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Article

Molecular Modeling and Anticholinesterasic Activity of Novel 2-Arylaminocyclohexyl *N,N*-Dimethylcarbamates

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O presente trabalho reporta um estudo teórico e experimental detalhado das séries inéditas de isômeros *cis*- e *trans*-*N,N*-dimetilcarbamatos de cicloexila 2-arilaminossubstituídos como potenciais inibidores de colinesterases. Os testes de inibição *in vitro*, realizados através do método de Ellman em amostras de sangue humano, mostraram que os novos carbamatos apresentaram boa seletividade frente à inibição da enzima butirilcolinesterase (BuChE), com um máximo de inibição de 90% e IC_{50} de 6 e 8 mmol L^{-1} para os compostos mais ativos da série. Os estudos de modelagem molecular apontaram significantes diferenças entre as orientações destes compostos nos sítios ativos das enzimas BuChE e acetilcolinesterase (AChE). Os resultados mostraram que os compostos interagem de forma mais efetiva com o sítio ativo da enzima BuChE, pois o grupo carbamato está próximo aos resíduos chave da tríade catalítica.

This work reports a detailed theoretical and experimental study of the novel isomer series *cis*- and *trans*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates as potential inhibitors of cholinesterases. *In vitro* inhibition assay by Ellman's method with human blood samples showed that the new carbamates are selective to the inhibition of enzyme butyrylcholinesterase (BuChE) with maximum inhibition of 90% and IC_{50} of 6 and 8 mmol L^{-1} for the more actives compounds of the series. Molecular modeling studies point to significant differences for the conformations of the compounds in the active sites of enzymes BuChE and acetylcholinesterase (AChE). The results show that the compounds interact more effectively with the active site of enzyme BuChE since the carbamate group is close to the key residues of the catalytic triad.

Keywords: carbamate derivatives, cholinesterase inhibitors, molecular docking, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive loss of memory and other functions. It was first described by Alois Alzheimer in 1906.[1] This type of mental deterioration is the most common in individuals aged over 60 and its incidence has increased as the world population has grown older. Currently, it is estimated that 26 million people suffer from AD in the world.[2]

Despite the numerous studies done, the causes of AD are not fully elucidated and no cure has been found yet. Nevertheless, its damages and progression can be minimized. The most efficient therapy for the symptomatic treatment of AD is based on the cholinergic hypothesis in which the cognitive deficiency is a consequence of an acetylcholine (ACh) deficiency with a resulting decrease in cholinergic neurotransmission.[3-6]

In physiological conditions, the activity of neurotransmitter ACh is known to be related mainly to acetylcholinesterase (AChE). However, substantive evidence points to the importance of the function of

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butyrylcholinesterase (BuChE) in the cholinergic system.[7-10] Recent studies have shown that due to a progressive decrease in the AChE activity in certain regions of the brain in AD,[4,11] the BuChE activity increases, suggesting that BuChE may act as a compensatory mechanism in the hydrolysis of ACh.[12,13]

Due to the cholinergic hypothesis and numerous reports on the multiple functions of cholinesterases on the pathogenesis and evolution of AD, AChE and BuChE are interesting targets for the development of new anti-AD drugs.[14-19]

The action of carbamates as cholinesterase inhibitors has been known for decades and several studies have been conducted in search of anticholinesterastic carbamates that are active, also safe and better tolerated.[20-24] Rivastigmine, a drug from the carbamate class, is one of four drugs that act as cholinesterase inhibitors currently approved by ANVISA, the Brazilian Health Surveillance Agency, for the treatment of AD.

This study describes the synthesis and evaluation of the *in vitro* activity of two novel series of potential cholinesterase inhibitors (Figure 1). Computational calculations were performed to determine the conformational preference of the isomers and molecular docking was performed for the proposal of a binding model for the compounds synthesized to the active site of enzymes AChE and BuChE. We hope this study may contribute to increase the number of therapeutic agents against AD.

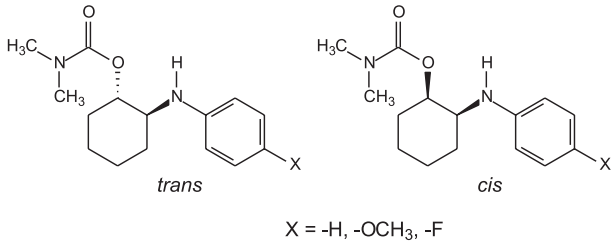


Figure 1. Structures of the two novel series of carbamate derivatives *cis*- and *trans*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates.

Results and Discussion

Synthesis

New *cis*- and *trans*-carbamate derivatives were synthesized via the route outlined in Scheme 1. *trans* Carbamates were synthesized in two steps. First, cyclohexene oxide was submitted to aminolysis with arylamines[25] to obtain *trans*-2-arylaminocyclohexanols **1a**-**1c** with yields from 50 to 80%. Next, the alcohols **1a**-**1c** were carbamoylated[26] with dimethylcarbamoyl chloride to give carbamates **2a**-**2c** with yields ranging from 35 to 50%.

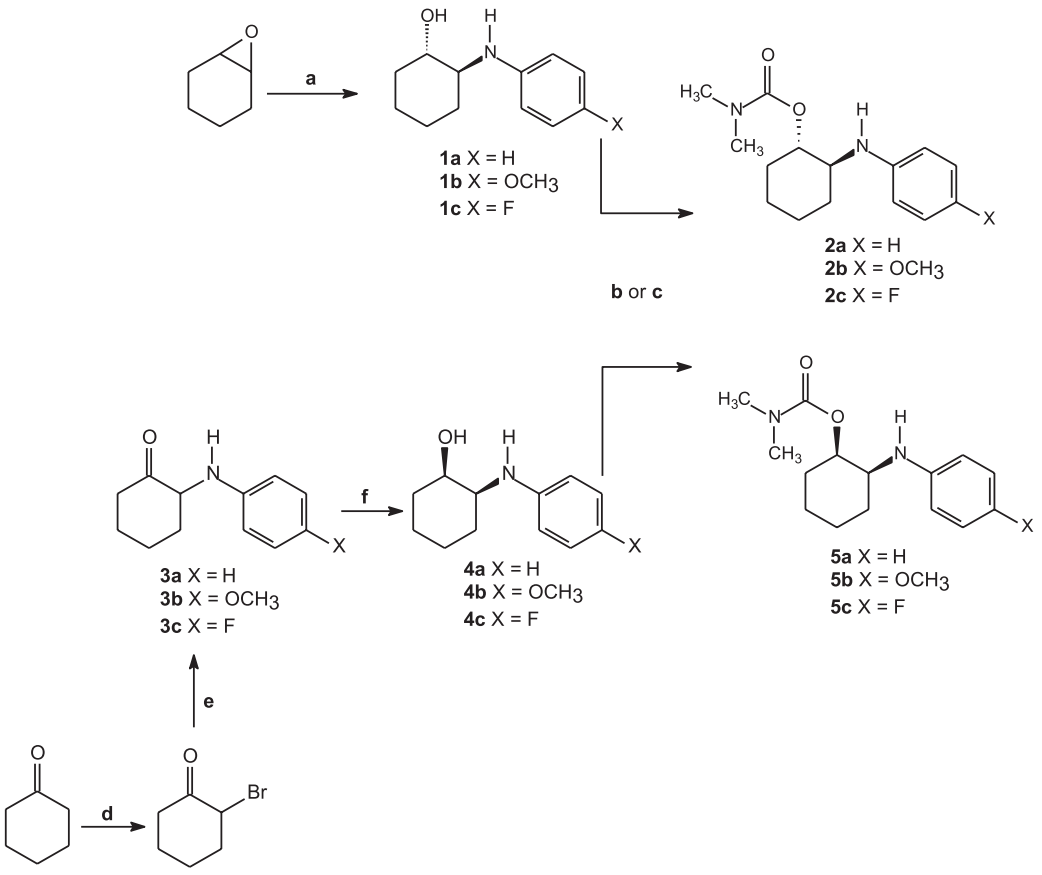
The cyclohexene oxide aminolysis was performed without a catalyst and with water as a solvent. Bonollo *et al.*[25] demonstrated that the reaction efficiency depends on a rigorous pH control. The ideal reactional condition is attained when 1.0 mmol cyclohexene oxide is mixed with 1.05 mmol amine in 2.0 mL deionized water; the resulting pH of 8.30 varies little during the reaction.

The *trans*-2-arylaminocyclohexanols **1a**-**1c** were carbamoylated with metallic sodium for the preparation of the corresponding alkoxide and later reacted with dimethylcarbamoyl chloride. However, the joint analysis of the nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) data in the preparation of derivative **2c** showed the formation of a mixture of compounds, 25% of the defluorinated compound **2a** and 75% of the desired product **2c**. Attempts to separate these compounds by column chromatography were unsuccessful. As an alternative, metallic sodium was substituted with sodium hydride in the synthesis of compound **2c**, which was obtained as a single product. The mechanism for hydrodefluorination was reported previously[27,28] as a sequence of steps, beginning with the electron transfer from metal to the substrate and ending with the formation of the hydrodefluorination product. Therefore, the product **2c** was successfully obtained when the metallic sodium was substituted by sodium hydride since in this case it is not possible the occurrence of electron transfer.

The *cis* carbamates were synthesized in four steps. First, cyclohexanone was reacted with bromide to produce 2-bromocyclohexanone[29] with a yield of 70%. Next, racemic 2-arylaminocyclohexanones **3a**-**3c** were prepared with 30% yield by substitution of 2-bromocyclohexanone with appropriate arylamines,[30] using quinoline as a catalyst and ethoxyethanol as a solvent. In the third step, ketones **3a**-**3c** were stereoselectively reduced in the presence of *N*-selectride,[31] giving the respective *cis*-2-arylaminocyclohexanols **4a**-**4c** in moderate yields, 36-50%. Temperature control (–78 °C) was very important in this step to ensure that only the *cis* isomer was obtained. Finally, compounds **4a**-**4c** were carbamoylated[26] under the same conditions used in the synthesis of the *trans* carbamates to obtain compounds **5a**-**5c**.

