ELSEVIER

Biophysical Chemistry 129 (2007) 212–217

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Interactions of *Lycopodium* alkaloids with acetylcholinesterase investigated by ^{1}H NMR relaxation rate

Yiming Li [a,1], Guowei Yin [b,1], Wei Wei [b], Hengbin Wang [a], Shanhao Jiang [a], Dayuan Zhu [a], Weihong Du [b,*]

[a] *State Key Laboratory for Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 201203, China*

[b] *Department of Chemistry, Renmin University of China, Beijing 100872, China*

Received 26 March 2007; received in revised form 31 May 2007; accepted 31 May 2007
Available online 7 June 2007

Abstract

In order to further understand the interaction processes between the *Lycopodium* alkaloids and acetylcholinesterase, the binding properties of *N*-acetyl huperzine A (**1**), huperzine B (**2**) and huperzine F (**3**) with *Torpediniforms Nacline* acetylcholinesterase (TnAchE) were investigated by ^{1}H NMR methods. The nonselective, selective and double-selective spin-lattice relaxation rates were acquired in the absence and presence of TnAchE at a ratio of [ligand]/[protein] = 1:0.005. The selective relaxation rates show protons of **1–3** have dipole–dipole interaction with protons of TnAchE at the binding interface. The molecular rotational correlation time of bound ligands was calculated by double-selective relaxation rate at 298 K, which showed that **1–3** had high affinity with the protein. The results indicate that investigation of ^{1}H NMR relaxation data is a useful method to locate the new *Lycopodium* alkaloids as AchE inhibitors.

Keywords: Acetylcholinesterase; *Lycopodium* alkaloids; ^{1}H spin-lattice relaxation rate; Ligand–macromolecules interaction

1. Introduction

The enzyme acetylcholinesterase (AchE) catalyzes the hydrolysis of the ester bond of acetylcholine (Ach) to terminate the impulse transmitted action of Ach through cholinergic synapses [1]. The Torpedo Acetylcholinesterase contains 14 α-helices and 12 stranded mixed β-sheet. The mixed structures of AchE with its ligands have showed that AchE contains 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 (**4**) (Fig. 1) is a natural compound first isolated from Chinese medicine *Huperzia serrata* (Thumb.) in 1986 [4]. **4** 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, which are also AchE inhibitors used as drugs in the market, **4** possesses a longer duration of action and higher therapeutic index as well as less peripheral cholinergic side effects at therapeutic dose [5]. To find more powerful inhibitors of AchE, many alkaloids have been isolated from *H. serrata*.

Nuclear magnetic resonance (NMR) has been widely used for studying interactions of small molecules (i.e. ligands) with macromolecules (i.e. receptors and enzymes) [6], due to the large number of spectral parameters that can be measured and analyzed (chemical shift [7], relaxation rates and line width [8], diffusion coefficients [9] or intermolecular magnetization transfer techniques such as NOE [10,11]. Among them, the proton spin relaxation rate of the small molecule has proved to be a very suitable parameter in the ligand–macromolecule complex studies.

* Corresponding author. Tel.: +86 10 62512660; fax: +86 10 62516444.
E-mail addresses: dyzhu@mail.shcnc.ac.cn (D. Zhu), whdu@ruc.edu.cn (W. Du).
[1] These authors contribute equally to this paper.

0301-4622/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2007.05.020

In these studies, NMR investigation is based on the comparison of selective (R^{se}) and non-selective (R^{ns}) proton spin-lattice relaxation rate of the ligand in the presence and absence of the macromolecular receptor. The formation of intermolecular adducts affects R^{ns} and R^{se} at different extents, depending on the dynamical parameters (i.e. molecular rotational correlation time t_c), assuming fast chemical exchange between the bound and the free environments. In particular, the slower rotational tumbling of the ligand–macromolecule complex mainly affects R^{se}. Even if the macromolecule concentration is just 0.5% of a ligand, the R^{se} value of the ligand's protons will sensitively change due to the binding process [12,13].

