ChemComm
Chemical Communications

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: H. R. Lim, S. Y. Kim, E. H. Jeon, Y. L. Kim, Y. M. Shin, T. Koo, S. J. Park, K. B. Hong and S. Choi, *Chem. Commun.*, 2019, DOI: 10.1039/C9CC04172A.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/chemcomm

View Article Online
DOI: 10.1039/C9CC04172A

COMMUNICATION

A highly sensitive fluorescent probe that quantifies transthyretin in human plasma as an early diagnostic tool of Alzheimer's disease

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Hye Rim Lim,[a,d] Seo Yun Kim,[a,d] Eun Hee Jeon,[a] Yun Lan Kim,[a] Yu Mi Shin,[a] Tae-Sung Koo,[a] Sung Jean Park,[c] Ki Bum Hong, [b,*] Sungwook Choi [a,*]

The development of sensitive and reliable fluorescent probes for the early diagnosis of Alzheimer's disease (AD) is highly challenging and plays an important role in achieving effective treatments. Herein, we designed and synthesized an indole-based fluorophore for TTR in human plasma, an important hallmark of AD pathogenesis. This robust and simple fluorescent method allows quantification of TTR in the complex biological matrix.

Alzheimer's disease (AD) is an age-associated neurodegenerative disorder and the most common form of dementia, affecting more than 50 million people worldwide. AD is characterized by progressive loss of memory and cognitive function, eventually leading to death. The major neuropathological hallmarks of the disease are the progressive accumulation of extracellular senile plaques, consisting of amyloid β peptide (Aβ) aggregates and intraneuronal neurofibrillary tangles constituted by hyperphosphorylated forms of tau protein.[1] Aβ peptides are produced by sequential proteolytic cleavage of the amyloid precursor protein (APP) by β– and γ–secretases.[2] Under abnormal conditions, this eventually leads to the accumulation of Aβ monomers, oligomers, and fibrillary aggregates, triggering neurodegeneration. The underlying mechanisms for AD remain elusive but substantial genetic, pathological and biochemical evidence suggest that the ultimate therapy would involve lowering Aβ peptide levels through regulating the balance between Aβ production and clearance.[3]

Transthyretin (TTR) is a homotetrameric protein composed of four 15 kDa, β-sheet rich subunits and circulates in the blood and cerebrospinal fluid (CSF). TTR is independently synthesized in the liver and choroid plexus, after which is secreted into the blood (3-7 μM) and CSF (0.1-0.4 μM), respectively.[4] While CSF TTR is the primary carrier of thyroid hormone thyroxine (T_4), serum TTR transports mainly the *holo*-retinol binding protein (RBP), with more than 99% of its T_4 binding sites being unoccupied.[4a] Interestingly, although TTR is known to be one of the human amyloidogenic proteins representative of pathological conditions, TTR has also been implicated in the neuroprotection of AD. Evidence for the latter emerged from the following results: (1) TTR is one of the major Aβ-binding proteins in human CSF, suppressing Aβ aggregation[5] and is involved in brain Aβ efflux[6] and peripheral clearance through proteolytic functions;[7] (2) overexpression of WT-TTR in an APP23 transgenic mouse model showed improved cognitive functions;[8] (3) TTR levels are reduced both in the CSF and the blood of AD patients, compared with age-matched controls.[9] Therefore, investigations on diagnostic tool and therapeutic drug developments using TTR's unique biological role are actively underway.

