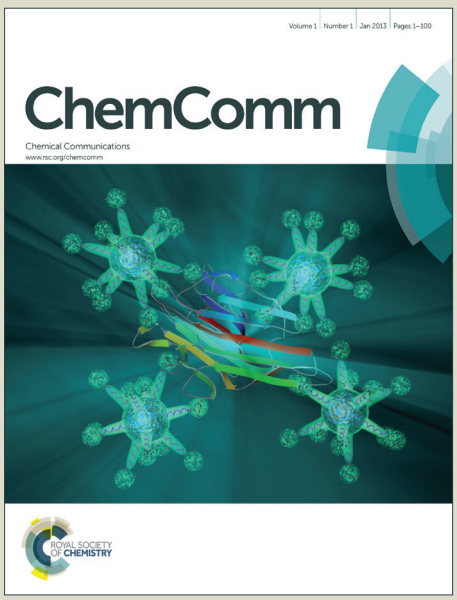
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

Solubilisation of 2,2-diphenyl-1-picrylhydrazyl radical to water by *β*-cyclodextrin to evaluate the radical-scavenging activity of antioxidants in aqueous media

Cite this: DOI: 10.1039/x0xx00000x

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

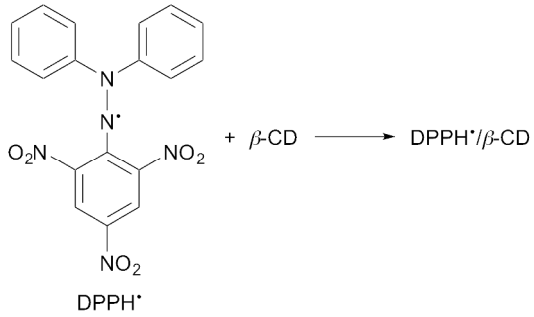
www.rsc.org/

Ikuo Nakanishi,*[a] Kei Ohkubo,[b,c] Kohei Imai,[d] Masato Kamibayashi,[e] Yasuo Yoshihashi,[f] Ken-ichiro Matsumoto,[a] Kiyoshi Fukuhara,[d] Katsuhide Terada,[f] Shinobu Itoh,[b] Toshihiko Ozawa[g] and Shunichi Fukuzumi*[b,c,h,f]

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH•) was successfully solubilised to water by *β*-cyclodextrin (*β*-CD). DPPH•/*β*-CD thus obtained was demonstrated to be a powerful tool to evaluate antioxidative activity of water-soluble antioxidants, such as ascorbate and Trolox, in aqueous buffer solutions.

A relatively stable radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (Scheme 1), is frequently used as a reactivity model of reactive oxygen species (ROS) to evaluate the radical-scavenging activity of antioxidants.[1–6] DPPH• has a characteristic absorption band at around 520 nm, which disappears upon addition of compounds with radical-scavenging activity. However, alcoholic cosolvents, such as methanol and ethanol, are required to use DPPH• in aqueous systems due to its little solubility to water.[7–9] In such a case, concentrated buffer solutions cannot be used to control the pH of the reaction systems, because buffer salts are precipitated in the alcoholic reaction media. Cyclodextrins (CDs) are cyclic oligosaccharides that have hydrophobic internal cavity and hydrophilic external surface. Thus, CDs form inclusion complexes with a wide range of hydrophobic molecules and solubilise them in water.[10,11] We report herein the solubilisation of DPPH• in water using *β*-cyclodextrin (*β*-CD: Scheme 1), which consists of 7 glucopyranoside units. The scavenging reaction of *β*-CD-solubilised DPPH• (DPPH•/*β*-CD) by water-soluble antioxidants in phosphate buffer solution (0.1 M, pH 7.4) demonstrated that DPPH•/*β*-CD would be a powerful tool to evaluate antioxidative activity in aqueous media without alcoholic cosolvents.