The parameters R^{se} and t_c have been used in evaluating the tacrine derivatives and huperzine A interacting with AchE [14,15]. In this paper, the characterization of the interaction between AchE and three *lycopodium* alkaloids: *N*-acetyl huperzine A (**1**), huperzine B (**2**) and huperzine F (**3**) (Fig. 1) were investigated using NMR spectroscopy.

2. Materials and methods

2.1. Sample preparation and chemical characterization

The huperzine A and three *lycopodium* alkaloids were isolated from the whole plant of *H. serrata* (Thumb.) Trev. (Huperziaceae) by using the same procedure as described previously [16]. Chromatography of the crude alkaloids of the whole plant of *H. serrata* on SiO_2 and elution with $CHCl_3$–Me_2CO followed by MeOH afforded two alkaloid-rich fractions. Repeated chromatography of the MeOH fraction over neutral Al_2O_3 and SiO_2 afforded *N*-acetyl huperzine A, huperzine B and huperzine F, whose structures were identified using various NMR spectroscopy data [4,16]. AchE from *Torpedo californa* was obtained from Sigma and used without further purification. Solutions were prepared in 99.9% deuterium oxide (CIL) buffered at pH 7.0 (phosphate saline buffer). All solutions were carefully deoxygenated by sealing off the NMR tube after filling the nitrogen. In all the experiments ligand concentration was 1.0 mM and the protein concentration was 5.0 μM.

2.2. NMR spectroscopy

All measurements were performed on a Bruker Avance 400 MHz NMR spectrometer operating at 400.13 MHz for hydrogen. All experiments were carried out at a temperature of 298 K. ^{1}H spectra were recorded using a BBO broadband probe. 16 scans were collected into 32 K data points giving a digital resolution of 0.13 Hz/point at a spectral width of 4006 Hz. To evaluate the data and calculate relaxation times, the XWINNMR program package (Version 3.5) was used on a Microsoft Windows PC. Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the water resonance calibrated at 298 K. The spin-lattice relaxation rates were measured using the $(180°\text{-}\tau\text{-}90°\text{-}t)_n$ sequence. The τ values used for the selective and nonselective experiments