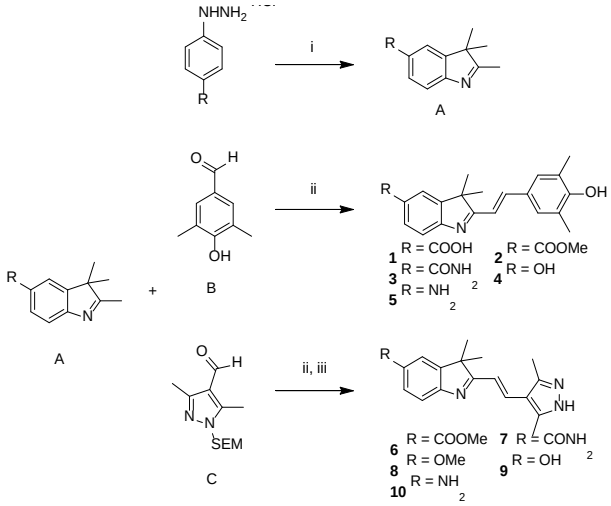
Various methods have been employed to determine TTR levels in the CSF and blood, including mostly immunoassays and 2D gel electrophoresis–mass spectrometry.[9a-c, 10] Most methods suffer from a lack of consistency and reliability due to the difficulty of standardizing detection methods. However, fluorescence methods present as powerful tools by virtue of their excellent sensitivity, simplicity, rapid response, and cost-effective instrumentation.[11] More importantly, TTR biomarkers that can be detected in the plasma have greater diagnostic value and are easier to access than CSF-based biomarkers. In this study, we designed and synthesized indole-based fluorescent probes for early detection of AD through measurements of blood concentrations of TTR because indole possess desirable photophysical properties and high sensitivity to its environment.[12]

Indole-based compounds **1-10** were synthesized following the synthetic route depicted in Scheme 1. Indole derivatives **A** were obtained from direct nitration and reduction of

[a] *Graduate School of New Drug Discovery and Development, Chungnam National University, Daejon, 305-764, Republic of Korea. E-mail: swchoi2010@cnu.ac.kr; Fax: +82 42-821-8927; Tel: +82 42-821-8625*
[b] *New Drug Development Center (NDDC), Daegu-Gyeongbuk Medical Innovation Foundation (DGMIF), 80 Cheombok-ro, Dong-gu, Daegu 701-310, Republic of Korea. E-mail: kbhong@dgmif.re.kr; Fax (+82)-53-790-5219; Tel: +82 53-790-5272*
[c] *College of Pharmacy, Gachon University, 534-2 Yeonsu 3-dong, Yeonsu-gu, Incheon 406-799, Republic of Korea*
[d] *These authors contributed equally to this work*

† Electronic Supplementary Information (ESI) available: General synthetic procedures, spectral data and other supplementary information. See DOI:

View Article Online
DOI: 10.1039/C9CC04173A



Scheme 1. Synthesis of the probes **1**-**10**. (i) 3-methyl-2-butanone, AcOH, 118 °C; (ii) HCl, EtOH, 80 °C; (iii) 4 M HCl in dioxane, 60 °C

unsubstituted indole or the reaction of substituted phenylhydrazine with 3-methyl-2-butanone. Compounds **1-5** were synthesized by reacting the HCl salt of substituted indoles **A** with compounds **B**. Compounds **6-10** were synthesized by reacting the HCl salt of substituted indole **A** with compounds **C**, followed by the deprotection of their SEM group using 4 M HCl in dioxane.

The absorption and fluorescence properties of **1**-**10** in PBS and in the presence of WT-TTR in PBS were assessed as shown in Table 1, Figure 1 and Figure S1 (see ESI). Probes **2**, **4** and **10** exhibited a strong increase in fluorescence intensity (turn-on response) upon binding to WT-TTR in PBS relative to other probes. Incubation of probes **2**, **4**, and **10** (4 μM) with WT-TTR

Table 1 Spectroscopic properties of probes **1**-**10** (4 μM) upon binding to WT-TTR (2 μM) in PBS

Comp	λ_{ex} (nm)	λ_{em} (nm)	Fold[a]	Stokes shift (nm)	RFI [b]	Φ_f
1	336	480	5	144	0.04	
2	460	566	920	106	0.35	0.178[c]
3	459	558	210	99	0.21	0.024[c]
4	389	513	11	124	0.14	0.057[c]
5	388	513	13	125	0.62	0.267[c]
6	329	450	3	121	0.01	
7	324	452	2	128	0.14	
8	334	457	4	123	0.03	
9	382	468	26	86	0.23	0.065[d]
10	357	476	111	119	1.00	0.379[d]

