted with ethyl acetate was added, filtered and concentrated under reduced pressure. Compounds 2b to 2d were obtained in 96-98\% yield.

Finally, the productions of the azides, 3b-3d, were performed by transferring the azido group of TfN\textsubscript{3} to the amino acid ethyl esters (2b-2d). In a round-bottom flask, 3 mL of TfN\textsubscript{3} solution in DCM were added dropwise to a solution of the corresponding amino acid ester (0.24 mmol), NaHCO\textsubscript{3} (2.4 mmol), CuSO\textsubscript{4}.5H\textsubscript{2}O (0.024 mmol) in 2 mL of distilled water and methanol 1:1 (v/v). The reaction was kept for 24 h at room temperature and then extracted with ethyl acetate or DCM (3 × 5 mL), dried, filtered, and concentrated to 2 mL for subsequent use in the respective triazole synthesis.\textsuperscript{54}

\textbf{4-(2-Azidoethyl)phenol (3e) preparation}

Azide 3e was obtained from 4-(2-bromoethyl)phenol (2e), which was previously prepared through bromination of tyrosol according to the methodology described by Bousada \textit{et al.}\textsuperscript{52} Briefly, to produce 2e, 2.49 mmol of tyrosol and 25 mL of 48\% HBr (m/m) were added to a reaction flask and kept to react under stirring for 17 h at 70-80 °C. Then, the reaction mixture was extracted with DCM (4 × 20 mL); the organic phases were combined, dried over anhydrous MgSO\textsubscript{4}, filtered, and concentrated on a rotary evaporator. The solid residue was subjected to column chromatography on silica gel eluted with hexane/ethyl acetate (5:1 v/v), affording compound 2e, in the form of a white solid in 68\% yield. Then, compound 2e (0.55 mmol), sodium azide (0.55 mmol), and 1 mL of dimethylacetamide (DMAC) were allowed to react in a round-bottomed flask under stirring for 5 h at room temperature. The reaction was then worked-up using ethyl acetate (3 × 5 mL), dried, and filtered to afford a solution of 3e (2 mL in DCM).

\textbf{Coumarin propargylation}

Synthesis of the alkyne (5) was performed by propargylation of coumarin (4) according to the method reported by Bousada \textit{et al.}\textsuperscript{52} For this, anhydrous potassium carbonate (2 mmol) was added over a solution of 7-hydroxy-4-methylcoumarin 4 (1 mmol) in anhydrous acetonitrile (2 mL) in a round-bottomed flask. The mixture was kept under stirring at room temperature and under nitrogen atmosphere. After 5 to 10 min, 165 μL of propargyl bromide solution in toluene (80% m/m, 1.5 mmol) was added and the mixture was kept under stirring at 50 °C for 24 h. After cooling to room temperature, acetonitrile was evaporated; ethyl acetate was added, filtered and concentrated under reduced pressure.

4-Methyl-7-(prop-2-yn-1-yl)-2H-chromen-2-one (5)

Beige solid; yield 77\%; Rf (hexane/EtOAc 1:1) 0.70; mp 131.5-133.1 °C; IR (ATR) ν / cm\textsuperscript{-1} 3302, 2140, 1717, 1605, 1261, 1011; 1 H NMR (300 MHz, CDCl\textsubscript{3}) δ 2.39 (d, 3H, J 1.1 Hz), 2.57 (t, 1H, J 2.4 Hz), 4.75 (d, 2H, J 2.4 Hz), 6.14 (d, 1H, J 1.1 Hz), 6.90-6.94 (m, 2H), 7.51 (d, 1H, J 9.5 Hz); 13 C NMR (75 MHz, CDCl\textsubscript{3}) δ 161.2; HRMS (ESI) m/z 215.0703, found: 215.0712.

General procedure for the preparation of triazoles

Coumarin-triazole-amino acid esters hybrids (6-10) were produced by reacting propargylated coumarin (5) with amino acid esters according to the methodology described by Kumari \textit{et al.}\textsuperscript{42} with modifications.