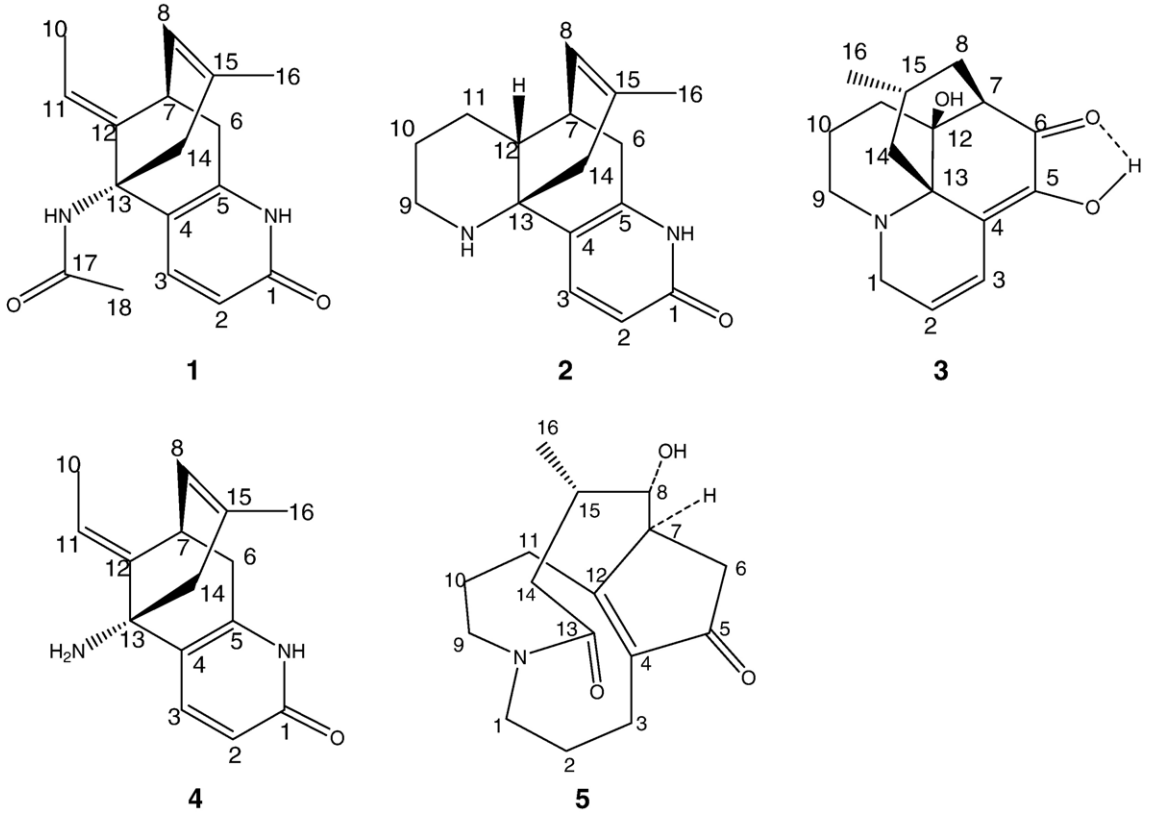

Fig. 1. Chemical structures of **1**–**5**.

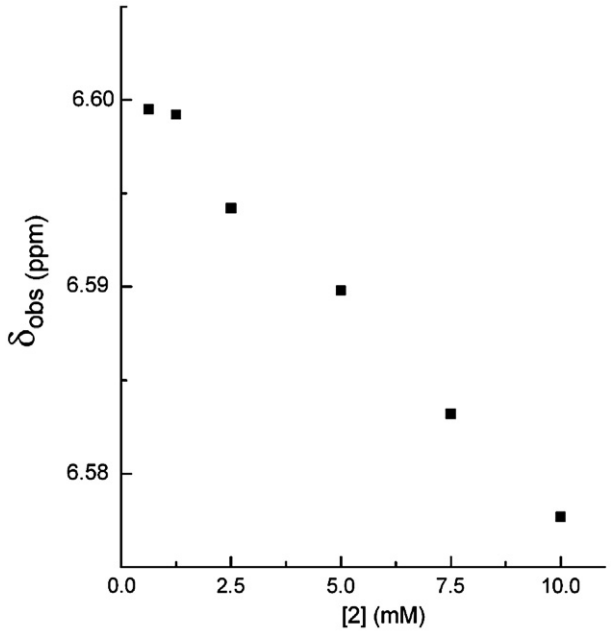

Fig. 2. Dependence of ^{1}H NMR chemical shifts (ppm) of H_2 in **2** upon concentration in deuterium oxide buffered at pH 7.0, T=298 K.

were: 0.01, 0.1, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.5, 2.0, 2.5, 3.0 and 5.0 s respectively, and the delay time t in this case is 10 s. The 180° selective inversion of the proton spin population was obtained by a selective Gauss1.100 shape pulse with a length of 47 ms and a power of 60 dB corresponding to an excitation width of about 45 Hz. Relaxation times were calculated by exponential regression analysis of recovery curves of longitudinal magnetization components. Thereby an area fit of the individual peak was performed. Single- and double-selective proton spin-lattice relaxation rates were measured with inversion recovery pulse sequences implemented with DANTE or double-DANTE sequences [17,18]. All relaxation rates were calculated in the initial rate approximation [19].

