Photochromic Azoderivatives for Acetylcholinesterase Inhibition †

Brunella Biscussi, Maria Alejandra Sequeira * and Ana Paula Murray

Instituto de Química del Sur (INQUISUR-CONICET), Chemistry Department, Universidad Nacional del Sur, Av. Alem 1253, B8000CPB Bahía Blanca, Argentina; brunella.biscussi@uns.edu.ar (B.B.); apmurray@uns.edu.ar (A.P.M.)
* Correspondence: alejandra.sequeira@uns.edu.ar
† Presented at the 23rd International Electronic Conference on Synthetic Organic Chemistry, 15 November–15 December 2019; Available online: https://ecsoc-23.sciforum.net/.
Published: 14 November 2019

Abstract: We present microwave-assisted synthesis and in vitro acetylcholinesterase inhibition of di-PRLC4OAzo, a new azoderivative designed on the basis of aza-stilbene active compounds, already reported by the group. From the total series of azoderivatives synthetized, di-PRLC4OAzo showed the powerful in vitro enzymatic response for its (E) isomer (IC50: 1.08 µM) by Ellman’s assay, beside a stable photostationary state monitored by UV/Vis absorption spectroscopy, indicating it might be an efficient photo-responsible probe to remote control the activity of the enzyme.

Keywords: microwave-assisted synthesis; acetylcholinesterase; azobenzene; photomodulation

1. Introduction

Cholinesterase inhibitors (ChEI) play a significant role in enhancing synaptic cholinergic activity avoiding cholinergic poisoning, and consequently have therapeutic relevance related to Alzheimer’s disease (AD), myasthenia gravis, and glaucoma [1]. In this sense, photochromic ChEI have become interesting due to the expansion of photopharmacological approach towards the remote control of acetylcholinesterase (AChE), a main target for studying the activity of neural networks due to its fast diffusion-controlled kinetics [2]. Nowadays, the control of synaptic communication with light has become a prime topic of investigation due to its noninvasive application, with very high temporal and spatial precision [3,4]. The Trauner group demonstrated photocontrol of AChE using an azobenzene derivative based on the drug Tacrine, approved for early stage AD [5]. The “azologization” approach, derived from “azobenzene” combined with “analogization”, support that azobenzene can mimic structural motifs (“azosteres”) found in drugs or drug candidates such as stilbenes, (heterocyclic) N-aryl benzamides, benzyl phenyl (thio)ethers, benzyl anilines, and 1,2-diaryl ethanes, offering the possibility for light sensitization and light-dependent control of its functions [6]. Inspired in this concept of “azologization”, we improved the rational introduction of a disubstituted azobenzene group into the structure of a designed aza-stilbene compound active as dual ChEI, previously reported by our group [7] (Figure 1). Electron-donating substituents in the azobenzene moiety allow efficient photoisomerization in aqueous solutions with slow thermal back-isomerization [8].
Figure 1. Application of the azologization principle to 11a, a reported aza-stilbene active as dual ChEI.

Versatile microwave-assisted synthesis allows us the generation of molecular diversity from the same azobenzene precursor in a fast way. In this work, we present microwave-assisted synthesis and structural characterization of a new azobenzene-based compound with potential application as reversible photochromic AChE inhibitor: 1,2-bis(4-(4-(pyrrolidin-1-yl)butoxy)phenyl)diazene (di-PRLcOAz), which showed in vitro enzymatic response for its (E) isomer. Ellman’s assay was performed for measuring AChE activity in the presence of the reversible photoswitchable blocker, whose in vitro photoisomerization efficiency was evaluated by UV/Vis absorption spectroscopy. The aim of this work is to contribute to the development of new synthetic photomodulable drugs for the treatment of AD.

2. Experimental Procedure

2.1. Materials and Synthesis

All solvents used were purified by distillation, after drying over a specific agent. The drying agents used were previously activated by heating in an oven. Acetonitrile was dried over K2CO3 and distilled immediately before use. DMF was distilled and dry by keeping it over 4Å molecular sieves under nitrogen atmosphere. The progress of the reactions was controlled using silica gel-60 F 254 chromatofoils (Merck). The development of thin layer chromatograms was performed by visualization with ultraviolet light of wavelengths 254 nm and/or in p-anisaldehyde developing stain.