Boiling 15 mL water (Milli-Q) or a phosphate buffer solution (0.1 M, pH 7.4) was added to the mixture containing DPPH• (0.23 mmol) and *β*-CD (0.35 mmol), and the suspension was cooled to room temperature. The filtration of the suspension with a membrane filter (pore size: 0.22 *μ*m) gave a deep violet solution. This solution showed an absorption band at 527 nm, which is diagnostic of DPPH• (Fig. 1). Thus, DPPH• could be solubilised in water by *β*-CD. A significant red shift of the band due to DPPH•/*β*-CD as compared to



Scheme 1 Incorporation of DPPH• into *β*-cyclodextrin (*β*-CD)

those of free DPPH• in *n*-hexane (509 nm), MeOH (516 nm), EtOH (517 nm) and acetonitrile (519 nm) suggests that the >N–N•– moiety of DPPH• may exist outside of the *β*-CD cavity and strongly interact with water. The concentration of DPPH• was estimated to be 5.9×10^{-5} M by using the ε value of 11000 M^{-1} cm^{-1} determined for DPPH• in a 1:1 ethanol–buffer solution.[9] When *β*-CD was replaced by *α*- or *γ*-CD, which consists of 6 or 8 glucopyranoside units and thus has a smaller or bigger hydrophobic cavity than *β*-CD, DPPH• was not solubilised to water. 2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl radical (DOPPH•) could not be solubilised to water by *β*-CD in the same manner, either. Fig. 2a shows an optimized structure of the inclusion complex of DPPH• with *β*-CD calculated by the density functional theory (DFT) (UB3LYP/3-21G:C-PCM solvation model parameterised for water) (see the ESI†). The picryl moiety of DPPH• is incorporated into the hydrophobic cavity of *β*-CD. DOPPH• is also incorporated into *β*-CD as shown in Fig. 2b. The calculated association energy between DPPH• and *β*-CD (–31 kcal mol^{-1}) by DFT is significantly less negative than that between DOPPH• and *β*-CD (–44 kcal mol^{-1}). These theoretical results suggest that DOPPH• may not be solubilised by *β*-CD due to the hydrophobic *tert*-octyl groups. DPPH• solubilised by *β*-CD to water or the phosphate buffer solution (0.1 M, pH 7.4) is stable at least

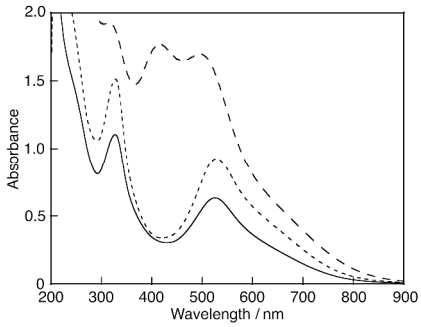


Fig. 1 UV-vis spectra of extracts of the mixture of DPPH$^{\bullet}$ (0.23 mmol) and β-CD (0.45 mmol) by 15 mL of boiling phosphate buffer (0.1 M, pH 7.4) (—), acetate buffer (50 mM, pH 4.4) (- - -) and borate buffer (14 mM, pH 9.1) (– – –).

for several days at room temperature. When a boiling acetate buffer solution (50 mM, pH 4.4) was used instead of the phosphate buffer, DPPH$^{\bullet}$ could also be solubilised by β-CD (Fig. 1). On the other hand, a brown solution with absorption bands at 416 and 505 nm was obtained with use of the boiling borate buffer solution (14 mM, pH 9.1) as shown in Fig 1. This suggests that DPPH$^{\bullet}$ is unstable under basic conditions as reported previously.[9] In fact, an addition of 0.75 mL of a borate buffer solution (0.1 M, pH 9.1) to DPPH$^{\bullet}$/β-CD in water (Milli-Q) (2.3 mL) resulted in a gradual decrease in the absorption band at 527 nm, accompanied by an increase in the band at 412 nm with clear isosbestic points at 252, 304, 346 and 491 nm as shown in Fig. 3.