3. Results and discussion

3.1. Chemical shifts

If there was self-stacking occurrence in a ligand, at different concentration, the ligand molecule would exist as a mixture with varying ratio of dimer over monomer in solution. 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 H_2 in compounds **1–3** were investigated under different concentrations ranging 0.2–10.0 mM. The concentration dependence of the chemical shifts of the proton H_2 in **2** at 298 K was shown in Fig. 2. The H_2 resonance was upfield shifted with the concentration raising, which suggested the occurrence of cooperative auto-aggregation of solute molecules. The result showed that the monomer was the predominant species in solution at the concentration of 1 mM. At this concentration, we could get the actual proton spin-lattice relaxation rates, which would not be affected by the self-aggregation of ligand.

3.2. Proton spin-lattice relaxation rates

The relaxation rates of protons in **1–3** were measured in non-selective (R^{ns}) and selective (R^{se}) manner in the absence (R_{free}) and in the presence of AchE (R_{obs}) state. The non-selective (R^{ns}) and selective (R^{se}) manner were decided by the following equations [14]:

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

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

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

The explicit forms of R_i^{ns} and R_i^{se} are:

$$R_i^{ns} = \frac{1}{10}\frac{\gamma_H^4\hbar^2}{r_{ij}^6}\left[\frac{3\tau_c}{1+\omega_H^2\tau_c^2} + \frac{12\tau_c}{1+4\omega_H^2\tau_c^2}\right] \quad (3)$$

$$R_i^{se} = \frac{1}{10}\frac{\gamma_H^4\hbar^2}{r_{ij}^6}\left[\frac{3\tau_c}{1+\omega_H^2\tau_c^2} + \frac{6\tau_c}{1+4\omega_H^2\tau_c^2} + \tau_c\right] \quad (4)$$

Eqs. (3) and (4) indicate: at the fast molecular reorientation time regime ($\omega_H\tau_c \ll 1$), which is typical for the free ligand, it gives $R_i^{ns} > R_i^{se}$, and at the slow motion time regime ($\omega_H\tau_c \gg 1$), which is typical for a ligand in complex with a macromolecule, it gives $R_i^{ns} < R_i^{se}$.

After detailed investigation of ^{1}H NMR spectra of **1–3**, the well resolved peaks were chosen for observation. For the overlap peaks there were not suitable 180° shape pulses to do the selective T_1 for them.

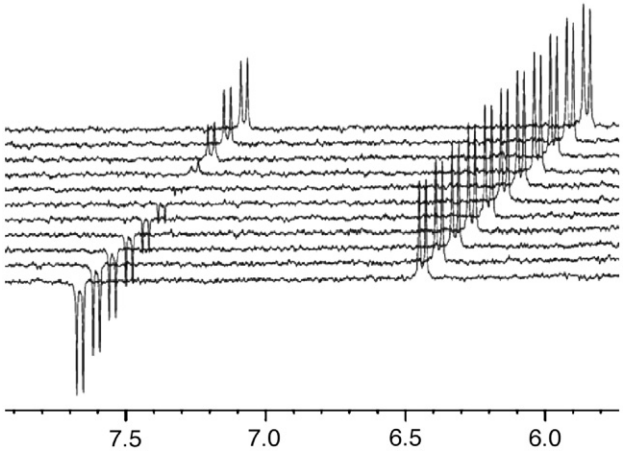

Fig. 3. Selective relaxation spectra of aromatic proton of *N*-acetyl huperzine A solution in 1 mM. The selective measurements refer to the H_3 proton.