Conventional synthesis of 4,4'-dihydroxyazobenzene (1). Synthesis of 4,4'-dihydroxyazobenzene was carried out according to the method described in bibliography [9] of Willstatter and Benz [10]. In a two-mouth ball containing a magnetic stirrer, 5.00 g of p-nitrophenol (35.95 mmol) was added to a solution containing 25 g of KOH (380 mmol) in 5 mL of MilliQ water. The reaction mixture was refluxed at 120 °C and the temperature was increased slowly for 1 h to 195–200 °C. Once the reaction was completed, it began vigorously to give a brown viscous liquid with a large number of developing bubbles. The crude product was dissolved in 40 mL of water. The solution was acidified to pH 3 by adding a solution of concentrated HCl, a dark red reaction crude was observed, and extraction was carried out with diethyl ether. The combined ether extracts were dried over Na2SO4 overnight. The solvent was removed by reduced pressure, and the crude product was obtained as a solid. It was recrystallized from 50% (v/v) aqueous solution of ethanol. When observing differences between experimental BP (120 °C) and the theoretical one (204–214 °C) [11], recrystallization was repeated up to a total of 10 times, to give yellow crystals of compound 1. The purification was monitored by TLC silica gel, using as eluents hexane:ethyl acetate (1:1). R.f. = 0.32. Compound 1, soluble in acetonitrile and acetone, was obtained in 10% yield. 1H-RMN (CD3CN, 300 MHz) δ (ppm): 6.95 (d, 4H, $J_3 = 8.90$ Hz, Ar–H), 7.77 (d, 4H, $J_3 = 8.91$Hz, Ar–H), 8.12 (s, 2H). 13C-RMN (CD3CN) δ (ppm): 116.71, 118.24, 125.21, 160.42.

Microwave-assisted synthesis of 1,2-bis(4-(4-bromobutoxy)phenyl)diazene (2). To a solution containing 0.1 mmol of 4,4'-dihydroxyazobenzene and 0.35 mmol of K2CO3 in 4 mL of acetonitrile placed in a microwave reaction glass tube with a magnetic stirrer included, 0.4 mmol of 1, 4-dibromobutane was added. The tube was placed in the microwave reactor (CEM Discover), at 150 W and 80 °C for 20 min.
The obtaining of the desired product was confirmed by TLC. The solvent was removed by reduced pressure, and the crude product was obtained as a solid. This solid was further purified by column chromatography on neutral aluminum oxide (Fluka AG, Bursch SG), using as eluents hexane: diethyl ether, and 2 was recovered as a yellowish solid in 65% yield. 1H-RMN (CDCl3, 300 MHz) δ (ppm): 2.08 (m, 8H, CH2), 3.50 (t, 4H, CH2), 4.07 (t, 4H, CH2), 6.99 (d, 4H, J = 8.89 Hz, Ar−H); 7.88 (d, 4H, J = 8.90 Hz, Ar−H). 13C-RMN (CDCl3) δ (ppm): 27.99, 29.56, 33.51, 67.28, 114.78, 124.50, 147.22, 161.00.

Microwave-assisted synthesis of 1,2-bis(4-(4-(pyrrolidin-1-yl)butoxy)phenyl)diazene (di-PRLC4OAzo).

To a solution containing 0.1 mmol of 2 and 0.5 mmol of K2CO3 in 4 mL of dry DMF placed in a microwave reaction glass tube containing a magnetic stirrer, it was added 0.4 mmol of pyrrolidine. The glass tube was placed in the microwave reactor at 150 W, 80 °C, for 20 min until observing total conversion by TLC. The solvent was evaporated under reduced pressure using a SpeedVac Concentrator (SPD111V, Thermo Savant) to give di-PRLC4OAzo as a yellow solid without further purification in 99% yield. 1H-RMN (CDCl3, 300 MHz) δ (ppm): 1.79 (m, 16H, CH2), 2.53 (m, 12H, CH2), 4.05 (t, 4H, CH2), 6.99 (d, 4H, J = 8.89Hz, Ar−H), 7.84 (d, 4H, J = 8.90Hz, Ar−H). 13C-RMN (CDCl3) δ (ppm): 23.71, 25.69, 27.51, 54.35, 56.29, 68.20, 114.81, 124.44, 147.12, 161.22.

2.2. Methods

NMR Spectroscopy. NMR spectra were recorded on a Bruker ARX 300 spectrometer (300 MHz and 75 MHz for 1H and 13C, respectively). CDCl3 was used as solvent with TMS as internal standard.

UV−Vis Spectroscopy. Switching experiments were done with an 8 W mercury arc lamp with filter of 360 nm from Pleuger Antwerp Brussels and a white light bulb of 60 W. UV−vis spectroscopy data were recorded on a JASCO V-630BIO (Tokyo, Japan) Spectrophotometer equipped with an EHCS-760 Peltier.

