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^{1}H NMR relaxation investigation of acetylcholinesterase inhibitors from huperzine A and derivative

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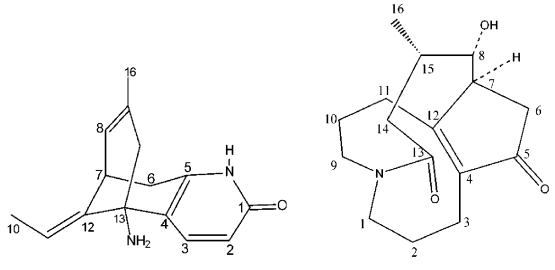
Abstract—The binding properties of huperzine A (**1**) with *Torpediniforms Nacline* acetylcholinesterase (TnAChE) were investigated by ^{1}H NMR methods. The noselective, selective and double-selective spin-lattice relaxation rates were acquired in absent and present of TnAChE at a ratio [ligand]/[protein] = 1:0.005. The selective relaxation rates shown protons of **1** had dipole–dipole interaction with protein active site protons. The motional correlation time of bound ligand was calculated by double-selective relaxation rate at **1** $\tau_{2,3} = 40.5$ ns at 298 K, which showed **1** had high affinity with TnAChE. The experiments give a possible method to use TnAChE to locate the new huperzine A derivatives as AChE inhibitors.

1. Introduction

The enzyme acetylcholinesterase (AChE) catalyzes the hydrolysis of the ester bound of acetylcholine (ACh) to terminate the impulse transmitted action of ACh through cholinegic synapses.[1] The Torpedo Acetylcholinesterase contains 14 α-helices and 12 stranded mixed β-sheet. The mixed structure of AChE with its ligands showed it contained an 'active-site gorge'.[2] When ACh binds to this gorge, it is quickly hydrolyzed into acid and choline. Although the basic reason of Alzheimer's disease (AD) is not clear so far, AD is firmly associated with impairment in Cholinergic transmission by present study. A number of AChE inhibitors have been considered as candidates for the symptomatic treatment of AD as the most useful relieving strategy.[3] (−)-huperzine A (**1**) (Fig. 1) is a natural compound first isolated from Chinese medicine *Huperzia serrata* (Thumb.) in 1986.[4] Compound **1** is a potent, reversible and selective inhibitor of AChE with a rapid absorption and penetration into the brain in animal tests. It exhibits memory-enhancing activities in animal and clinical trials. Compared to tacrine and donepezil, **1** possesses a longer duration of action and higher therapeutic index, and the peripheral cholinergic side effects are minimal at therapeutic doses.[5] NMR is one of the most suitable methods capable of determining the conformation of the bound ligands. The small molecule protons spin-relaxation rates have been proved as a very suitable parameter in the ligand–macromolecules complexes studies. Even if the macromolecules concentration is just 0.5% of the ligands, the protons spin-relaxation rates of ligands will sensitively change to the binding process.[6,7] The parameters have been used in evaluating the tacrine derivatives interacting with AChE.[8] Here, we present NMR data of **1** and its analogue interacting with TnAChE to evaluated the possibility of using this method to procedure new AChE inhibitor of **1** derivatives.



1 **2**

Figure 1. Structure of huperzine A (**1**), and 8-*exo*-hydroxyphlegmariurine B (**2**).

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1 was isolated from *H. serrata* (Thumb.) as previously reported.[4] TnAChE was isolated from the electric organs of *Torpediniforms Nacline timelei* by affinity chromatography.[9] Its activity was assayed using a colorimetric method.[10] Solutions were prepared in deuterium oxide 100% (Sigma) buffered at 50 mM pH 7.0 (sodium phosphate buffer). All solutions were carefully deoxygenated by sealing off the sample after filling the nitrogen. All NMR experiments were carried out on Varian Inova 600 spectrometer at the controlled temperature of 298K.

Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the water resonance calibrated at 298 K. Proton spin-lattice relaxation rates were measured with inversion recovery pulse sequences and calculated by exponential regression analysis of recovery curves of longitudinal magnetization components. Single- and double-selective proton spin-lattice relaxation rates were measured with inversion recovery pulse sequences implemented with DANTE or double-DANTE sequences.[11,12] All relaxation rates were calculated in the initial rate approximation.[13]