Biological activity

Inhibitory potencies of the novel synthesized carbamates against cholinesterases from fresh human blood were evaluated by Ellman's modified spectroscopic method.[32] This test is based on the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid), known as DTNB or as Ellman's reagent, with the sulfhydryl group of acetylthiocholine



Scheme 1. Synthesis route for isomers *cis* and *trans*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates. Reagents and conditions: (a) arylamine, H_2O, 60 °C, 48 h; (b) Na°/THF, 80 °C, 8 h; *N,N*-dimethylcarbamoyl chloride, reflux, 16 h; (c) NaH/THF, 80 °C, 8 h; *N,N*-dimethylcarbamoyl chloride, reflux, 16 h; (d) Br_2, H_2O, 0-5 °C; (e) Na_2CO_3, quinoline, methoxyethanol, reflux, 2 h; and (f) *N*-selectride, THF, –78 °C, 4 h.

or butyrylthiocholine, which results in the formation of a yellow-colored product, 2-nitro-5-thiobenzoic acid (TNB). This product has maximum absorbance at 412 nm, thus the lower the measured absorbance, the greater the activity of the compounds tested, since the enzyme is inhibited. Since this method requires water soluble compounds, chlorohydrates **2a'**-**2c'** and **5a'**-**5c'** were prepared.

The assays were performed at five concentrations (0.01, 0.025, 0.05, 0.1 and 0.2 mol L^{-1}) of the potential inhibitors. Exelon®, a drug that contains the active principle rivastigmine, was used as a standard. In these experiments, the inhibition potential of each compound against butyrylcholinesterase (found in plasma) and acetylcholinesterase (found in red blood cells) was assessed.

The inhibition results are shown in Figure 2a-2d. The IC_{50} (the testing compound concentration that inhibits the hydrolysis of substrates by 50%) values (Table 1) of each compound were obtained through the plot of percent inhibition *versus* compound concentration.

The analysis of Figure 2 shows that all compounds were more active in the butirylcholinesterase inhibition for any evaluated concentration (Figures 2a and 2c). The *cis* and *trans* salts produced maximum activity inhibition for BuChE, around 90% at 0.2 mol L^{-1}. Compounds **5a'** and **2b'** were the most active, with IC_{50} values of 6.0 and 8.0 mmol L^{-1}, respectively.

Relative to the acetylcholinesterase inhibition (Figures 2b and 2d), compounds **2a'**-**2c'** and **5b'**-**5c'** followed the same tendency and did not inhibit 50% of the enzyme activity in any of the investigated concentrations. Only compound **5a'** inhibited AChE significantly, with a maximum inhibition of 79% at 0.2 mol L^{-1}.

Likewise, the investigated compounds had good selectivity in relation to the BuChE inhibition. Derivative **2b'** was the most active selective compound, with an IC_{50} of 8.0 mmol L^{-1}. Among the tested compounds, only derivative **5a'** was active against both enzymes, with an IC_{50} of 6.0 mmol L^{-1} against plasma cholinesterase (BuChE) and 59.2 mmol L^{-1} against erythrocyte cholinesterase (AChE).

The analysis of the data shows that the *cis-trans* isomerism does not influence the activity of the compounds significantly. However, the presence of donor or acceptor substituents in the *para* position of the aromatic ring may

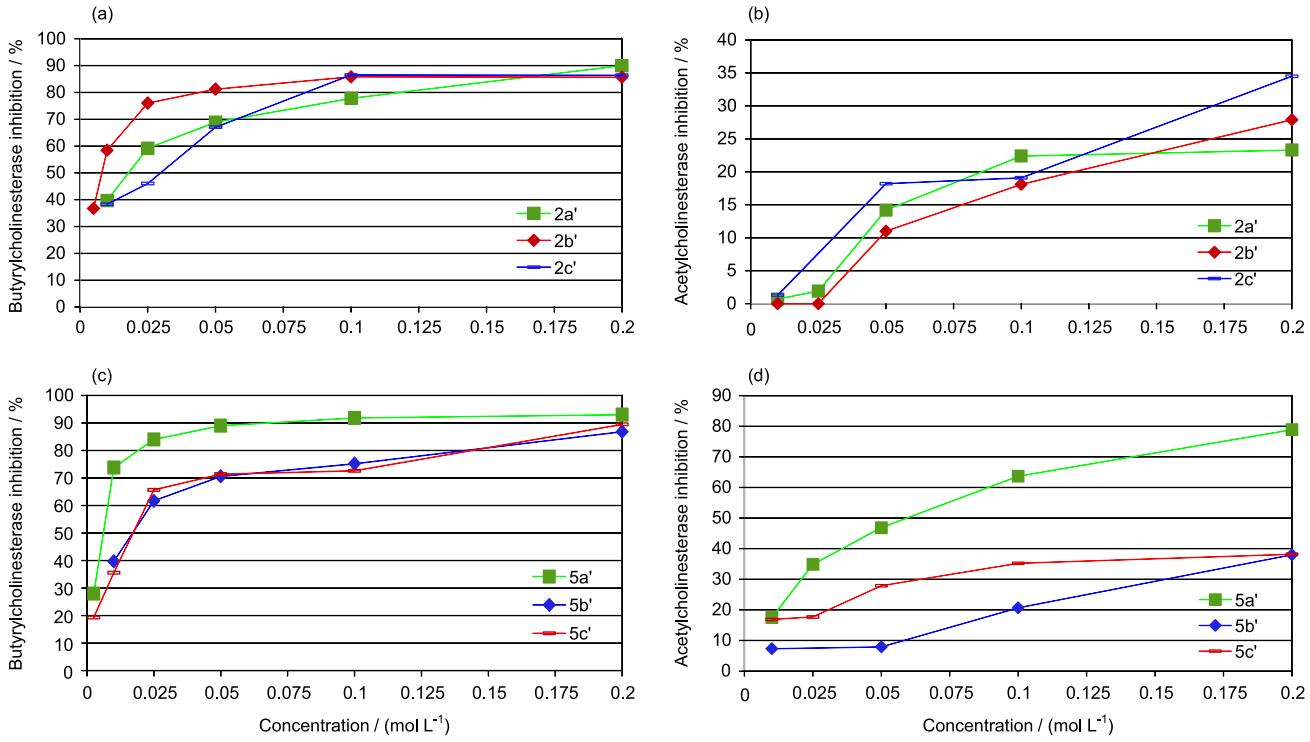


Figure 2. Concentration effects (0.01, 0.025, 0.05, 0.1 and 0.2 mol L^{-1}) of **2a'-2c'** and **5a'-5c'** on the BuChE (a and c) and AChE (b and d) activities from fresh human blood.

Table 1. IC_{50} values of carbamate chlorohydrates and reference compound, rivastigmine, as inhibitors of cholinesterases

Compound	BuChE IC_{50}[a] / (mmol L^{-1})	AChE IC_{50}[a] / (mmol L^{-1})
2a'	17.9	n.a.
2b'	8.0	n.a.
2c'	29.8	n.a.
5a'	6.0	59.2
5b'	17.0	n.a.
5c'	17.2	n.a.
Rivastigmine	0.031	1.29

[a]IC_{50} values are representative of three independent experiments performed in triplicate, standard deviations within 10% of mean values reported for each compound. n.a.: not active (IC_{50} > 100 mmol L^{-1}).

either increase or decrease the activity of the compounds, depending on the series that is analyzed. For the *trans* series (compounds **2a'-2c'**), the presence of the methoxyl group in **2b'** potentialized the anticholinesterasic activity and was approximately two times more active than derivative **2a'**. For the *cis* series, the presence of activator (**5b'**) and deactivator (**5c'**) substituents in the aromatic ring significantly reduced the activity of the compounds in relation to the non-substituted derivative **5a'**.

Bocca *et al.*[23] studied the inhibitory properties of 2-*N*,*N*-dimethylaminecyclohexyl 1-*N'*,*N'*-dimethyl-carbamate isomers and their methyl sulfate salts, and found an IC_{50} of 59 mmol L^{-1} for the most active compound of the series against BuChE. Comparatively, all compounds tested in this work (**2a'-2c'** and **5a'-5c'**) were more active against BuChE, with IC_{50} values ranging from 6 to 29.8 mmol L^{-1}. These data demonstrate that the presence of the arylamine group potentialized the activity of these carbamates in the inhibition of plasma cholinesterase.

Conformational equilibrium analysis

The molecular modeling calculations were performed with Gaussian 03 package programs.[33] To investigate the *cis* and *trans* isomer preferential conformations and the influence of the substituents on the conformational equilibrium, surface energy potential calculations were performed at HF/6-31G level. The most stable structures were then optimized at B3LYP/6-311++G(d,p) level, and the obtained energy values were used to determine the contribution of each conformer to the equilibrium. Table 2 shows the relative energies and calculated populations of each conformer of the studied compounds. Figure 3 presents the optimized structure for the *cis* and *trans* carbamate derivates of aniline (**2a** and **5a**). Similar conformations were obtained for the other compounds.

According to Table 2, all *trans* isomers presented **ee** as major conformers. This preference can be attributed to

Table 2. Relative energy (E_{rel}) and population for conformers of isomers *cis*- and *trans*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates

trans				*cis*					
Compound	ee / %	E_{rel} / (kcal mol^{-1})		Compound	ae / %	E_{rel} / (kcal mol^{-1})			
		ee	aa			ae1	ae2	ea1	ea2
2a	98.6	0	2.52	**5a**	90.1	0	0.19	1.13	1.90
2b	97.2	0	2.10	**5b**	90.2	0	0.27	1.17	1.94
2c	98.2	0	2.37	**5c**	87.6	0	0.03	0.86	1.89