Alkyne 5 (1.0 eq.), sodium ascorbate (0.40 eq.) and Cu\textsubscript{5}SO\textsubscript{4}.5H\textsubscript{2}O (0.20 eq.) were transferred to a round-
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Ethyl 2-(4-(((4-methyl-2-oxo-2H-chromen-7-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)acetate (6)

White solid; yield 86%; Rf (hexane/EtOAc 2:1) 0.40; [α]D25.5°+29.2 (c 1.3, CHCl3); IR (ATR) ν/cm−1 3272, 1689, 1611, 1529, 1430, 1365, 1232, 1146, 1141, 1106, 1059; 1H NMR (300 MHz, CDCl3) δ 1.18 (t, 3H, J 7.3 Hz), 2.35 (s, 3H), 3.33 (d, 2H, J 7.2 Hz), 4.19 (q, 2H, J 7.0 Hz), 5.18 (d, 2H, J 7.2 Hz), 5.49 (t, 1H, J 7.2 Hz), 6.11 (s, 1H), 6.60 (d, 1H, J 2.5 Hz), 6.63 (d, 2H, J 8.6 Hz), 6.72 (d, 1H, J 8.6 Hz), 6.88 (dd, 2H, J 8.8, 2.5 Hz), 7.45 (d, 1H, J 8.8 Hz), 7.52 (s, 1H); 13C NMR (75 MHz, CDCl3) δ 14.1, 18.8, 29.7, 62.5, 62.6, 64.3, 101.7, 111.9, 113.7, 114.0, 116.0, 123.3, 125.8, 130.0, 142.7, 153.5, 154.8, 155.8, 161.3, 162.1, 168.2; HRMS (ESI) m/z, calcd. for C17H17N3O5 [M + H]+: 344.1241, found: 344.1229.

(S)-Ethyl 3-(1H-indol-3-yl)-2-(4-(((4-methyl-2-oxo-2H-chromen-7-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)propanoate (9)

Beige solid; yield 27%; Rf (hexane/EtOAc 1:1) 0.17; mp 181.0-182.6 °C; IR (ATR) ν/cm−1 3500-3000, 2926, 1705, 1608, 1188; 1H NMR (300 MHz, CDCl3) δ 1.19 (t, 3H, J 7.1 Hz), 2.32 (d, 3H, J 1.1 Hz), 3.59-3.69 (m, 2H), 4.17 (q, 2H, J 7.1 Hz), 5.14 (s, 2H), 5.61-5.64 (m, 2H), 6.09 (d, 1H, J 1.1 Hz), 6.70-6.71 (m, 1H), 6.85 (dd, 1H, J 8.8, 2.5 Hz), 7.02-7.13 (m, 2H), 7.29 (d, 1H, J 8.0 Hz), 7.40 (d, 1H, J 3.1 Hz), 7.42-7.43 (m, 1H), 7.70 (s, 1H); 13C NMR (75 MHz, CDCl3) δ 14.0, 18.8, 29.1, 31.7, 62.2, 63.5, 101.9, 108.5, 111.6, 112.1, 113.0, 113.3, 117.9, 119.7, 122.4, 123.4, 125.8, 126.7, 136.1, 142.8, 153.4, 154.9, 161.1, 161.7, 168.5; HRMS (ESI) m/z, calcd. for C25H24N4O5 [M + H]+: 473.1819, found: 473.1819.

General procedure for the synthesis of amino acid-coumarin derivatives

The synthesis of the hybrids of coumarin and amino acids using the triazole ring as linker (11-14) was carried out through hydrolysis of the corresponding amino acid esters derivatives 6-9 according to the methodology described by Kumari et al.42 To the triazole (0.23-0.32 mmol) and ethanol solution at 0 °C, a 20% (m/v) NaOH solution (5 mL) was added dropwise.
The reaction was stirred for 3 h at room temperature. The ethanol was completely concentrated under reduced pressure to afford the corresponding salt of the acid and the pH was adjusted to 1.0 using concentrated HCl to obtain the corresponding carboxylic acid. The aqueous layer was extracted with ethyl acetate (3 × 5 mL). The organic layers were combined, dried over MgSO₄, filtered, and concentrated under reduced pressure.