[a] Fold increase of fluorescence intensity after incubation with WT-TTR in PBS, compared to probe alone in PBS.
[b] Relative fluorescence intensity ratio was defined as the ratio of the fluorescence intensity of probes over that of **10**.
[c] Coumarin 503 was used as reference for quantum yield measurements.
[d] Quinine sulfate used as reference for quantum yield measurements.

(2 μM) in PBS showed 920-, 210- and 111-fold increases in fluorescence intensity, compared to each compound alone in PBS, respectively. A blue shift in emission maximum was also observed. The quantum yields were also measured using coumarin 503 and quinine sulfate as the reference compounds. Probes **2**, **5**, and **10** display significant values (0.178, 0.252, and 0.379) in the quantum yield relative to other probes. A large stokes shift is a beneficial photophysical property for imaging and sensing applications because these probes can show a low possibility of self-quenching.[13] From this perspective, almost all designed indole probes in this study may be promising TTR sensing probes as they exhibit large stokes shifts ($\Delta\lambda$ = 100 ~ 120 nm), whereas typical fluorophore dyes such as fluorescein dyes, rhodamine, and BODIPY all have small stokes shift ($\Delta\lambda \leq$ 70 nm).[14] Therefore, based on their relative fluorescence intensity, stokes shift, and quantum yield, probe **10** was comprehensively selected for further examination and assessment as a TTR sensing probe.

In order to confirm that the probe selectively binds to the two T_4 binding sites of WT-TTR, probe **10** was incubated with a double mutant TTR (F87M, L110M) that adopts a monomeric structure (M-TTR).[15] and measured for its fluorescence spectrum (Fig. 1). The fluorescence intensity of probe **10** changed from 4 to 476, with a 111-fold increase in the presence of 2 μM WT-TTR, whereas the same concentration of M-TTR did not induce any significant changes, similarly to the results with probe **10** alone in PBS. These observations indicated that probe **10** detects only the tetramerically folded TTR. Furthermore, Job plot experiments were carried out to determine the binding stoichiometry of the WT-TTR–probe **10** complexes (Fig. 1); the results suggest that the formation of the TTR–probe **10** complex followed a 1:1.42 stoichiometry.

The medium effect in different organic solvents was then examined to gain a better insight into the environmental sensitivity of probe **10**'s fluorescence (Fig. 2a). While slight fluorescence changes in aprotic solvents (toluene, THF, ACN, DCM) were observed, a significant bathochromic shift of its emission spectrum was observed in protic solvents (EtOH and water). The environmental sensitivity of probe **10** was observed in which the fluorescence intensity of probe **10** in a relatively less polar solvent (acetonitrile) was gradually decreased by an

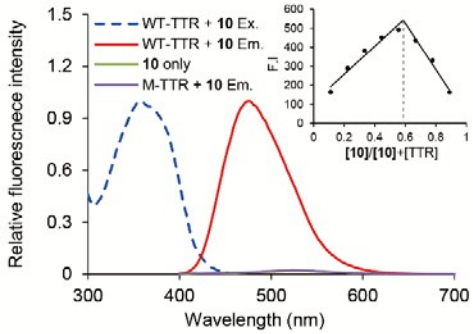


Fig. 1 Fluorescence emission spectra of probe **10** (4 μM) after incubation with WT-TTR (2 μM, red trace) and M-TTR (8 μM, solid purple trace) in PBS (10 mM sodium phosphate, 100 mM KCl, 1 mM EDTA, pH 7.0). The inset shows a Job plot analysis of TTR–probe **10** complexes recorded by the mixture of TTR and probe **10** at different ratios, at a constant total concentration of 4.5 μM.