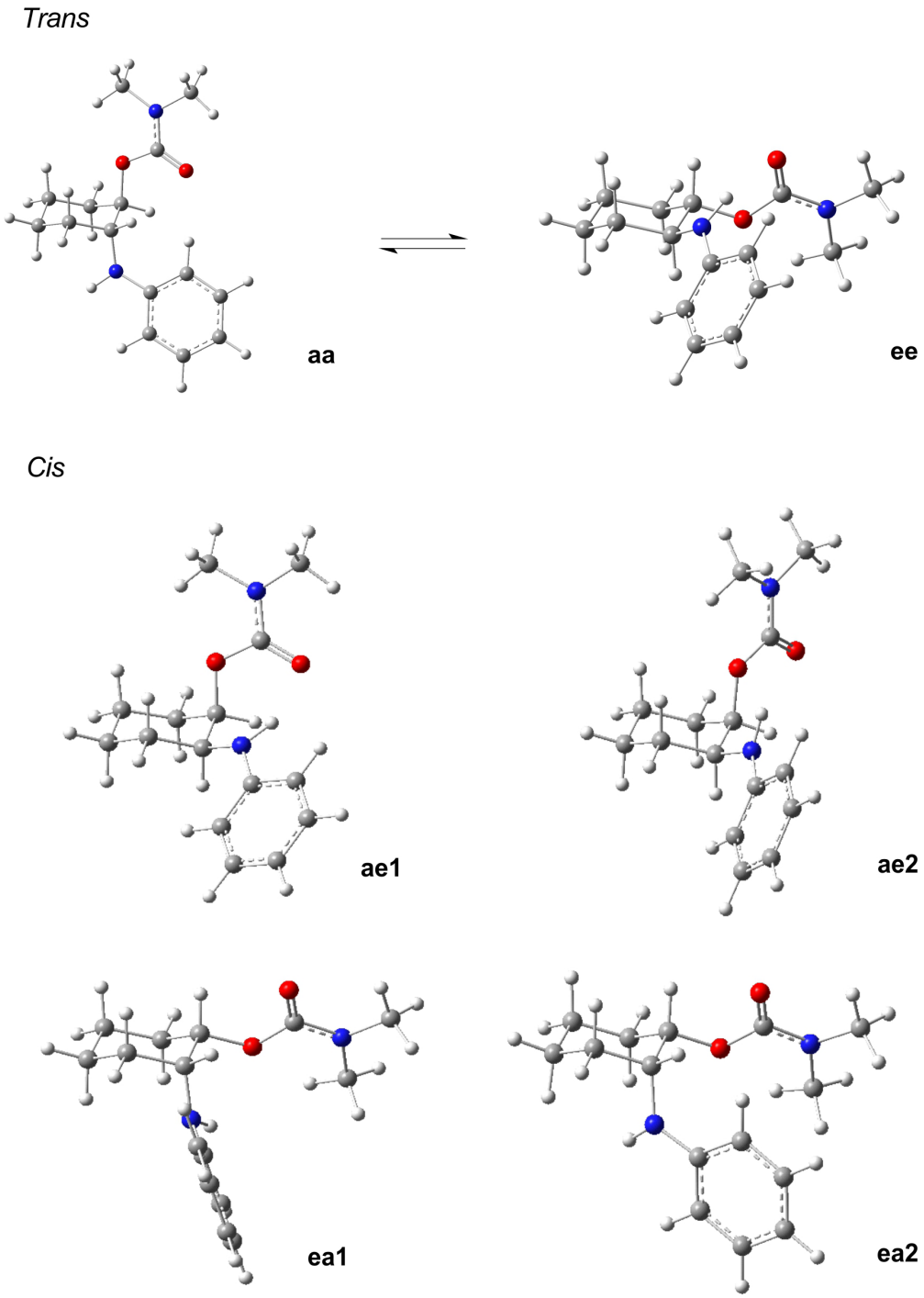


Figure 3. Structures optimized at B3LYP/6-311++G(d,p) for *trans*- and *cis*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates.

steric repulsion between the individual substituents and the hydrogens in 1 and 3 arrangements (syn, 1,3-diaxial interactions). When the substituents adopted equatorial positions (ee), these repulsions were avoided and otherwise, when the substituents adopted axial positions (aa), these interactions were maximized.

These results agree with the coupling constant values $^3J_{HH}$ obtained from the ^{1}H NMR spectra, with $^3J_{HH}$ greater than 9.3 Hz for hydrogens H-1 and H-2, indicating that they are in axial position and the substituents (carbamate and arylamine) in equatorial position.

For isomers *cis*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates, two conformations are possible, one with the carbamate group in axial position and the arylamine group in equatorial position (ae), and another with the carbamate group in equatorial position and the arylamine group in axial position (ea). Each of the conformations had two minimum energy structures, represented by **ae1**, **ae2**, **ea1** and **ea2** (Figure 3). The analysis of the data in Table 2 shows that the conformers with the carbamate group in the axial position (ae) have lower energy than the conformers with the arylamine group in axial position (ea). These results agree with the values of the coupling constant $^3J_{HH}$ obtained from the ^{1}H NMR spectra. In this case, values of $^3J_{HH}$ over 9.3 Hz were observed only for hydrogen H2, indicating that it is in axial position and, consequently, the arylamine group is in equatorial position.

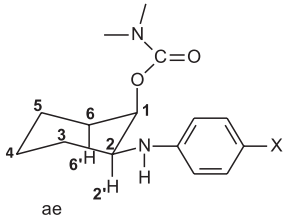
The preference of conformer **ae** can also be explained by the steric repulsion between the individual substituents and the hydrogens in 1 and 3 arrangements. In the **ae** conformation, the atoms of the carbamate group (despite its volume being greater than that of the arylamine group) are relatively distant from the cyclohexane ring, which decreases the *syn* 1,3-diaxial interactions. In contrast, in the **ea** conformation, the arylamine group is close to the ring, causing a greater repulsion between the hydrogen in the amine group and the 1,3-diaxial hydrogens. These results are consistent with previous studies published on these compounds.[34,35]

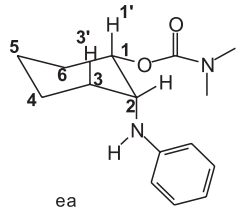
The effect of hyperconjugative interactions on conformers **ae** and **ea** was assessed by means of NBO analysis, using B3LYP/6-311++G(d,p) theory level.

Table 3 shows the main interactions involving the substituent groups. When the carbamate group is in axial position (ae), a stabilizing orbital interaction occurs between $\sigma_{C2\text{-}H2'} \rightarrow \sigma^*_{C1\text{-}O}$, which has considerably more energy than the interaction between $\sigma_{C1\text{-}H1'} \rightarrow \sigma^*_{C2\text{-}N}$, which occurs when the arylamine group is in axial position (ea). This occurs because antibonding orbital $\sigma^*_{C1\text{-}O}$ is a better electron acceptor than antibonding orbital $\sigma^*_{C2\text{-}N}$. The sum of the main hyperconjugative interactions of the substituent groups shows that they are more effective in the **ae** conformation, with a difference of about 1.50 kcal mol^{-1} in relation to the **ea** conformation. The conformational preference of the *cis* isomers can be assigned to both steric and electronic effects.

The molecular modeling calculations were also applied to the protonated form of the carbamates. The differences of energy between the conformers (E_{ee}-E_{ea} and E_{ae}-E_{ea}) were higher than 5.0 kcal mol^{-1}, so **ee** and **ae** are the dominant conformers in the equilibrium.

Table 3. Hyperconjugative interactions obtained through NBO analysis for derivatives *cis*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates





Conformer	NBO donor	NBO acceptor	E / (kcal mol^{-1})		
			5a	**5b**	**5c**
ae	$\sigma_{C2\text{-}H2'}$	$\sigma^*_{C1\text{-}O}$	5.90	5.87	5.88
	$\sigma_{C6\text{-}H6'}$	$\sigma^*_{C1\text{-}O}$	4.91	4.92	4.91
	$\sigma_{C3\text{-}C4}$	$\sigma^*_{C2\text{-}N}$	2.71	2.68	2.71
	$\sigma_{C1\text{-}C6}$	$\sigma^*_{C2\text{-}N}$	2.56	2.55	2.57
Total			16.08	16.02	16.07
ea	$\sigma_{C1\text{-}H1'}$	$\sigma^*_{C2\text{-}N}$	3.98	3.95	3.98
	$\sigma_{C3\text{-}H3'}$	$\sigma^*_{C2\text{-}N}$	4.27	4.21	4.29
	$\sigma_{C5\text{-}C6}$	$\sigma^*_{C1\text{-}O}$	3.04	3.06	3.03
	$\sigma_{C2\text{-}C3}$	$\sigma^*_{C1\text{-}O}$	3.29	3.33	3.29
Total			14.58	14.55	14.59

Enzyme-inhibitor interactions

Molecular docking calculations were performed using the AutoDock 4.2.3 program implemented at the interface PyRx 0.9[36] to assess the enzyme-inhibitor interactions and propose a bonding model based on the experimental results. All the experimentally tested compounds were docked to both enzymes (AChE and BuChE) and their interactions were analyzed. Due to the similarity of the obtained results, the most active selective compound **2b'** was chosen as model in the present discussion. It is important to point out that only the most stable conformer of each isomer was used in the docking calculations.

Figure 4a shows the *trans*-2-(4-methoxyphenylamino) cyclohexyl *N,N*-dimethylcarbamate hydrochloride **2b'** complexed with enzyme AChE. One can observe that the ligand is stabilized by residues of the catalytic anionic site Glu199 and Phe330 and by one of the residues of the anionic subsite, Gly118. There is a hydrogen bond between Hsd440 and the protonated amino group. Residues Trp84, Tyr334 and Tyr121 contribute to the stabilization through van der Waals interactions. The hydrolysis mechanism of acetylcholine involves mainly the catalytic triad residues (Ser200, His440 and Glu327). Upon analysis of the enzyme-substrate complex, one can observe that the carbamate group is distant from the catalytic triad residues, decreasing the possibility of the enzyme inhibition.

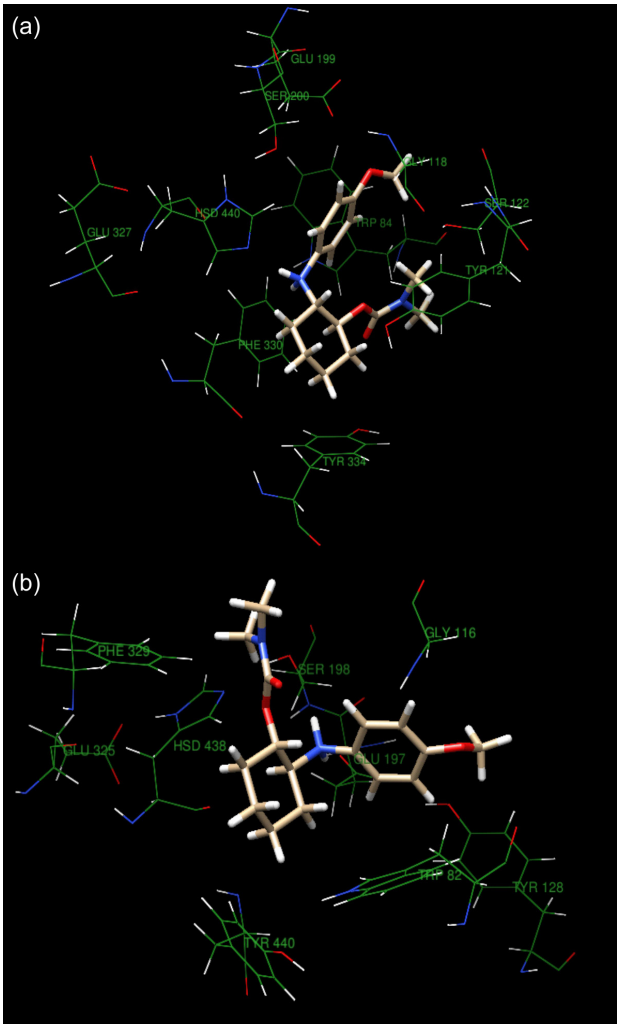


Figure 4. Active sites of enzymes (a) AChE and (b) BuChE complexed with *trans*-2-(4-methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate hydrochloride **2b'**.