We measured the relaxation rate constants of H_2, H_3, H_7, H_8, M_{10} and M_{16} protons in **1**, H_2, H_3 and M_{16} in **2**, H_2, H_3, H_7 and M_{16} in **3**. Experimental spectra of H_3 proton in **1** used for R^{se} measurement were shown in Fig. 3. The chemical shifts, non-selective and selective proton relaxation rates of the ligands **1**–**3** in the absence and presence of TnAchE were summarized in Table 1. At a ratio of [protein]/[ligand]= 0.005:1, it showed that all chemical shifts were almost unaffected in the absence or presence of macromolecule; while almost all proton selective relaxation rates were enhanced in the presence of macromolecule. R^{se} is much more affected than R^{ns}, although most detected protons also displayed positive increasing of R^{ns} in the presence of TnAchE. Upon binding to a macromolecular, the increase of the auto-relaxation was concomitantly cancelled mostly by the negative cross-relaxation due to the slow molecular tumbling, resulting R^{ns} is less affected by the binding process.

In Table 1, for most protons of **1**–**3**, $R_i^{ns} > R_i^{se}$ was obtained in the absence of TnAchE, while $R_i^{ns} < R_i^{se}$ was obtained in the presence of TnAchE. Correspondingly, the value of R_i^{ns}/R_i^{se} changed from being larger than 1 in the absence of macromolecule to being less than 1 in the presence of macromolecule. The exceptions were H_2, H_3 and M_{16} in **2**, where the values of R_2^{ns}/R_2^{se} (without TnAchE) were less than 1, suggesting a negative value of σ^{ij} in free ligand. However, when TnAchE was added, a higher increase of R_2 value than that of R_2^{ns} was still observed, resulting the decrease of the value of R^{ns}/R^{se} in **2** too. Therefore, the decrease of the value of R_i^{ns}/R_i^{se} was an important indication of the occurring of the interaction of **1**–**3** with TnAchE.

Table 1
400 MHz ^{1}H NMR parameters of 1–4 (1 mM) in D_2O buffered at pH 7.0, T=298 K in the absence and presence of TnAchE (5 μM)

Protons	Free ligand				Ligand+TnAchE			
	δ (ppm)	R^{ns} (s^{-1})	R^{se} (s^{-1})	R^{ns}/R^{se}	δ (ppm)	R^{ns} (s^{-1})	R^{se} (s^{-1})	R^{ns}/R^{se}
N-acetyl huperzine A (**1**)								
H_2	6.45	0.46	0.36	1.27	6.45	0.52	0.58	0.90
H_3	7.67	0.65	0.52	1.25	7.67	0.75	0.79	0.95
H_7	3.74	1.60	1.13	1.42	3.74	1.05	1.12	0.93
H_8	5.56	0.83	0.67	1.24	5.56	1.00	1.01	0.99
M_{10}	1.66	1.29	1.17	1.10	1.66	1.36	1.40	0.97
H_{11}	5.35	0.46	0.39	1.18	5.35	0.57	0.60	0.96
M_{16}	1.55	1.44	1.36	1.06	1.55	1.47	1.57	0.94
Huperzine B (**2**)								
H_2	6.60	0.56	0.65	0.87	6.60	0.59	0.73	0.81
H_3	7.78	0.89	1.03	0.86	7.78	0.92	1.12	0.82
M_{16}	1.57	1.22	1.34	0.91	1.57	1.25	1.38	0.91
Huperzine F (**3**)								
H_2	5.80	0.81	0.65	1.24	5.80	0.92	0.91	1.01
H_3	6.85	0.50	0.45	1.12	6.85	0.64	0.72	0.89
H_7	2.67	0.90	0.74	1.21	2.67	1.00	1.00	1.00
M_{16}	0.92	2.25	2.18	1.03	0.92	2.21	2.24	0.99
Huperzine A (**4**)[a]								
H_2	6.57	0.51	0.36	1.42	6.57	0.54	0.57	0.90
H_3	7.89	0.66	0.48	1.38	7.89	0.68	0.74	0.93
H_7	3.82	1.24	0.95	1.31	3.82	1.27	1.31	0.96
M_{10}	1.76	1.26	1.04	1.21	1.76	1.21	1.23	0.98
M_{16}	1.60	1.27	1.15	1.09	1.60	1.22	1.37	0.90

[a] Data were published in Ref. [15].