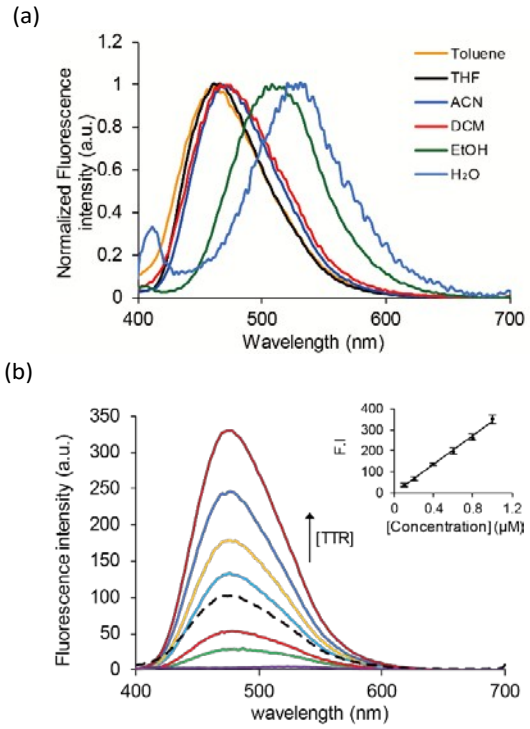


Fig. 2 (a) Normalized emission spectra of probe **10** (4 μM) in various solvents. (b) The fluorescence spectra of probe **10** (4 μM) in the presence of different TTR concentrations (0-1 μM) in PBS and 18-fold diluted human plasma (dotted line). Insert shows the linear relationship between fluorescence enhancement of probe **10** and TTR concentrations in PBS. λ_{ex} = 357 nm

increase of polar solvent (water) ratio along with bathochromic shifts (54 nm, Fig S2a, see ESI). In addition, a similar phenomenon was observed in the nonpolar methylene chloride and the polar methanol system (Fig. S2b, see ESI). Collectively, these observations indicate that a dramatic increase in the fluorescence intensity and the strong dependence of emission maximum of probe **10** upon binding to WT-TTR in PBS results from the intramolecular charge transfer[16] and occupancy of hydrophobic T_4-binding pockets on WT-TTR.

To examine our accuracy, the linearity of fluorescence change in response to TTR concentration was examined. As shown in Figure 2b and S2c, fluorescence intensity gradually increased with TTR concentrations, obtaining a good linearity relationship (R^2 = 0.997). Considering that the average concentration of TTR in human blood is around 4 μM, probe **10** for TTR in PBS showed a low detection limit (limit of detection = 0.007 μM) based on the $3\sigma/k$ rule, indicating that the quantification of TTR was quite sensitive and highly reliable.

Prior to the complex biological application, we examined the selectivity of probe **10** in the presence of a series of biological samples existing in human serum plasma. Only slight fluorescent enhancement was observed upon addition of highly concentrated albumin, whereas no substantial fluorescence increase was observed when probe **10** was incubated with other biological species (Fig. 3). As shown in Fig. S3, we tested the anti-interference of the probe toward various inorganic substances (Na^+, K^+, Cu^{2+}, Zn^{2+}, and Fe^{3+}) and pH changes. Various metal ions in the serum plasma led to negligible

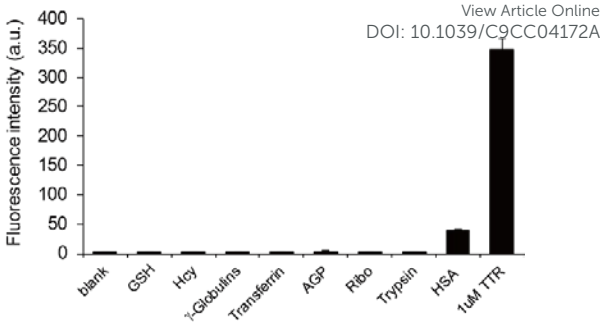


Fig. 3 The fluorescence intensity of probe **10** in the presence of various biological species, including glutathione (GSH, 10 μM), homocystein (Hcy, 10 μM), γ-globulins (10 mg/L), transferrin (10 mg/L), α-1 acid glycoprotein (AGP, 10 mg/L), ribonuclease A (Ribo, 10 mg/L), trypsin (10 mg/L), human serum albumin (HSA, 2000 mg/L), and TTR (1 μM).