Figure 4b shows the ligand complexed with enzyme BuChE. In this case, one can observe that the carbamate group is close to the catalytic triad residues of the enzyme (Ser198, Hsd438 and Glu325), enabling the nucleophilic attack of the oxygen of Ser198 to the carbonyl of the carbamate group. It can also be observed that the arylamine group is stabilized by hydrogen bonds with Glu197, Gly116 and Ser198, and those residues Tyr128, Trp82 and Tyr440 contribute to stabilize the ligand through van der Waals interactions.

In general, the molecular docking results show that the assessed compounds are located in the active site of both enzymes, but in butyrylcholinesterase, the carbamate group is close to the catalytic triad residues, whereas in acetylcholinesterase this group is farther away. These results agree with the experimental data, which showed that the tested compounds are selective inhibitors of enzyme butyrylcholinesterase.

Conclusions

New selective inhibitors of enzyme BuChE may serve as biological investigative tools of its role in AD and its treatment. This study resulted in a series of new cholinesterase inhibitors, selective BuChE inhibitors (**2a'**-**2c'**, **5b'** and **5c'**), and one non-selective AChE/BuChE inhibitor (**5a'**). The *in vitro* inhibition test by Ellman's modified method showed that the *cis-trans* isomerism does not influence the activity of the compounds significantly, but that the presence of donor and acceptor substituents in the *para* position of the aromatic ring may either increase or decrease the activity of the compounds, depending on the series being analyzed.

According to the DFT (density functional theory) calculations, the *trans* carbamates presented diequatorial substitution as major conformers, which can be attributed to steric repulsion between the substituents and the hydrogens in 1 and 3 arrangements (*syn* 1,3-diaxial interactions). For the *cis* isomers, the most stable conformer is the one with the carbamate group in axial position and the arylamine group in equatorial position. In this case, the conformational preference is explained both by steric and electronic effects.

Molecular modeling studies attributed the selectivity of the compounds toward BuChE to the distance between the carbamoyl group and the catalytic site of the enzyme. In the BuChE, the carbamoyl group is oriented towards the catalytic triad, creating import interactions between the triad and the active site. In contrast, in AChE, the long distance between the carbamoyl group and the catalytic triad of enzyme makes difficult any interaction between them. Overall, these results indicate that the novel carbamate derivatives are interesting structures for the development of selective and more potent BuChE inhibitors.

Experimental

General experimental procedures

NMR spectra were acquired in a Varian Mercury Plus BB apparatus operating at 300.059 MHz for ^{1}H and 75.457 MHz for ^{13}C. The spectra were recorded in 20 mg cm^{-3} solutions of $CDCl_3$, with a probe temperature of ca. 300 K and TMS (tetramethylsilane) as reference. Melting points were determined with a Micro-Química apparatus model MQAPF-301 and are uncorrected.

HRMS analyses were performed for new 2-arylamino-cyclohexyl *N,N*-dimethylcarbamates. The compounds were dissolved in a solution of 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water and 0.1% formic acid. The solutions were infused directly and individually into the ESI (electrospray ionization) source by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 μL min^{-1}. ESI(+)-MS and tandem ESI(+)-MS/MS were acquired using a hybrid high-resolution and high accuracy (5 μL L^{-1}) MicrOTOF-Q II mass spectrometer (Bruker Daltonics) under the following conditions: capillary and cone voltages were set to +3500 and +40 V, respectively, with a de-solvation temperature of 200 °C. For data acquisition and processing, Micro-TOF software (Bruker Daltonics) was used. The data were collected in the *m/z* range of 50-400 at the speed of two scans *per* s, providing the resolution of 50,000 (FWHM) at *m/z* 200.

Procedures for the synthesis and purification of all compounds are described in the Supplementary Information section.

Cholinesterase inhibition bioassay

The inhibitory activity of cholinesterases (AChE and BuChE) was evaluated in fresh human blood by Ellman's modified spectrophotometric method.[32] For measurement, 10 mL of a 100 mmol L^{-1} phosphate buffer solution pH 8.0 and 10 μL of heparinized fresh blood were used. To determine the total amount of cholinesterases, 3.0 mL of solution (erythrocyte + plasma) were transferred to vials and 40 μL of tested compounds were preincubated with the enzymes for 10 min at 30 ºC before starting the reaction by adding of the substrate (acetylthiocholine iodide). Next, 50 μL of 10 mmol L^{-1} DTNB (Ellman's reagent, Sigma-Aldrich Co.) solution and 20 μL of 75 mmol L^{-1} acetylthiocholine iodide (Sigma-Aldrich Co.) solution were added. Enzyme activity was determined by measuring the absorbance at 412 nm for 5 min with a Shimadzu UV-1061PC apparatus. An inhibitor-free sample was used (100% enzyme activity) as a reference. Each compound was assayed at five concentrations in triplicate. The reaction rates were compared and the percent inhibition due to the presence of the tested compounds was calculated. The IC_{50} values were determined by spectrophotometric measurement of the effect of increasing compound concentrations on the enzyme activity.

To determine the plasma cholinesterase (BuChE), heparinized fresh blood was centrifuged at 2000 rpm for 10 min for the sedimentation of red blood cells, and 20 μL of the supernatant (plasma) were added to 12 mL of a 100 mmol L^{-1} phosphate buffer solution at pH 8.0. Amounts of 3.0 mL of this solution were transferred to vials and 40 μL of test compounds were preincubated with the enzymes for 10 min at 30 ºC. Next, 25 μL of 10 mmol L^{-1} DTNB solution and 20 μL of 75 mmol L^{-1} acetylthiocholine iodide solution were added. Enzyme activity was measured as for total cholinesterase.

Ligand modeling

The molecular modeling calculations were performed with Gaussian 03 package programs.[33] Energy potential surfaces were obtained for all the compounds at theory level HF/6-31G to determine the position of lowest energy adopted by the groups in space. The lowest energy structures were optimized at a high level of theory [B3LYP/6-311++G(d,p)] and characterized as absolute minima by vibrational frequency calculations. NBO calculations (version 5.0) were performed to evaluate the hyperconjugative interactions using theory level B3LYP/6-311++G(d,p).

Molecular docking

Molecular docking studies were performed using the AutoDock 4.2.3 program implemented at the interface PyRx 0.9.[36] The compounds under study were docked to 1GQR crystallographic structures, AChE complexed with rivastigmine, and 1POM, BuChE complexed with choline

ions, selected from the Protein Data Bank (PDB). For each PDB file, molecules of water and other ligands (except the main ligands rivastigmine and choline ion) were removed. Redocking calculations were performed to validate the parameters that had been chosen. The compound structures were drawn and optimized with the Gaussian 09 program[33] as described in the Ligand Modeling section.

The docking calculations for the enzymes and compounds were performed by building a 50 × 50 × 50 Å box centered at coordinates x = 8.058, y = 64.887 and z = 61.330 for AChE and x = 132.478, y = 114.974 and z = 38.835 for BuChE with a grid space of 0.375 Å. The genetic algorithm (GA) was used as a standard protocol of 50 poses obtained for the ligand, an initial population of 150 random individuals, a maximum number of 2.5×10^5 energy evaluations and a maximum of 2.7×10^4 generations. The docked results within an RMSD (root mean square deviation) of 2.0 Å were clustered and the final results of each ligand were selected considering both the embedded empirical binding free energy evaluation and the clustering analysis.

The best results were submitted to energy minimization with the NAMD2 program.[37] The force field adopted for proteins was CHARMM C35b2-C36a2, and for the ligands, they were generated in the same format by the SwissParam server.[38] Energy minimization was simulated with the complexes immersed in a box with water measuring at least 10 Å from the outermost surface of the protein. Either Na^+ or Cl^- counter ion was added in appropriate amounts to neutralize the charges of the system. The temperature and the pressure were adjusted to 300 K and 1 atm. After energy minimization, the protein-ligand complexes were redocked using the same docking parameters, which gave a maximum RMSD of 1.5 Å.

Acknowledgments

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Supplementary Information

Experimental details and supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Supplementary Information

Molecular Modeling and Anticholinesterasic Activity of Novel 2-Arylaminocyclohexyl *N*,*N*-Dimethylcarbamates

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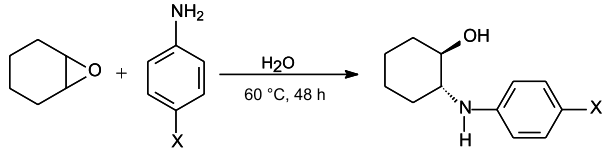
(A) Preparation of *trans*-2-arylaminocyclohexanols **1a-1c**[1]



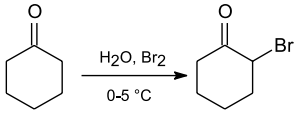
In a two-necked flask equipped with a magnetic stirrer, 51 mmol of appropriate arylamine (4.65 mL of aniline, 6.3 g of *p*-anisidine or 4.8 mL of *p*-fluoraniline) and cyclohexene oxide (49 mmol) were consecutively added in water (100 mL) and the resulting mixture was left under vigorous stirring at 60 °C for 48 h. The mixture was basified with 5 mol L^{-1} NaOH until pH 10 and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4. The solvent was evaporated and the product pure was isolated.

trans-2-Phenylaminocyclohexanol **1a**: white solid; mp 58-59 °C; 50% yield.

trans-2-(4-Methoxyphenylamino)cyclohexanol **1b**: brown solid; mp 67-68 °C; 80% yield.