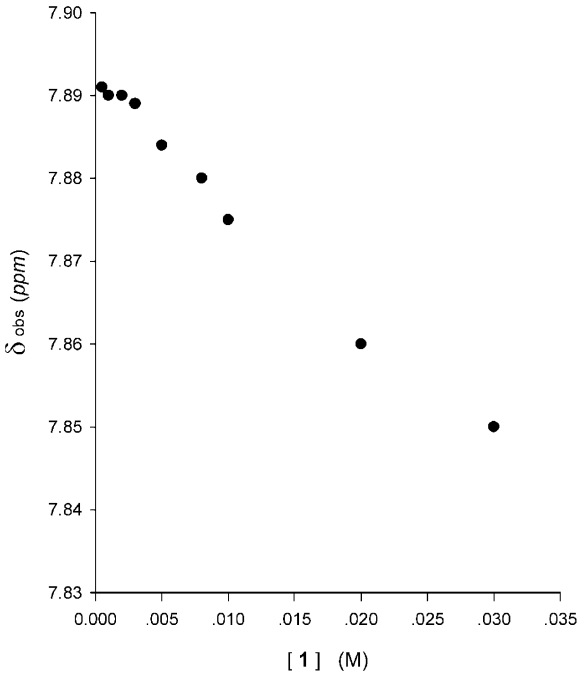

Figure 2. Dependence of ^{1}H NMR chemical shifts (ppm) of H3 in **1** upon concentration in deuterium oxide buffered at pH 7.0, T = 298 K.

2. Chemical shifts

If there were self-stacking occurrence in a ligand, the molecules of the ligand would exist in the solution in mixture of dimer and monomer forms in different concentration. Then the chemical shifts of ligand protons would be affected by concentration of the ligand. In order to observe the self-aggregation state of the sample, the chemical shifts of protons in **1** were investigated under different concentrations 0.2–30.0 mM. The concentration dependence of the ^{1}H NMR chemical shifts H-3 of **1** at 298 K was shown in Figure 2. The H-3 was upfield shifts by raising the concentration, which suggested the occurrence of co-operative auto-aggregation of solute molecules. The result showed at concentration $\leq$1 mM, the monomer was the predominant species in solution. At this concentration, we could get the actual proton spin-lattice relaxation rates, which would not affect by the self-aggregation of ligand.

3. Proton spin-lattice relaxation rates

The relaxation rates of protons in **1** were recorded in non-selective (R^{ns}) and selective (R^s) meaner in the absent (R_{free}) and in the present of AchE (R_{obs}) state. The non-selective (R^{ns}) and selective (R^s) meaner were decided by the following equations:

$$R_i^{ns} = \sum_{j\neq i}\rho_{ij} + \sum_{j\neq i}\sigma_{ij} + \rho_i^* \quad (1)$$

$$R_i^{s} = \sum_{j\neq i}\rho_{ij} + \rho_i^* \quad (2)$$

in the equations, ρ_{ij} and σ_{ij} are the direct- and cross-relaxation rates for any H_i–H_j interaction and sum is extended to all the dipolarly connected protons. ρ^* is contributions of other relaxation mechanisms.

Here we observed the parameter of H2, H3, H7, M10 and M16 protons in **1**. The data of H6, H8, H11 and H14 protons in **1** were ignored since the chemical shifts of H8, H11 was too closed and H6, H14 were AB system protons. There were not suitable shape 180 pulses to do the selective T1 for them. The proton relaxation rates were summarized in Table 1. The results showed, in [protein]:[ligand] = 1:0.005, all chemical shifts were almost unaffected (data were not shown) where almost all proton relaxation rates were selectively enhanced, R^s being much more affected than R^{ns}. Although most detected protons shown positive increasing in the present of TnAchE, the R^{ns} values were not affected much more, since binding to a macromolecular site, the increase R^{ns} part by dipole interaction was averaged by the negative value of σ_{ij} coming from slowing down of molecular motion. R^s showed **1** had strong intermolecular dipole–dipole interactions. The most affected protons were H7 > H3 > H2 > M16 > M10. The ΔR^s of aromatic ring protons were larger than methyl group protons.

4. The molecular motion rate

In order to ascertain **1** binding to TnAChE tightly to induce strong intermolecular dipole–dipole interactions. The molecular motion rate τ was calculated by the cross-relaxation rate, which obtained by double-selective relaxation method. Comparing the eqs 1 and 2, the main difference of R^{ns} and R^s was the absent of cross-relaxation rates:

$$\sigma_{ij} = R_i^{ij} - R_i^s \quad (3)$$

Table 1. 600 MHz ^{1}H NMR Parameters of **1** (1 mM) in D_2O buffered at pH 7.0, T = 298 K in the free and in the present of TnAChE (5 μM)