changes in the fluorescence intensity of the WT-TTR–**10** complexes and the responses of the probe toward the pH changes from 5 to 8 were inconsequential. All of these findings suggest that probe **10** could selectively bind to WT-TTR and generate strong fluorescence responses.

Next, we investigated the plasma stability of probe **10** to evaluate any susceptibility to degradation and modification by enzymes, particularly hydrolases and esterase. Probe **10** exhibited high plasma stability in freshly obtained rat plasma (93.3%) and commercially available human plasma (96.5%) after 1hr of incubation at 37°C (Fig S4, see ESI).[17]

To demonstrate the practical usefulness of the studied system for TTR detection in real biological samples, we evaluated the sensing potential of probe **10** for measuring the exact concentration of TTR in two types of samples (human male AB plasma and human male AB clotted serum, Sigma-aldrich). Samples were analyzed by SDS-PAGE, followed by western-blotting using anti-TTR antibody as a control method (Fig. 4 and Fig. S5, see ESI). Human serum albumin is the most abundant blood protein (ca. 0.63 mM) and carrier for many neutral and weak acidic endogenous and exogenous molecules,[18] whose properties may affect the output of fluorescence signals. Therefore, there is a need to develop a practical fluorescence method for detecting TTR in a complex

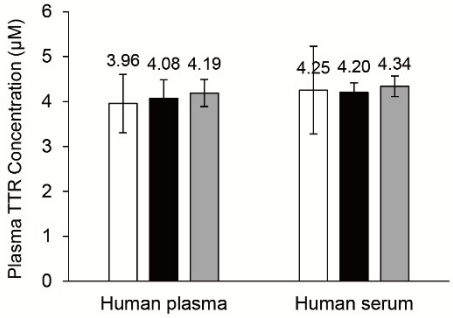


Fig. 4 Quantitative detection of TTR in human plasma- and human serum-employing immunoassay method (white bar, control method), a turn-on fluorescence method after subtracting the fluorescence intensity resulted from human serum albumin (black bar), and a turn-on fluorescence method of human albumin deleted samples (gray bar).

biological environment that can avoid interference from albumin. For our fluorescence detection method, excess probe **10** (4 μM) was incubated with 18-fold diluted human plasma sample in buffer medium (25 mM Tris, 192 mM Glycine, 0.1% SDS, 1 mM DTT, pH 8.6). We monitored the changes in emission intensity and the normalized fluorescence intensity was obtained by subtracting the fluorescence intensity resulted from 0.63 mM of human serum albumin. Then, the TTR concentration in this sample was estimated by using a standard calibration plot (Fig. 2b and Fig. S6, see ESI). As shown in Figure 4, the TTR level (4.08 μM) determined by probe **10** was much close to the results (3.96 μM) obtained using SDS-PAGE/ western blot technique. To further show the reliability of the fluorescence detection method, the fluorescence change induced by probe **10** was also monitored with albumin-deprived human plasma using a commercially available albumin-depletion kit. As expected, the obtained result for TTR level (4.19 μM) is in very good agreement to the range of that determined through the aforementioned methods. The newly developed fluorescence method in this study was further validated by measuring the exact concentration of TTR in human clotted serum, which suggested that different sample preparation methods do not interfere with the sensing ability of probe **10**.