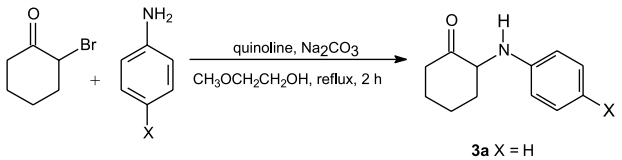
trans-2-(4-Fluoropheyilamino)cyclohexanol **1c**: white solid; mp 94-95 °C; 50% yield.

(B) Preparation of 2-bromocyclohexanone[2]



Cyclohexanone (19 mL, 180 mmol) and water (70 mL) were placed in a three-necked flask equipped with a stirrer and a dropping funnel. Bromine (9.5 mL, 180 mmol) was added dropwise to the stirred heterogeneous mixture during 1 h, and a water-ice bath (0-5 °C) was employed to cool the reaction. When addition was completed, stirring was continued until the reaction mixture was colorless (30-60 min). The mixture was extracted with ethyl ether (3 × 30 mL), the combined organic layers were dried over anhydrous Na_2SO_4. The filtrate was concentrated under reduced pressure (73 °C, 2 mmHg) to give the product (70% yield).

(C) Preparation of 2-arylaminocyclohexanones **3a-3c**[3]



0.2 mol of the appropriate arylamine (18.2 mL of aniline, 24.6 g of *p*-anisidine or 19.0 mL of *p*-fluoroaniline), 2-bromocyclohexanone (23 mL, 0.2 mmol), quinoline (2.4 mL, 0.02 mmol), 0.3 mol sodium carbonate (31.8 g, 0.3 mmol) and 150 mL of 2-methoxyethanol were

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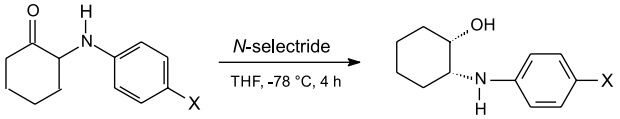
added to a dry flask and the resulting reaction mixture was heated to reflux for 2 h. The reaction mixture was cooled to room temperature. The solid was removed by filtration and washed with chloroform. The filtrate was concentrated under reduced pressure to give crude product. Pure racemic α-arylaminocycloalkanone was obtained by recrystallization from anhydrous methanol.

2-(Phenylamino)cyclohexanone **3a**: white solid; mp 81-82 °C; 30% yield.

2-(4-Methoxyphenylamino)cyclohexanone **3b**: brown solid; mp 91-93 °C; 30% yield.

2-(4-Fluorophenylamino)cyclohexanone **3c**: white solid; mp 75-76 °C; 30% yield.

(D) Preparation of *cis*-2-arylaminocyclohexanols **4a-4c**[4]



3a X = H
3b X = OCH_3
3c X = F

4a X = H
4b X = OCH_3
4c X = F

4.55 mmol of the appropriate 2-arylaminocyclohexanone (0.9 g of **3a,** 1.0 g of **3b** and 1.0 g of **3c**) were dissolved in dried THF (tetrahydrofuran, 25 mL) in a round-bottom flask under nitrogen atmosphere and magnetic stirring. After lowering the temperature to −78 °C, *N*-selectride (9.1 mL, 9.1 mmol) was added, and the reactor was kept under stirring for 4 h. The reaction mixture was allowed to attain room temperature, after which it was hydrolyzed with water (2.0 mL) and ethanol (5.0 mL). The organoborane was oxidized with 6.0 mol L^{-1} NaOH (4.0 mL) and 30% H_2O_2 (5.0 mL). The aqueous phase was than saturated with $CaCO_3$ and extracted with ethyl ether. The two organic portions were joined, dried with $MgSO_4$ and carried to a rotary evaporator where the solvent was removed.

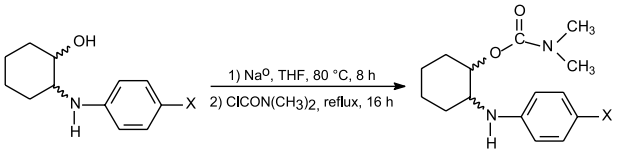
cis-2-Phenylaminocyclohexanol **4a**: white solid; mp 70-72 °C; 35% yield.

cis-2-(4-Methoxyphenylamino)cyclohexanol **4b**: brown solid; mp 51-52 °C; 45% yield.

cis-2-(4-Fluorophenylamino)cyclohexanol **4c**: white solid; mp 73-74 °C; 50% yield.

(E) Preparation of new *cis*- and *trans*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates **2a, 2b, 5a** and **5b**[5]

23 mmol of the appropriate 2-arylaminocyclohexanol (4.4 g of **1a** or **1b,** 5.1 g of **4a** or **4b**) were dissolved in dried THF (30 mL) in a round-bottom flask under nitrogen atmosphere and magnetic stirring. Following metalic sodium (1.0 g, 45 mmol) addition, the resulting reaction



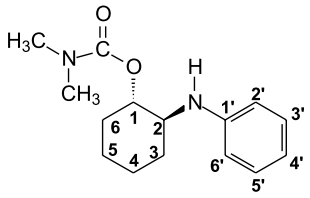
1a X = H
1b X = OCH_3
4a X = H
4b X = OCH_3

2a X = H
2b X = OCH_3
5a X = H
5b X = OCH_3

mixture was heated to 80 °C for 8 h. After this time, dimethylcarbamyl chloride (3.1 mL, 34 mmol) was added and the reaction was heated to reflux for 15 h.

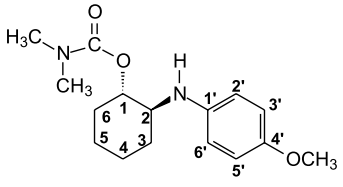
The reaction mixture was cooled to room temperature, added cold solution of sodium bicarbonate 1% (50 mL), and extracted with ethyl ether (3 × 30 mL) and cold water (2 × 20 mL). The organic layers were dried over anhydrous Na_2SO_4. The solvent was evaporated and the crude product was obtained. Compounds **2a** and **5a** were purified washing the obtained solid repeatedly with cold hexane. Pure compounds **2b** and **5b** were obtained by silica gel column chromatography (hexane/ether 8:2).

trans-2-(Phenylamino)cyclohexyl *N,N*-dimethylcarbamate **2a**



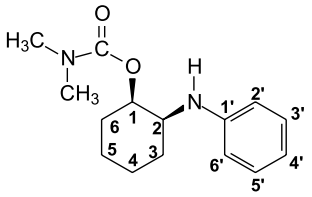
White solid; mp 72.9-73.5 °C; 50% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.16-1.30 (m, 1H, H_a6), 1.32-1.42 (m, 2H, H_a4 and H_a5), 1.40-1.52 (m, 1H, H_a3), 1.68-1.91 (m, 2H, H_e4 and H_e5), 1.94-2.06 (m, 1H, H_e3), 2.10-2.22 (m, 1H, H_e6), 2.67 (s, 1H, CH_3), 2.81 (s, 1H, CH_3), 3.32 (ddd, 1H, *J* 9.3, 9.3, 4.2 Hz, H2), 4.64 (ddd, 1H, *J* 9.3, 9.3, 4.2 Hz, H1), 6.54-6.64 (m, 2H, H2' and H6'), 6.58-6.66 (m, 1H, H4'), 7.12 (ddd, 2H, *J* 8.7, 7.2, 4.2 Hz, H3' and H5'); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.3 (C5), 24.4 (C4), 31.7 (C3), 32.2 (C6), 35.9 (CH_3), 36.5 (CH_3), 57.2 (C2), 76.8 (C1), 113.1 (C2' and C6'), 116.9 (C4'), 129.2 (C3' and C5'), 148.2 (C1'), 157.0 (C=O); ESI-HRMS calcd. for $C_{15}H_{23}N_2O_2$ ([M + H]$^+$): 263.1760; found: 263.1837.

trans-2-(4-Methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate **2b**



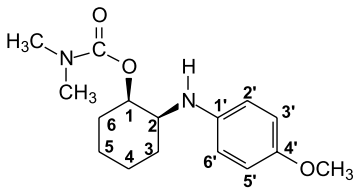
Brown oil; 50% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.17-1.28 (m, 1H, H_a6), 1.28-1.39 (m, 2H, H_a4 and H_a5), 1.38-1.50 (m, 1H, H_a3), 1.60-1.76 (m, 2H, H_e4 and H_e5), 1.94-2.04 (m, 1H, H_e3), 2.07-2.18 (m, 1H, H_e6), 2.67 (s, 1H, CH_3), 2.82 (s, 1H, CH_3), 3.28 (ddd, 1H, *J* 9.3, 9.3, 4.2 Hz, H2), 3.72 (s, 1H, OCH_3), 4.62 (ddd, 1H, *J* 9.3, 9.3, 4.2 Hz, H1), 6.57 (d, 2H, *J* 9.0 Hz, H2' and H6'), 6.73 (d, 2H, *J* 9.0 Hz, H3' and H5'); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.2 (C5), 24.4 (C4), 31.5 (C3), 32.3 (C6), 35.8 (CH_3), 36.4 (CH_3), 55.9 (OCH_3), 58.1 (C2), 76.9 (C1), 114.5 (C2' and C6'), 114.8 (C3' and C5'), 142.5 (C1'), 151.8 (C4'), 156.9 (C=O); ESI-HRMS calcd. for $C_{16}H_{25}N_2O_3$ ([M + H]$^+$): 293.1865; found: 293.1946.

cis-2-(Phenylamino)cyclohexyl *N,N*-dimethylcarbamate **5a**



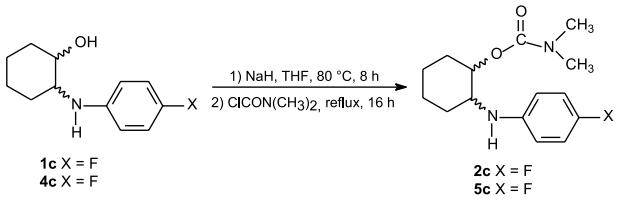
White solid; mp 68.1-70.0 °C; 39% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.34-1.44 (m, 1H, H_a4), 1.44-1.58 (m, 2H, H_a5 and H_e5), 1.50-1.62 (m, 1H, H_a6), 1.52-1.66 (m, 1H, H_a3), 1.69-1.77 (m, 1H, H_e4), 1.85-1.94 (m, 1H, H_e3), 1.94-2.06 (m, 1H, H_e6), 2.91 (s, 2H, CH_3), 3.50 (ddd, 1H, *J* 9.9, 3.3, 3.3 Hz, H2), 5.00-5.06 (m, 1H, H1), 6.55-6.64 (m, 2H, H2' and H6'), 6.62-6.70 (m, 1H, H4'), 7,14 (ddd, 2H *J* 6.9, 6.9, 1.8 Hz, H3' and H5'); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.0 (C5), 23.9 (C4), 28.3 (C3), 29.6 (C6), 36.1 (CH_3), 36.6 (CH_3), 53.6 (C2), 73.3 (C1), 113.4 (C2' and C6'), 117.4 (C4'), 129.4 (C3' and C5'), 147.3 (C1'), 156.4 (C=O); ESI-HRMS calcd. for $C_{15}H_{23}N_2O_2$ ([M + H]$^+$): 263.1760; found: 263.1842.