Table 2
The molecular motional correlation time measured for selected proton pairs in D_2O buffered at pH 7.0, T=298 K in the absence and presence of TnAChE (5 μM)

Proton pair	σ_{free}^{ij} (s^{-1})	σ_{obs}^{ij} (s^{-1})	$\Delta\sigma^{ij}$ (s^{-1})	σ_{bound}^{ij} (s^{-1})	τ_{free}^{ij} (s)	τ_{bound}^{ij} (s)
N-acetyl huperzine A (**1**)						
H_2–H_3	0.106	−0.016	−0.122	−24.4	1.95×10^{-11}	2.21×10^{-8}
H_7–H_8	0.359	0.178	−0.181	−36.2	7.68×10^{-11}	3.27×10^{-8}
Huperzine B (**2**)						
H_2–H_3	−0.023	−0.043	−0.020	−4.0	4.57×10^{-10}	3.66×10^{-9}
Huperzine F (**3**)						
H_2–H_3	0.086	0.036	−0.05	−10.0	1.57×10^{-11}	9.05×10^{-9}
Huperzine A (**4**)[a]						
H_2–H_3	0.05	−0.006	−0.056	−11.2	3.30×10^{-10}	4.05×10^{-8}

[a] Data were published in Ref. [15].

3.3. Molecular rotational correlation time

Comparing Eqs. (1) and (2), the main difference of R^{ns} and R^{se} was the absence of cross-relaxation rates σ^{ij} in R^{se}. Therefore, the σ^{ij} can be calculated from double-selective relaxation rate as:

$$\sigma^{ij} = R_i^{ij} - R_i^{se} \tag{5}$$

where R_i^{ij} is the double-selective relaxation rate measured for H_i upon selective excitation of H_i and H_j, R_i^{se} is the single-selective relaxation rate measured for H_i. Assuming a fast exchange between the ligand in the free state and in the bound state with the macromolecule, the observed cross-relaxation rate of the ligand in the presence of the macromolecule, s_{obs}^{ij} can be well approximated as:

$$\sigma_{obs}^{ij} = p_{free}\sigma_{free}^{ij} + p_{bound}\sigma_{bound}^{ij} \tag{6}$$

in Eq. (6), σ_{free}^{ij} and σ_{bound}^{ij} are the cross-relaxation rates of the ligand in free and in bound state, respectively. the fraction of ligand in bound state, p_{bound} can be approximated by p_{bound}= $[protein]_T/[ligand]_T$, $[protein]_T$ and $[ligand]_T$ are the total concentrations of the macromolecule and the ligand in solution, respectively. When the protein concentration was very low, $[ligand]_T \gg [protein]_T$, and the fraction of ligand in free state, $p_{free}=1-p_{bound}\sim 1$. The cross-relaxation rate of the ligand in bound state, σ_{bound}^{ij} can then be calculated by Eq. (7):

$$\sigma_{bound}^{ij} = \left(\sigma_{obs}^{ij} - \sigma_{free}^{ij}\right)/p_{bound} = \Delta\sigma^{ij}/p_{bound}. \tag{7}$$

In order to ascertain if **1**–**3** tightly bind to TnAchE, the molecular rotational correlation time t_c in the free and bound

state, (τ_{free}^{ij} and τ_{bound}^{ij} in Table 2, respectively) were calculated using Eq. (8) from the cross-relaxation rate,

$$\sigma^{ij} = \frac{1}{10}\frac{\gamma^4\hbar^2}{r_{ij}^6}\left[\frac{6\tau_c}{1+4\omega^2\tau_c^2} - \tau_c\right] \quad (8)$$

where γ is the proton magnetogyric ratio, $\hbar$ is the reduced Plank's constant ($=h/2\pi$), r_{ij} is the $H_i - H_j$ internuclear distance, ω is the proton Larmor frequency, σ^{ij} is the cross-relaxation rate (σ_{free}^{ij} and σ_{bound}^{ij} for the ligand in free and in bound state, respectively) and the obtained t_c is the molecular rotational correlation time.