Quantitative AChE inhibition assay. AChE from electric eel (500 U, Sigma, Buenos Aires, Argentina) was used as a source of acetylcholinesterase. The inhibitory activity of AChE was determined in vitro using the Ellman’s spectrophotometric method with minor modifications [12,13]. The absorbance was recorded at 405 nm for 120 s at 27 °C. Enzymatic activity was calculated by comparing the reaction rates between the sample and the blank. The sample concentration reflecting 50% inhibition (IC50) was calculated by nonlinear regression of the response curve versus log (concentration), using GraphPadPrism 5. Tacrine was used as the reference inhibitor.

2.3. Results and Discussion

In order to obtain azoderivatives with potential application as reversible photochromic AChE inhibitor, we decided to exchange the aza-stilbene structure by a fotomodulable azobenzene core, preserving the successfully linker design for the catalytic site (CAS) of the enzyme, consisting in a hydrocarbon chain connected to a tertiary amine already developed by the group [14]. The peripheral anionic site (PAS) of AChE would be the possible target of interaction with the azobenzene structure being the potential photomodulable site. Herein, the azodervative di-PRLC4OAzo presented is disubstituted at 4, 4′ position with four-hydrocarbon chain connected to pyrrolidine.

The preparation of di-PRLC4OAzo was carried out using the procedures shown in Scheme 1. The first synthetic step was conducted in a conventional manner, because microwave reactor was not initially available. Otherwise, at the next steps azoderivatives were prepared in high yields and in shorter reaction times using the optimized microwave heating method, showing great yield advantages and reducing purifications stages over the conventional method.

Scheme 1. Synthetic Pathway to di-PRLC4OAzo. (a) KOH/H2O, 120 °C, 1 h. (b) Br(CH2)4Br, K2CO3, CH3CN, MW 20’. (c) pyrrolidine, K2CO3, DMF, MW 25’.
The duplication of the linker at 4, 4′ position, as well as the introduction of oxygen atoms as connectors between the azobenzene moiety and the linkers, were designed for the propose of allowing efficient photoisomerization with slow thermal back-isomerization. Upon isomerization from the E-form to the Z-form, the distance between the 4 and 4′ positions shortens by about 3 Å. This remarkably large change (the E azobenzene molecule itself is 9 Å long) can be amplified with appropriate substitution (Figure 2a).

In methanol, yellow solution of di-PRLC4OAzo (E) changed to orange upon UV-light illumination (360 nm, 8 watts, 5 min), when the stabilized photostationary state (pss) of E:Z (12:88) was reached, named di-PRLC4OAzo (Z) (orange solution). The photoconversion ratio from E to Z isomer was evidenced by UV–Vis spectroscopy (Figure 2b). The UV–Vis spectra of di-PRLC4OAzo (E) showed a characteristic π–π* transition centered in 355 nm and a small band at 446 nm corresponding to n–π* transition. For di-PRLC4OAzo (Z), the band of π–π* transition was blue-shifted to 314 nm, and the band corresponding to n–π* transition at 446 nm was more evident. Thermal Z → E isomerization was slow enough to allow the evaluation of Z isomer at the stabilized pss (stable 10 h in darkness). This experiment suggested that di-PRLC4OAzo could be used as optically controlled probe [14].

![Figure 2. (a) Photoisomerization of di-PRLC4OAzo. (b) UV–Vis spectra of di-PRLC4OAzo before ( ) and after ( ) UV-light illumination (360 nm, 8 watts, 5 min) in methanol (50 µM).](image)

The AChE inhibitory activity of di-PRLC4OAzo was evaluated in vitro by the Ellman spectrophotometric method with slight modifications. Its E isomer showed a IC50 value of 1.08 µM (log IC50 ± DS: 0.037 ± 0.030) for AChE inhibition, displaying a sharply more powerful in vitro enzymatic response than its aza-stilbene analogue, compound 11a, with a previous reported IC50 = 30.4 µM (log IC50 ± DS: 1.489 ± 0.0456) [15].