cis-2-(4-Methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate **5b**



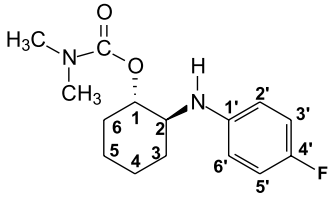
Brown oil; 23% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28-1.42 (m, 1H, H_a4), 1.34-1.42 (m, 1H, H_a5), 1.44-1.56 (m, 2H, H_e5 and H_a6), 1.47-1.62 (m, 1H, H_a3), 1.64-1.76 (m, 1H, H_e4), 1.80-1.92 (m, 1H, H_e3), 1.92-2.06 (m, 1H, H_e6), 2.90 (s, 2H, CH_3), 3.38 (ddd, 1H, *J* 9.6, 3.3, 3.3 Hz, H2), 3.72 (s, 1H, OCH_3), 4.96-5.04 (m, 1H, H1), 6.57 (d, 2H, *J* 9.3 Hz, H3' and H5'), 6.75 (d, 2H, *J* 9.0 Hz, H2' and H6'); ^{13}C NMR (75 MHz, $CDCl_3$) δ 20.8 (C5), 23.9 (C4), 28.4 (C3), 29.5 (C6), 36.0 (CH_3), 36.4 (CH_3), 54.7 (C2), 55.8 (OCH_3), 73.0 (C1), 115.0 (C2' and C6'), 115.1 (C3' and C5'), 141.3 (C1'), 152.1 (C4'), 156.1 (C=O); ESI-HRMS calcd. for $C_{16}H_{25}N_2O_3$ ([M + H]$^+$): 293.1865; found: 293.1944.

(F) Preparation of *cis*- and *trans*-2-(4-fluorophenylamino) cyclohexyl *N,N*-dimethylcarbamate **2c** and **5c**



12 mmol of the appropriate 2-arylaminocyclohexanol were dissolved in dried THF (30 mL) in a round-bottom flask under nitrogen atmosphere and magnetic stirring. Following, sodium hydride (0.6 g, 24 mmol) was added and the resulting reaction mixture was heated to 80 °C for 8 h. After this time, dimethylcarbamyl chloride (3.1 mL, 34 mmol) was added and the reaction was heated to reflux for 15 h. The reaction mixture was cooled to room temperature, added cold solution of sodium bicarbonate 1% (50 mL) and extracted with ethyl ether (3 × 30 mL) and cold water (2 × 20 mL). The organic layers were dried over anhydrous Na_2SO_4. The solvent was evaporated and the crude product was obtained. Pure compounds **2b** and **5b** were obtained by silica gel column chromatography (hexane/ether 8:2).

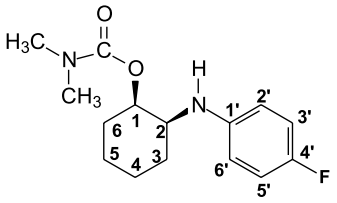
trans-2-(4-Fluorophenylamino)cyclohexyl *N,N*-dimethylcarbamate **2c**



White solid; mp 54.5-56.4 °C; 35% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19-1.29 (m, 1H, H_a6), 1.29-1.40 (m, 2H, H_a4 and H_a5), 1.41-1.52 (m, 1H, H_a3), 1.67-1.81 (m, 2H, H_e4 and H_e5), 1.96-2.05 (m, 1H, H_e3), 2.09-2.19 (m, 1H, H_e6), 2.67 (s, 1H, CH_3), 2.82 (s, 1H, CH_3), 3.25 (ddd, 1H, *J* 9.6, 9.6, 3.9 Hz, H2), 4.63 (ddd, 1H, *J* 9.3; 9.3; 4.0 Hz, H1), 6.49-6.56 (m, 2H, H2' and H6'), 6.79-6.87 (m, 2H, H3' and H5'); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.3 (C5), 24.5 (C4), 31.7 (C3), 32.3 (C6), 35.9 (CH_3), 36.5 (CH_3), 58.0 (C2), 76.9 (C1), 114.0 (C2' and C6'), 115.5 (C3' and C5'),

144.6 (C1'), 155.6 (C4'), 156.9 (C=O); ESI-HRMS calcd. for $C_{15}H_{22}FN_2O_2$ ([M + H]$^+$): 281.1665; found: 281.1741.

cis-2-(4-Fluorophenylamino)cyclohexyl *N,N*-dimethylcarbamate **5c**



White solid; mp 66.4-67.5 °C; 45% yield; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.30-1.40 (m, 1H, H_a4), 1.42-1.52 (m, 2H, H_e4 and H_a5), 1.46-1.55 (m, 1H, H_a6), 1.46-1.58 (m, 1H, H_a3), 1.64-1.74 (m, 1H, H_e5), 1.78-1.88 (m, 1H, H_e3), 1.90-2.02 (m, 1H, H_e6), 2.88 (s, 2H, CH_3), 3.38 (ddd, 1H, *J* 9.6;3.3;3.3 Hz, H2), 4.94-5.02 (m, 1H, H1), 6.44-6.52 (m, 2H, H2' and H6'), 6.76-6.87 (m, 2H, H3' and H5'); ^{13}C NMR (75 MHz, $CDCl_3$) δ 20.7 (C5), 23.8 (C4), 28.1 (C3), 29.4 (C6), 35.8 (CH_3), 36.3 (CH_3), 54.2 (C2), 72.7 (C1), 114.2 (C2' and C6'), 115.5 (C3' and C5'), 143.5 (C1'), 155.6 (C4'), 156.0 (C=O); ESI-HRMS calcd. for $C_{15}H_{22}FN_2O_2$ ([M + H]$^+$): 281.1665; found: 281.1847.

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(G) NMR spectra for new *cis*- and *trans*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates

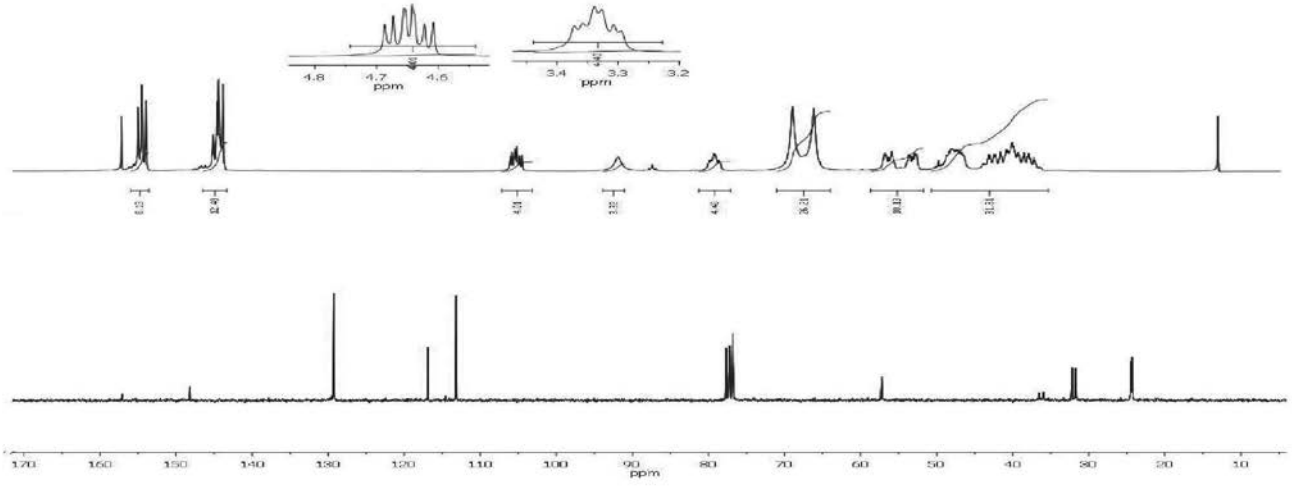

Figure S1. ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of *trans*-2-(phenylamino)cyclohexyl *N,N*-dimethylcarbamate **2a**.

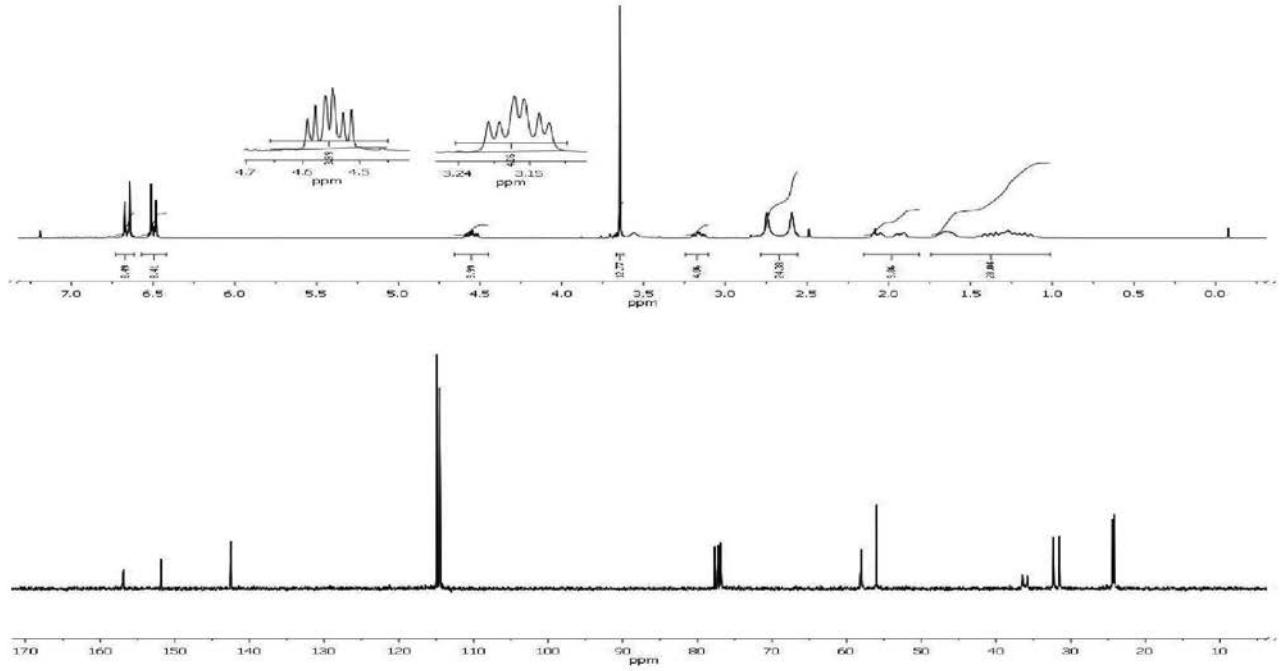

Figure S2. ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of *trans*-2-(4-methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate **2b**.