In **1**–**3**, we use the aromatic amidated ring H_2–H_3 proton pair and H_7–H8 proton pair to characterize the molecular rotational correlation time of the ligands in free and in bound state with TnAchE. In Table 2, σ_{obs}^{ij} was measured in the presence of TnAchE and σ_{free}^{ij} was measured for the free ligands. $\triangle\sigma^{ij}$ was the difference of the cross-relaxation rate of the ligands in presence and in the absence of TnAchE Eq. (7). The cross-relaxation rates of the bound ligands were calculated using Eq. (7) with p_{bound}=[protein]/[ligand]=0.005:1. In consideration of the distance between the protons r_{ij}=0.243 nm, the motional correlation time t_c (τ_{free}^{ij} and τ_{bound}^{ij} for the ligand in free and in bound state, respectively) was calculated by Eq. (8). In Table 2, it was showed that, in **1** and **3**, there were changes from relatively small positive values of σ_{free}^{ij} to large negative values of σ_{bound}^{ij}; and in **2**, there was change from relatively small negative value of σ_{free}^{ij} to large negative value of σ_{bound}^{ij}, resulting in the change of τ value from *ps* timescale to *ns* timescale when TnAchE was added in. These were consistent with slowing down of molecular motions which were observed in proton spin-lattice relaxation rates test.

3.4. Control experiment

When the ligand was bound to the active site of protein, the relaxation rate was affected by the slowing down of molecular motions, increasing in viscosity in a relatively high concentration of proteins, as well as by the occurrence of intermolecular ^1H–^1H dipole–dipole interaction of protons at the binding interface. If a ligand just sticks to the surface of a protein, it will also slow down the ligand's motion and give some contributions to the molecular dipole–dipole interaction. In the previous study [15], the small molecule 8-exo-hydroxyphlegmarine B (**5**), which was also an alkaloid obtained from *H. serreta*, was investigated on its interaction with TnAchE. The results showed that the R^{se} of H_7 and M_{16} in **5** did not change before and after adding TnAchE, which suggested that **5** be not easy to stick to the surface of TnAchE. Since **5** had the similar chemical properties with **1**–**3**, we were much sure that **1**–**3** bound in a selective manner to the TnAchE.

4. Conclusion

In presence of the TnAchE, the decrease of the value R_i^{ns}/R_i^{se} of the ligand compared to that in free state, and the decrease of the molecular rotational correlation time of the ligands as suggested by the negative change of the values of σ_{free}^{ij} σ_{bound}^{ij} undoubtedly revealed that *Lycopodium* alkaloids **1**–**3** bound to TnAchE at a ratio of [ligand]/[protein]=1:0.005. These results demonstrated that investigation of NMR relaxation rate is a very useful method to locate the new huperzine derivatives as AchE inhibitors.

Acknowledgments

We thank Prof. Zhifen Li from Institute of Physical Chemistry, Peking University and Dr. Kaifeng Hu from Laboratory of Chemical Physics, NIDDK, NIH for helpful discussion. The authors are grateful for supports by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Ministry Education; and by the National Natural Science Foundation of China grant nos. 90409015 and 20473013.