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\text{2-((4-Methyl-2-oxo-2H-chromen-7-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (11)}
\]

Light beige solid; yield 57%; RF (methanol) 0.0; mp 110.5-111.2 °C; IR (ATR) ν / cm⁻¹ 1387, 1347, 1301, 1239, 1148, 1120, 1038, 746; ¹H NMR (300 MHz, DMSO-d₆) δ 3.86 (s, 3H), 4.15 (s, 1H), 6.40-6.42 (m, 1H), 6.77 (d, 1H, J = 1.1 Hz), 6.84 (d, 1H, J = 2.5 Hz), 6.90-6.92 (m, 1H), 6.94-6.96 (m, 1H), 7.00 (d, 1H, J = 8.9 Hz), 7.01-7.03 (m, 2H), 7.11 (d, 1H, J = 2.5 Hz), 7.17 (d, 1H, J = 2.5 Hz), 7.26-7.29 (m, 1H), 7.48 (d, 1H, J = 8.9 Hz), 8.40 (s, 1H), 10.81 (s, 1H); ¹³C NMR (75 MHz, DMSO-d₆) δ 18.7, 51.2, 62.0, 102.0, 111.7, 113.1, 113.9, 126.9, 127.0, 129.3, 135.9, 151.5, 161.5, 162.2, 169.1; HRMS (ESI) m/z 364.3, 62.0, 64.3, 102.0, 111.8, 113.2, 113.8, 115.5, 125.6, 126.6, 126.9, 130.2, 142.0, 153.9, 155.1, 156.5, 160.7, 161.4, 170.3; HRMS (ESI) m/z, calcd. for C₁₂H₁₀N₄O₆ [M + H]⁺: 445.1506, found: 445.1504.

Molecular docking

Molecular docking was performed using AutoDockVina® 1.1.2³ software following Ferreira et al.⁴⁸ with adaptations. The receptor was the acetylcholinesterase of the species *Torpedo californica*, obtained from the protein database (Protein Data Bank, code: 6G1U, Galdeano et al., 1.79 Å) in PDB format. The receptor edition, including removal of water molecules, the addition of non-polar hydrogen atoms and calculation of the protein charges was done with AutoDockTools® software.⁵⁰ The file was converted to the PDBQT (Protein Data Bank, partial charge (Q), and atom type (T)) format.

Ligands, compounds developed in the present work, were drawn in Marvin Sketch® 17.28.0⁶ software, with all hydrogens shown. The files were saved in 3D in PDB format. PyRx® Python Prescription 0.8⁶ software was used to convert the files to the PDBQT format.

The possible inhibitors were anchored in the enzyme using the AutoDockVina® 1.1.2⁶ software. A rectangular base prism was created so that the ligands could interact throughout the protein adopting the non-directed docking strategy. The dimensions of the prism were 66 × 70 × 74 Å (axes x, y, and z, respectively), with center at x = −3.333, y = 2.167 and z = 20.917 Å. Pharmacophore maps were designed in BIOVIA Discovery Studio 2016 16.1.0.15350.⁶²

**AChE inhibition assay**

AChE (*Electrophorus electricus*, type VI, Sigma Aldrich, Saint Louis, Missouri, USA) inhibition evaluation was performed by spectrophotometric assay in a 96-well microplate (TPP, Trasadingen, Schaffhausen, Switzerland).

Initially, triazole compounds 6 to 9 were tested. The samples were prepared at the concentration of
Inhibition of Acetylcholinesterase by Coumarin-Linked Amino Acids

10000 μmol L⁻¹ (HPLC grade methanol, Tedia®, São Paulo, Brazil). These samples were subjected to serial dilution in buffer A (Tris-HCl 50 mmol L⁻¹, pH 8.0), obtaining intermediate solutions at the concentrations of 2000, 1000, and 500 μmol L⁻¹. The concentration of methanol in these samples was adjusted so that all of them had 20% (v/v) of this solvent. The assay was performed for three consecutive days, with a triplicate for each compound, including the controls.