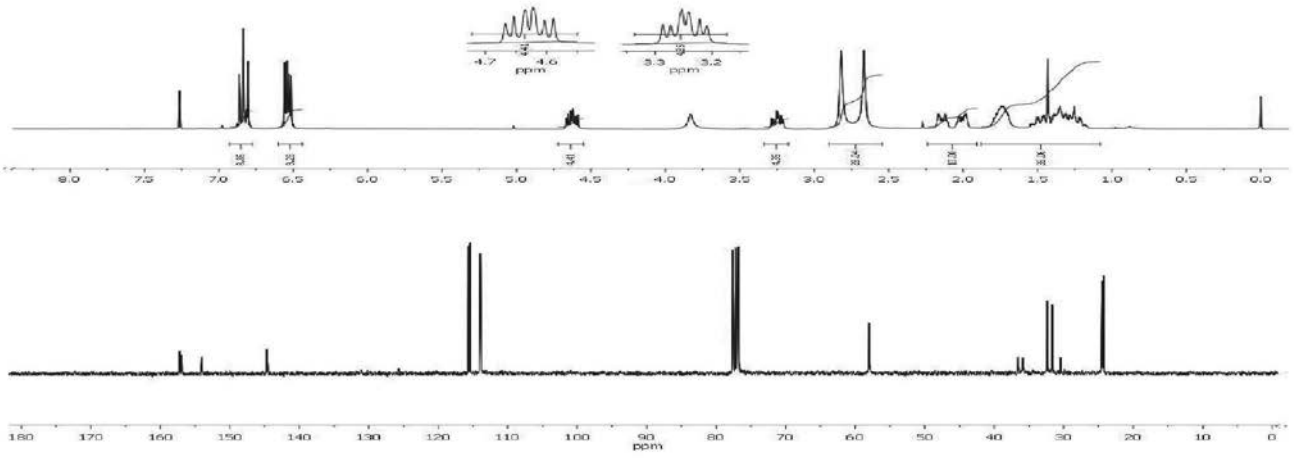

Figure S3. ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of *trans*-2-(4-fluorophenylamino)cyclohexyl *N,N*-dimethylcarbamate **2c**.

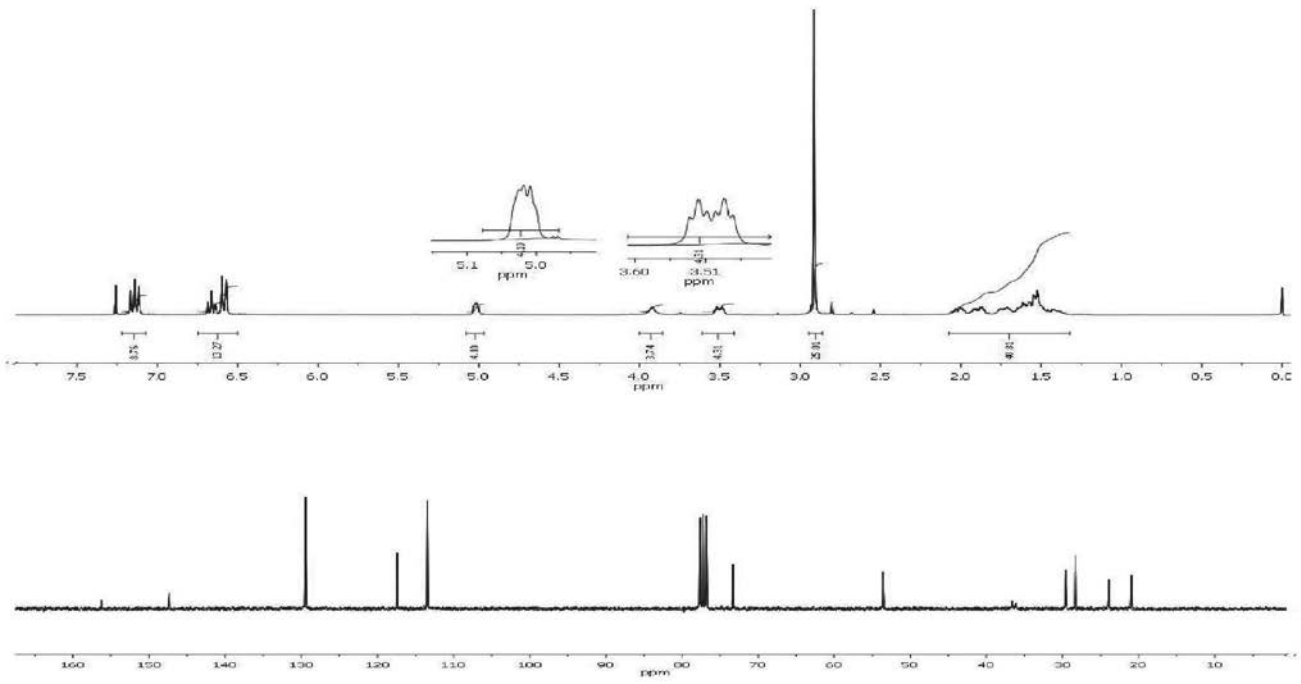

Figure S4. ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of *cis*-2-(phenylamino)cyclohexyl *N,N*-dimethylcarbamate **5a**.

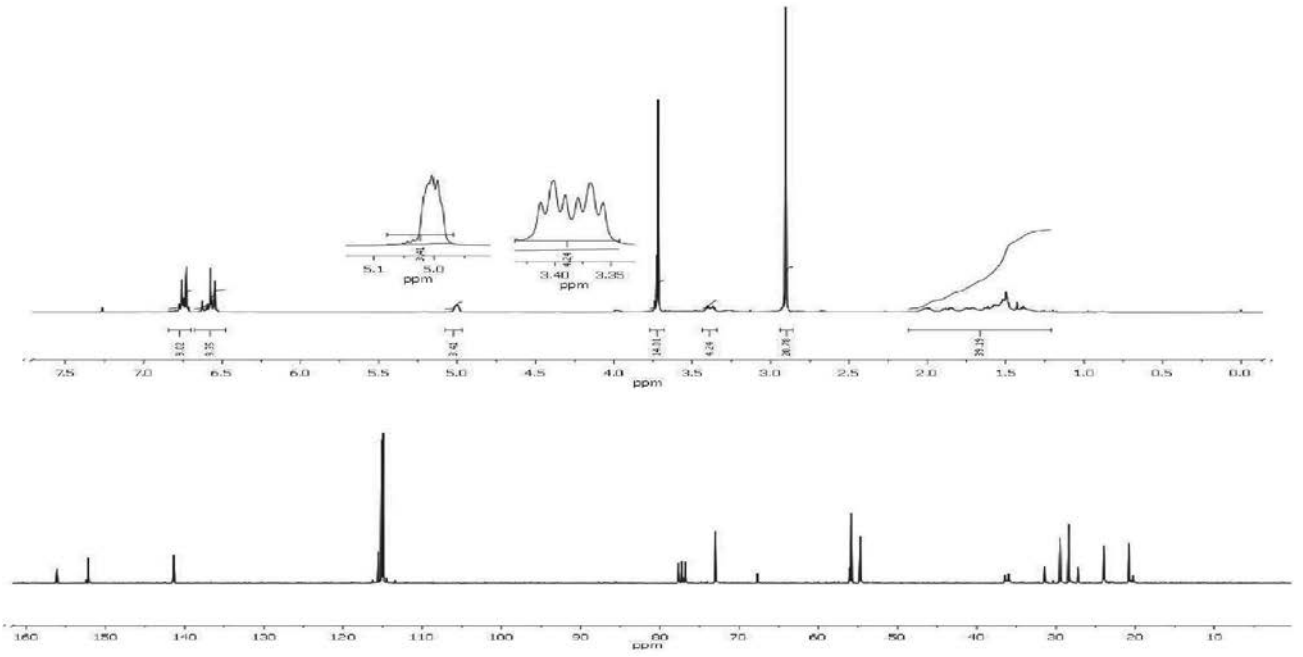

Figure S5. ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of *cis*-2-(4-methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate **5b**.