References

[1] L. Stryer, in: L. Stryer (Ed.), Biochemistry, 4th ed., WH Freeman, San Francisco, 1995, p. 1017.
[2] H. Dvir, D.M. Wong, M. Harel, X. Barril, M. Orozco, F.J. Luque, D. Munoz-Torrero, P. Camps, T.L. Rosenberry, I. Silman, J.L. Sussman, 3D structure of *Torpedo californica* acetylcholinesterase complexed with huprine X at 2.1 Å resolution: kinetic and molecular dynamic correlates, Biochemistry 41 (2002) 2970–2981.
[3] R.M. Howes, S.L.N. Perry, P.J. Houghton, Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders, Phytother, Res. 17 (2003) 1–17.
[4] J.S. Liu, C.M. Yu, Y.Z. Zhou, The chemical structures of huperzines A and B, Acta Chem. Sin. 44 (1986) 1035–1039.
[5] D. Bei, X. Tang, X. He, Huperzine A, a potential therapeutic agent for treatment of Alzheimer's disease, Curr. Med. Chem. 7 (2000) 355–374.
[6] B.J. Stockman, C. Dalvit, NMR screening techniques in drug discovery and drug design, Prog. Nucl. Magn. Reson. Spectrosc. 41 (2002) 187–231.
[7] M.P. Foster, D.S. Wuttke, K.R. Clemens, W. Jahnke, I. Radhakrishnan, L. Tennant, M. Reymond, J. Chung, P.E. Wright, Chemical shift as a probe of molecular interfaces: NMR studies of DNA binding by the three amino-terminal zinc finger domains from transcription factor IIIA, J. Biomol. NMR 12 (1998) 51–71.
[8] S. Ravindranathan, J.M. Mallet, P. Sinay, G. Bodenhausen, Transferred cross-relaxation and cross-correlation in NMR: effects of intermediate exchange on the determination of the conformation of bound ligands, J. Magn. Reson. 163 (2003) 199–207.
[9] T. Brand, E.J. Cabrita, S. Berger, S. Berger, Intermolecular interaction as investigated by NOE and diffusion studies, Prog. Nucl. Magn. Reson. Spectrosc. 46 (2005) 159–196.
[10] J. Clarkson, I.D. Campbell, Studies of protein–ligand interactions by NMR, Biochem. Soc. Trans. 31 (2003) 1006–1009.
[11] A.P. Zabell, C.B. Post, Docking multiple conformations of a flexible ligand into a protein binding site using NMR restraints, Proteins 46 (2002) 295–307.
[12] E. Gaggelli, G. Valensin, T. Kushnir, G. Navon, Determination of absolute values of dipolar cross-relaxation rates for ligands bound to macromolecules using double-selective T1, Magn. Reson. Chem. 30 (1992) 461–465.
[13] G. Veglia, M. Delfini, M.R. Del Giudice, E. Gaggelli, G. Valensin, ^1H NMR studies on the interaction of b-carboline derivatives with human serum albumin, J. Magn. Reson. 130 (1998) 281–286.
[14] M. Delfini, R. Gianferri, V. Dubbini, C. Manetti, E. Gaggelli, G. Valensin, ^1H NMR relaxation investigation of inhibitors interacting with *Torpedo californica* Acetylcholinesterase, J. Magn. Reson. 144 (2000) 129–133.
[15] Y. Li, Q. Li, M. Sun, G. Song, S. Shan, D. Zhu, ^1H NMR relaxation investigation of acetylcholinesterase inhibitors from huperzine A and derivative, Bioorg. Med. Chem. Lett. 14 (2004) 1585–1588.

[16] D.Y. Zhu, M.F. Huang, B.D. Wang, X.M. Kong, Y.Q. Yang, Study on the structures of the huperzine E and F, Chin. J. Appl. Environ. Biol. 2 (1996) 352–355.
[17] G.A. Morris, R. Freeman, Selective excitation in Fourier transform nuclear magnetic resonance, J. Magn. Reson. 29 (1978) 433–462.
[18] H. Geen, X.L. Wu, P. Xu, J. Friedrich, R. Freeman, Selective excitation at two arbitrary frequencies: the double-DANTE sequence, J. Magn. Reson. 81 (1989) 646–652.
[19] R. Freeman, H.D.W. Hill, B.L. Tomlinson, L.D. Hall, Dipolar contribution to NMR spin-lattice relaxation of protons, J. Chem. Phys. 61 (1974) 4466–4473.