In conclusion, we have designed, synthesized, and characterized a series of compounds **1**-**10** based on an indole moiety for detection of TTR, which serves as a potential biomarker for early diagnosis of AD. Spectroscopic and stability studies demonstrated that probe **10** exhibited the desired photo-physical properties (large stokes shifts, high quantum yield, and low limit of detection) and high plasma stability, which are beneficial for its biological application. The simple and sensitive fluorescence method developed according to the TTR level of human plasma and serum is highly sensitive and reliable, and can further enhance AD's diagnostic ability through composite analysis of different biomolecules (Aβ and tau) and neuroimaging studies.

We are grateful for the support of the National Research Foundation of Korea, funded by the Ministry of Education, Science and Technology (NRF-2018R1D1A3B07046990) and the Ministry of Science and ICT (NRF-2015M3A9B5053643).

Notes and references

1. (a) J. A. Hardy and G. A. Higgins, *Science*, 1992, **256**, 184; (b)K. S. Kosik, C. L. Joachim and D. J. Selkoe, *Proc. Natl. Acad. Sci. U. S. A.*, 1986, **83**, 4044.
2. B. De Strooper, R. Vassar and T. Golde, *Nat. Rev. Neurol.*, 2010, **6**, 99.
3. J. M. Tarasoff-Conway, R. O. Carare, R. S. Osorio, L. Glodzik, T. Butler, E. Fieremans, L. Axel, H. Rusinek, C. Nicholson, B. V. Zlokovic, B. Frangione, K. Blennow, J. Menard, H. Zetterberg, T. Wisniewski and M. J. de Leon, *Nat. Rev. Neurol.*, 2015, **11**, 457.
4. (a) S. M. Johnson, R. L. Wiseman, Y. Sekijima, N. S. Green, S. L. Adamski-Werner and J. W. Kelly, *Acc. Chem. Res.*, 2005, **38**, 911; (b) H. L. Monaco, M. Rizzi and A. Coda, *Science*, 1995, **268**, 1039.
5. (a) D. T. Yang, G. Joshi, P. Y. Cho, J. A. Johnson and R. M. Murphy, *Biochemistry*, 2013, **52**, 2849; (b) A. L. Schwarzman, L. Gregori, M. P. Vitek, S. Lyubski, W. J. Strittmatter, J. J. Enghilde, R. Bhasin, J. Silverman, K. H. Weisgraber, P. K. Coyle, M. G. Zagorski, J. Talafous, M. Eisenberg, A. M. Saunders, A. D. Roses and D. Goldgaber, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 8368.
6. M. Alemi, C. Gaiteiro, C. A. Ribeiro, L. M. Santos, J. R. Gomes, S. M. Oliveira, P. O. Couraud, B. Weksler, I. Romero, M. J. Saraiva and I. Cardoso, *Sci. Rep.*, 2016, **6**, 20164.
7. (a) C. S. Silva, J. Eira, C. A. Ribeiro, A. Oliveira, M. M. Sousa, I. Cardoso and M. A. Liz, *Neurobiol. Aging*, 2017, **59**, 10; (b) R. Costa, F. Ferreira-da-Silva, M. J. Saraiva and I. Cardoso, *Plos One*, 2008, **3**, 2899.
8. J. N. Buxbaum, Z. Ye, N. Reixach, L. Friske, C. Levy, P. Das, T. Golde, E. Masliah, A. R. Roberts and T. Bartfai, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 2681.