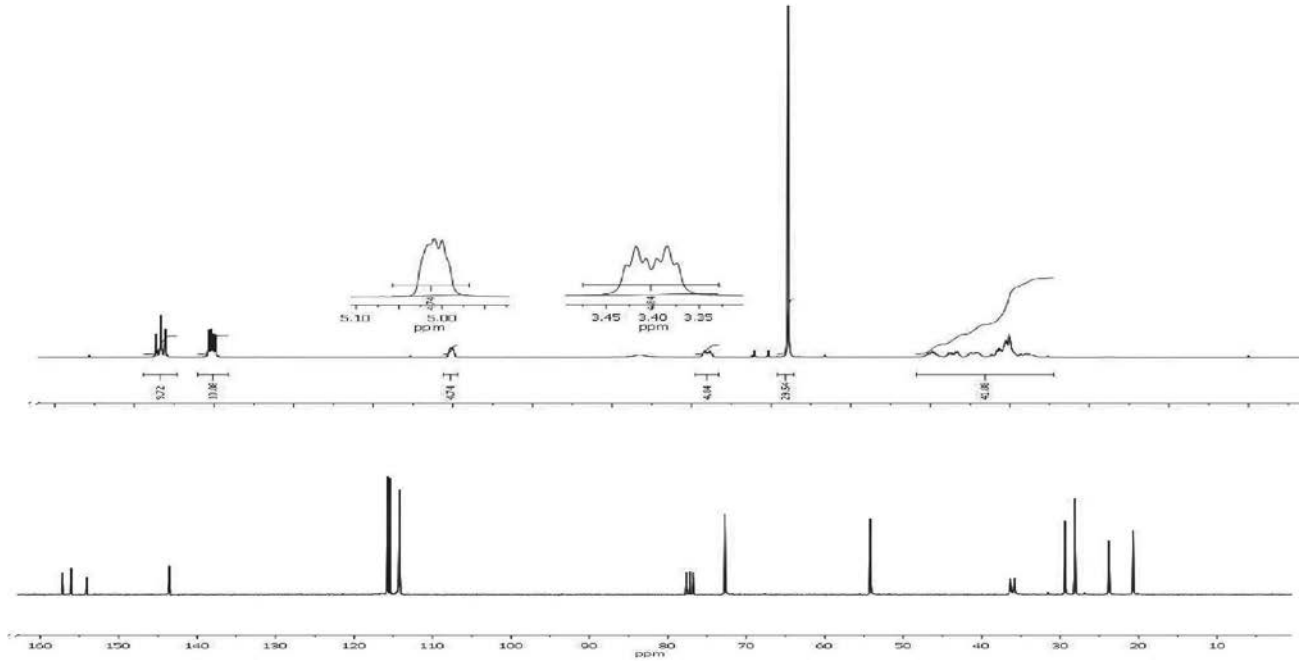

Figure S6. ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of *cis*-2-(4-fluorophenylamino)cyclohexyl *N,N*-dimethylcarbamate **5c**.

(H) ESI-HRMS spectra for new *cis*- and *trans*-2-arylaminocyclohexyl *N,N*-dimethylcarbamates

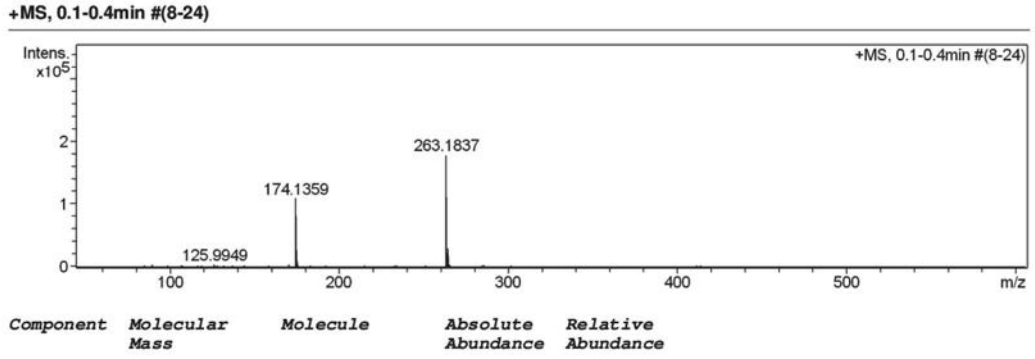

Figure S7. ESI-HRMS spectra of *trans*-2-(phenylamino)cyclohexyl *N,N*-dimethylcarbamate **2a**.

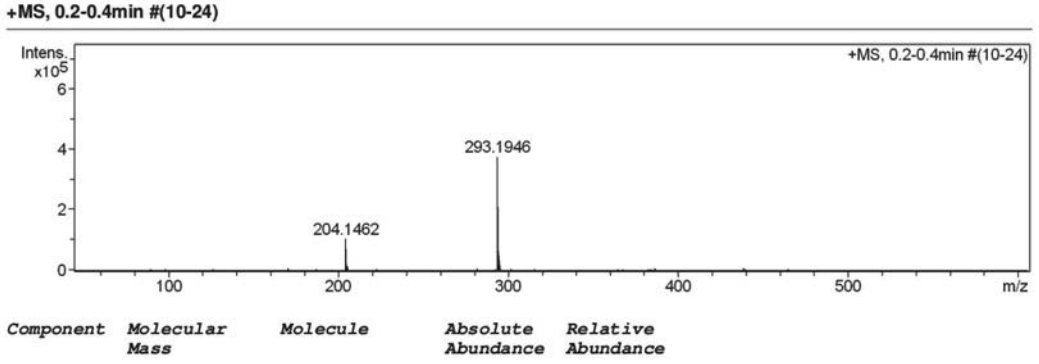

Figure S8. ESI-HRMS spectra of *trans*-2-(4-methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate **2b**.

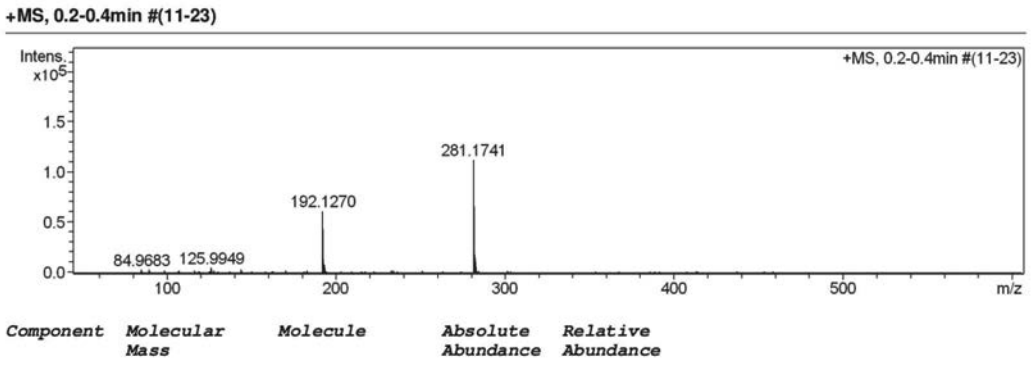

Figure S9. ESI-HRMS spectra of *trans*-2-(4-fluorophenylamino)cyclohexyl *N,N*-dimethylcarbamate **2c**.

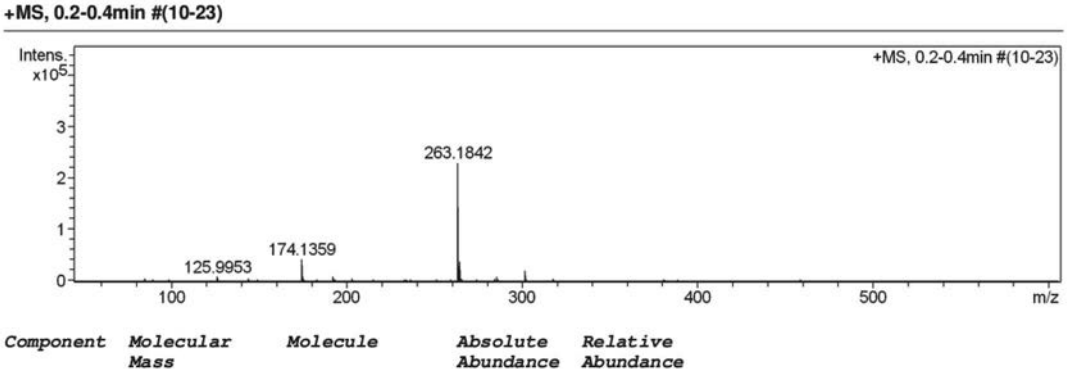

Figure S10. ESI-HRMS spectra of *cis*-2-(phenylamino)cyclohexyl *N,N*-dimethylcarbamate **5a**.

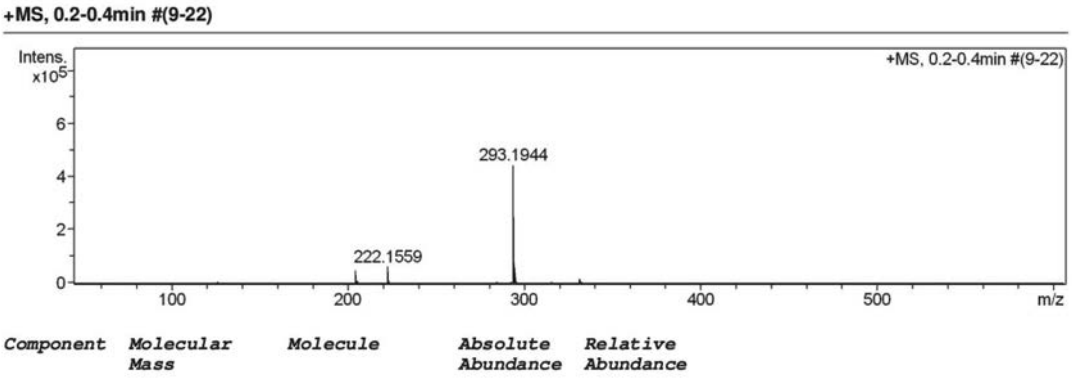

Figure S11. ESI-HRMS spectra of *cis*-2-(4-methoxyphenylamino)cyclohexyl *N,N*-dimethylcarbamate **5b**.

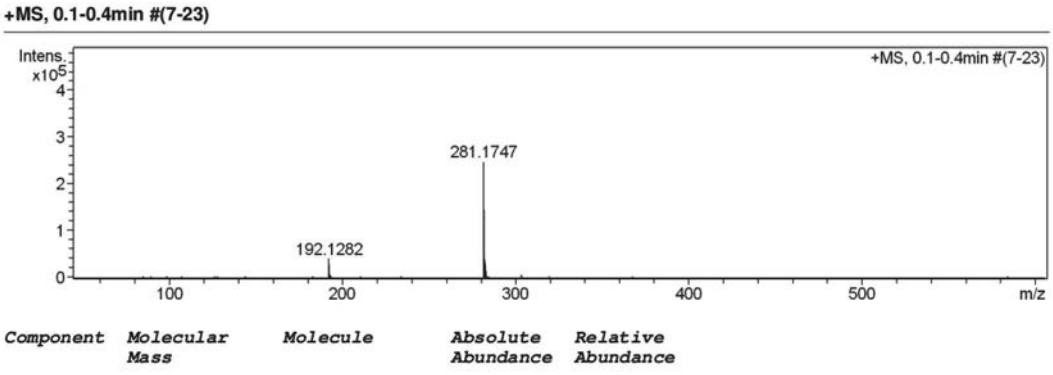

Figure S12. ESI-HRMS spectra of *cis*-2-(4-fluorophenylamino)cyclohexyl *N,N*-dimethylcarbamate **5c**.