Proton name	δ (ppm)	Without AchE			With AchE		
		R^{ns} (s^{-1})	R^s (s^{-1})	R^{ns}/R^s (s^{-1})	R^{ns} (s^{-1})	R^s (s^{-1})	R^{ns}/R^s (s^{-1})
H2	6.57	0.51	0.36	1.42	0.54	0.57	0.90
H3	7.89	0.66	0.48	1.38	0.68	0.74	0.93
H7	3.82	1.24	0.95	1.31	1.27	1.31	0.96
M10	1.76	1.26	1.04	1.21	1.21	1.23	0.98
M16	1.60	1.27	1.15	1.09	1.22	1.37	0.90

where R_i^{ij} is the double-selective relaxation rate measured for Hi upon selective excitation of Hi and Hj, R^{sel} is the single-selective relaxation rate measured for Hi. If we gave a reasonable assumption that a fast exchange existed between the bound **1** and it's free state, there was a following equation:

$$\sigma_{obs}^{ij} = p_{free}\sigma_{free}^{ij} + p_{bound}\sigma_{bound}^{ij} \quad (4)$$

in the eq 4, the p fractions of ligand in each environment can be approximated by p_{bound} = [protein]/[ligand], p_{free} = 1- p_{bound} ~1. When protein concentration was very small, it made it possible to evaluate the σ_{bound}^{ij} in the bound state. Then the molecular motion rate τ_{ij} was estimated by eq 5.[14]

$$\sigma_{bound}^{ij} = \left(\sigma_{obs}^{ij} - \sigma_{free}^{ij}\right)/p_{bound} = \frac{1}{10}\frac{\gamma^4\hbar^2}{r_{ij}^6}\left\{\frac{6\tau_{ij}}{1+4\omega^2\tau_{ij}^2} - \tau_{ij}\right\} \quad (5)$$

where γ is the proton magnetogyric ratio, h is the reduced Plank's constant (= h/2π), r_{ij} is the Hi–Hj internuclear distance, ω is the proton Larmor frequency, and τ_{ij} is the motional correlation time characterizing reorientation of the Hi–Hj vector. Table 2 compared σ^{ij} in the presence of TnAChE and those in the free solution state. In the Table 2, σ_{obs}^{ij} were got in the presence of TnAChE and σ_{free}^{ij} were got in the free solution state. $\Delta\sigma^{ij}$ were the difference cross-relaxation rates after and before adding TnAChE. By the eq 4, we got the cross-relaxation rates in bound TnAChE status as setting p_{bound} = [protein]/[ligand] = 0.005:1. The change from relatively small positive to large negative values of σ^{ij} was consistent with slowing down of molecular motions from a region where ωτ <1 to one where ωτ >1. Here we used H2–H3 proton pairs $\sigma_{bound}^{23} = -11.2\ s^{-1}$ in **1** to calculate the motional time. If considered the distance of H2–H3 proton pairs r_{23} = 0.243 nm, by the eq 5, the motional cross-correlation time was calculated as τ_{23} = 40.5 ns in **1** at T = 298 K. This showed **1** was high affinity with TnAChE.

Table 2. Dipolar interaction energies measured for selected proton pairs of 1 mM **1** in D_2O buffered at pH 7.0 in the free and in the present of TnAChE (5 μM), T = 298K

Proton pair	σ_{free}^{ij} (s^{-1})	σ_{obs}^{ij} (s^{-1})	$\Delta\sigma^{ij}$ (s^{-1})	σ_{bound}^{ij} (s^{-1})
H_2–H_3	0.050	−0.006	−0.056	−11.2
H_7–H_8	0.055	0.032	−0.023	−4.6
H_{10}–H_{11}	0.115	0.020	−0.095	−19.0

5. Controlled experiment

When the ligand was bound to the active site of protein, the relaxation rate was affected by the slowing down of molecular motions as well as by the occurrence of intermolecular ^{1}H–^{1}H dipole–dipole interaction of protons in the protein core. If a ligand just sticks to the surface of a protein, it will also slowing down of the ligand motion and give some contributes to the molecular dipole interacting change. In order to make it clear that **1** combined to the active core of TnAChE, another small molecule 8-*exo*-hydroxyphlegmarine B (**2**), which was also an alkaloid got from *H. serreta*, was investigated the interaction with TnAChE. The results showed the R^{sel} of H7 and M16 in **2** did not change before and after adding TnAChE, which suggested that **2** be not easy to stick to the surface of TnAChE. Since **2** had the similar chemical properties with **1**, we are more sure **1** was binding in a selective meaner to the TnAChE.

6. Conclusion

By enhancement of R^{ns}/R^s value, the molecular motion rate and comparison with controlled compound, **1** combined to the TnAChE was observed undoubtedly. Although the addition concentration of TnAChE was just 5 μM, we still observed the spin-lattice relaxation change of protons in **1**. The experiments give us a possible method to use TnAChE to locate the new huperzine A derivatives as AChE inhibitors.

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