The assays were performed according to the methodology proposed by Ellman et al. with modifications to decrease losses due to the solubility of the compounds. In microtubes, 200 μL of bovine serum albumin solution (0.1% BSA in Tris-HCl buffer), 100 μL of acetylthiocholine iodide solution in ultrapure water (14.5 mmol L⁻¹), 500 μL of 5,5'-dithiobis 2-nitrobenzoic acid solution (3 mmol L⁻¹ of DTNB in Tris-HCl buffer containing 10 mmol L⁻¹ of NaCl and 20 mmol L⁻¹ of MgCl₂) and 100 μL of the intermediate solutions were added. Negative control was performed using methanol, and both compound 10 and galantamine were used as positive controls. The tested concentrations of compounds were 200, 100, and 50 μmol L⁻¹, including the standard inhibitor 10. Galantamine as tested at 17 μmol L⁻¹.

Plate assembly was performed by removing a 225 μL aliquot from each microtube, in triplicate. The background was read at 405 nm wavelength and 30 ºC in a spectrophotometer (Thermoplate, model TP-reader). To perform the kinetic assay 25 μL (0.2 U mL⁻¹, 0.1% BSA in buffer A) of the enzyme AChE (Electrophorus electricus type VI) were added and the plate and were read (λ = 405 nm at 30 °C) every 5 min for 25 min. Inhibition of the enzymatic hydrolysis was calculated according to equation 1, from the difference in absorbance reading obtained in the background (without enzyme) and kinetic assay (20 min with enzyme).

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\text{Inhibition} \%(%) = 100 - \frac{[\text{Ab kinetic assay} - \text{Ab background}] \times 100}{\text{Ab negative control}} \tag{1}
\]

where Ab is the absorbance value.

Subsequently, the esterified triazole compounds 11 to 14 were tested according to the above methodology. However, the concentrations evaluated were only 100 and 50 μmol L⁻¹ due to the solubility.

Physico-chemical parameters

Molar mass (g mol⁻¹), MlogP, and van der Waals volume (Å³) were calculated by the MedChem Designer® 3.1.0.30, Marvin Sketch® 17.28.0, and ChemDraw Ultra® 12.0.2.1076, respectively.

Statistical analysis

Data were expressed as the mean ± standard deviation. Analysis of variance (ANOVA), and Tukey’s post-test were used to compare enzyme inhibition of amino acids connected to the coumarin nucleus via triazole. Nonparametric correlation (Spearman) was used to evaluate physicochemical parameters. t-test was used to compare derivatives esterified or not. The software used was GraphPad Prism® 5.0.

Supplementary Information

Supplementary information (IR, ¹H and ¹³C NMR and HRMS spectra) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Bianca L. de Sousa was responsible for conceptualization, methodology, investigation, resources, writing-original draft, writing-review and editing; João P. V. Leite for data curation, methodology, investigation, writing-review and editing; Tiago A. O. Mendes for data curation, investigation, visualization, software, validation, writing original draft, writing-review and editing; Eduardo V. V. Varejão for resources, investigation, writing-review and editing; Anna C. S. Chaves for methodology, resources, writing-review and editing; Júnio G. da Silva for methodology, resources, writing-original draft; writing-review and editing; Ana P. Agrizzi for data curation, methodology, investigation, writing-review and editing; Priscila G. Ferreira for data curation, investigation, visualization, software, validation, writing original draft, writing-review and editing; Eduardo J. Pilau for methodology, writing-review and editing; Evandro Silva for methodology, writing-review and editing; Marcelo H. Santos for project administration, funding acquisition, supervision investigation, resources, writing-original draft, writing-review and editing.
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