3. Conclusions

In conclusion, from several azoderivatives optimally synthesized by microwave-assisted synthesis, we obtained in high yields and short reaction times, di-PRLC4OAzo, a photomodulable azobenzene core disubstituted at 4, 4′ position with four-hydrocarbon chain connected to pyrrolidine. The rational design promoted conversion to the Z isomer with brief UV light exposure, showing efficient photoisomerization with slow thermal back-isomerization, which support its potential use as a suitable optical controlled probe. E isomer of PRLC4OAzo proved to be more effective ACE inhibitor (IC50 = 1.08 µM) than its aza-stilbene structural analogue. These preliminary studies suggest the importance in the rational design for the optimization of the interaction at the enzyme binding sites and performed azoderivatives as promising candidate compounds for the development of new multifunctional drugs for the treatment of AD. Further experiments are currently under progress to evaluate in vitro enzymatic response of Z isomer PRLC4OAzo.
Acknowledgments: This work was supported by CONICET (National Scientific and Technical Research Council), ANPCyT (National Agency for Promotion of Science and Technology) and UNS (Universidad Nacional del Sur).

References
1. McHardy, S.; Wang, H.; McCowen, S.; Valdez, M. Recent advances in acetylcholinesterase Inhibitors and Reactivators: An update on the patent literature (2012–2015). Expert Opin. Ther. Pat. 2017, 27, 455–476.
2. Chen, X.; Wehle, S.; Kuzmanovic, N.; Merget, B.; Holzgrabe, U.; König, B.; Sotriffer, C.A.; Decker, M. Acetylcholinesterase Inhibitors with Photoswitchable Inhibition of β-Amyloid Aggregation. ACS Chem Neurosci. 2014, 5, 377–389, doi:10.1021/cn500016p.
3. Reisinger, B.; Kuzmanovic, N.; Löffler, P.; Merkl, R.; König, B.; Sterner, R. Exploiting protein symmetry to design light-controllable enzyme inhibitors. Angew. Chem. Int. Ed. 2014, 53, 595–598.
4. Broichhagen, J.; Frank, J.A.; Trauner, D. A roadmap to success in photopharmacology. Acc. Chem. Res. 2015, 48, 1947–1960.
5. Broichhagen, J.; Jurastow, I.; Iwan, K.; Kummer, W.; Trauner, D. Optical Control of Acetylcholinesterase with a Tacrine Switch. Angew. Chem. Int. Ed. 2014, 53, 7657–7660.
6. Agnetta, C.; Decker, M. Photoresponsive Hybrid Compounds. In Design of Hybrid Molecules for Drug Development, 1st ed.; Decker, M., Ed.; Elsevier Ltd.: Amsterdam, The Netherlands, 2017; Volume 11, pp. 279–315.
7. Biscussi, B.; Menéndez, C.; Appignanesi, G.A.; Gerbino, D.C.; Murray, A.P. XXI SINAQO; Organizado por la Sociedad Argentina de Investigación en Química Orgánica (SAIQO): Potrero de los Funes, San Luis, Argentina, 2017; p. 239.
8. Sequeira, M.A.; Herrera, G.; Quirolo, Z.; Dodero, V. Easy Directed Assembly of only Nonionic Azoamphiphile Builds up Functional Azovesicles. RSC Adv. 2016, 6, 108132–108135, doi:10.1039/C6RA20933E.
9. Okuno, H.; Wei, W.H.; Tomohiro, T.; Kodaka, M. Selective Synthesis and Kinetic Measurement of 1:1 and 2:2 Cyclic Compounds Containing 1, 4, 7, 10-Tetraazacyclododecane and Azobenzene Units. J. Org. Chem. 2000, 65, 8979–8987.
10. Willstatter, R.; Benz, M. Zur Kenntniss der ‘Azophenole Chem. Ber. 1906, 339, 3492.
11. Jaeger, C. Ueber das Azophenol. Berichte Der Deutschen Chemischen Gesellschaft 1875, 8, 1499.
12. Ellman, G.L.; Courtney, K.D.; Andres, V.; Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 1961, 7, 88–95.
13. Alza, N.P.; Richmond, V.; Baier, C.J.; Freire, E.; Baggio, R.; Murray, A.P. Synthesis and cholinesterase inhibition of cativic acid derivatives. Org. Med. Chem. 2014, 22, 3838–3849.
14. Benedini, L.A.; Sequeira, M.A.; Fanani, M.L.; Maggio, B.; Dodero, V.I. Development of a Nonionic Azobenzene Amphiphile for Remote Photocontrol of a Model Biomembrane. J. Phys. Chem. B 2016, 120, 4053–4063.
15. Biscussi, B.; Murray, A.P. Síntesis de análogos de aza-resveratrol con actividad anticolinesterasa. In Proceedings of the XXVI Jornada de Jóvenes Investigadores AUGM, Universidad Nacional de Cuyo, Mendoza, Argentina, 17–19 October 2018. Available online: http://bdigital.uncu.edu.ar/13200 (accessed on 8 November 19).

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).