9. (a) I. Zerr and S. F. Gloeckner, *European Neurological Review*, 2009, **4**, 17; (b) M. A. Bradley-Whitman, E. Abner, B. C. Lynn and M. A. Lovell, *J. Alzheimers Dis.*, 2015, **47**, 761; (c) S. H. Han, E. S. Jung, J. H. Sohn, H. J. Hong, H. S. Hong, J. W. Kim, D. L. Na, M. Kim, H. Kim, H. J. Ha, Y. H. Kim, N. Huh, M. W. Jung and I. Mook-Jung, *J. Alzheimers Dis.*, 2011, **25**, 77; (d)H. Riisoen, *Acta. Neurol. Scand.*, 1988, **78**, 455.
10. (a) E. M. Castano, A. E. Roher, C. L. Esh, T. A. Kokjohn and T. Beach, *Neurol. Res.*, 2006, **28**, 155; (b) A. Biroccio, P. del Boccio, M. Panella, S. Bernardini, C. Di Ilio, D. Gambi, P. Stanzione, P. Sacchetta, G. Bernardi, A. Martorana, G. Federici, A. Stefani and A. Urbani, *Proteomics*, 2006, **6**, 2305.
11. (a) S. Choi, D. S. T. Ong and J. W. Kelly, *J. Am. Chem. Soc.*, 2010, **132**, 16043; (b) S. Choi and J. W. Kelly, *Bioorg. Med. Chem.*, 2011, **19**, 1505; (c) N. Myung, S. Connelly, B. Kim, S. J. Park, I. A. Wilson, J. W. Kelly and S. Choi, *Chem. Commun.*, 2013, **49**, 9188; (d) A. Baranczak, S. Connelly, Y. Liu, S. Choi, N. P. Grimster, E. T. Powers, I. A. Wilson and J. W. Kelly, *Biopolymers*, 2014, **101**, 484; (e) A. Baranczak, Y. Liu, S. Connelly, W. G. Du, E. R. Greiner, J. C. Genereux, R. L. Wiseman, Y. S. Eisele, N. C. Bradbury, J. Dong, L. Noodleman, K. B. Sharpless, I. A. Wilson, S. E. Encalada and J. W. Kelly, *J. Am. Chem. Soc.*, 2015, **137**, 7404; (f) N. P. Grimster, S. Connelly, A. Baranczak, J. Dong, L. B. Krasnova, K. B. Sharpless, E. T. Powers, I. A. Wilson and J. W. Kelly, *J. Am. Chem. Soc.*, 2013, **135**, 5656; (g) Y. Cen, Y. M. Wu, X. J. Kong, S. Wu, R. Q. Yu and X. Chu, *Anal. Chem.*, 2014, **86**, 7119; (h) Z. Chen, C. Wu, Z. F. Zhang, W. P. Wu, X. F. Wang and Z. Q. Yu, *Chin. Chem. Lett.*, 2018, **29**, 1601; (i) S. J. Sun, Q. W. Guan, Y. Liu, B. Wei, Y. Y. Yang and Z. Q. Yu, *Chin. Chem. Lett.*, 2019, **30**, 1051.
12. C. A. Royer, *Chem Rev*, 2006, **106**, 1769.
13. Z. Gao, Y. Hao, M. Zheng and Y. Chen, *RSC Adv.*, 2017, **7**, 7604.
14. (a) Y. Hayashi, N. Obata, M. Tamaru, S. Yamaguchi, Y. Matsuo, A. Saeki, S. Seki, Y. Kureishi, S. Saito, S. Yamaguchi and H. Shinokubo, *Org. Lett.*, 2012, **14**, 866; (b) B. K. Nunnally, H. He, L. C. Li, S. A. Tucker and L. B. McGown, *Anal. Chem.*, 1997, **69**, 2392; (c) K. Jia, Y. Wan, A. D. Xia, S. Y. Li, F. B. Gong and G. Q. Yang, *J. Phys. Chem. A*, 2007, **111**, 1593.
15. (a) A. R. Hurshman, J. T. White, E. T. Powers and J. W. Kelly, *Biochemistry*, 2004, **43**, 7365; (b) X. Jiang, C. S. Smith, H. M. Petrassi, P. Hammarstrom, J. T. White, J. C. Sacchettini and J. W. Kelly, *Biochemistry*, 2001, **40**, 11442.
16. P. Borowicz, J. Herbich, A. Kapturkiewicz and J. Nowacki, *Chem. Phys.*, 1999, **244**, 251.
17. F. M. Williams, *Pharmacol. Therapeut.*, 1987, **34**, 99.
18. X. M. He and D. C. Carter, *Nature*, 1992, **358**, 209.