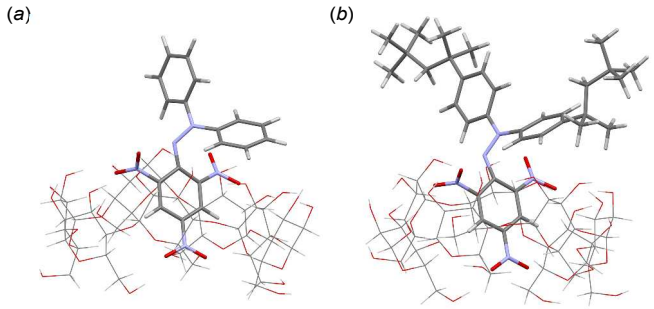


Fig. 2 Optimized structures of the inclusion complex of (a) DPPH$^{\bullet}$ with β-CD and (b) DOPPH$^{\bullet}$ with β-CD calculated by DFT (UB3LYP/3-21G:C-PCM solvation model parameterised for water).

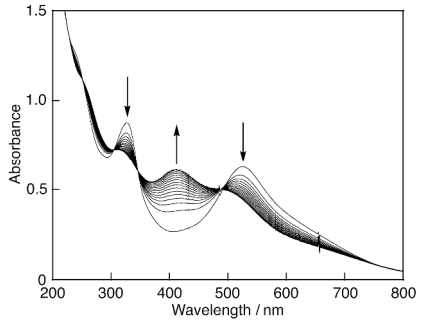


Fig. 3 Spectral change observed for DPPH$^{\bullet}$/β-CD (5.8×10^{-5} M) in borate buffer (25 mM, pH 9.1) at 298 K. Interval: 20 min.

The one-electron reduced DPPH$^{\bullet}$ (DPPH^{-}) is reported to have an absorption band at 426 nm in a 1:1 ethanol–buffer solution,[9] suggesting that DPPH^{-} may be included in the products. However, the detailed reaction mechanism of DPPH$^{\bullet}$ under basic conditions is under investigation and will be reported elsewhere.

The EPR spectrum of DPPH$^{\bullet}$/β-CD observed in water at room temperature has the same g value (2.0036) and hyperfine coupling constant (7.8 G) as those of DPPH$^{\bullet}$ in MeOH (2.0036 and 7.9 G, respectively) (See the ESI,† Fig. S1). The slightly small hyperfine coupling constant (14.6 G) was observed in water as compared to that in MeOH (15.2 G) for the EPR spectra measured at 77 K (Fig. 4).

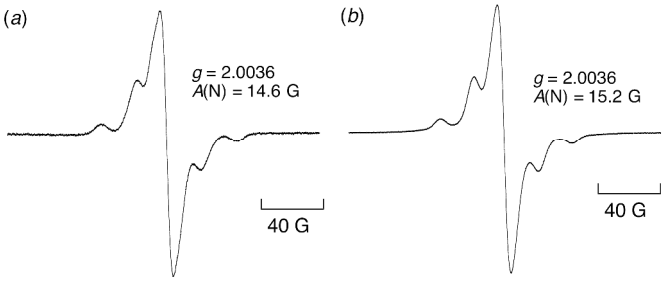


Fig. 4 EPR spectra of (a) DPPH$^{\bullet}$/β-CD (1.0×10^{-5} M) in distilled water at 77 K and (b) DPPH$^{\bullet}$ (1.0×10^{-5} M) in MeOH at 77 K.

When ascorbic acid ($AscH_2$) was added to the phosphate buffer solution (0.1 M, pH 7.4) of DPPH$^{\bullet}$/β-CD, the band at 527 nm disappeared immediately with clear isosbestic points at 320, 338 and 431 nm as shown in Fig. 5a. Since the pK_a value of $AscH_2$ is reported to be 4.1,[12] $AscH_2$ undergoes deprotonation and exists as its anion form, $AscH^{-}$, in phosphate buffer solution (0.1 M, pH 7.4). Thus, this spectral change indicates that $AscH^{-}$ efficiently scavenged DPPH$^{\bullet}$ in phosphate buffer [(Eq. (1)). When $AscH^{-}$ was replaced by Trolox, a water-soluble analogue of α-tocopherol (vitamin E), a similar spectral change was observed due to the scavenging reaction of DPPH$^{\bullet}$ by Trolox [Fig. 5b and Eq. (2)].[13]

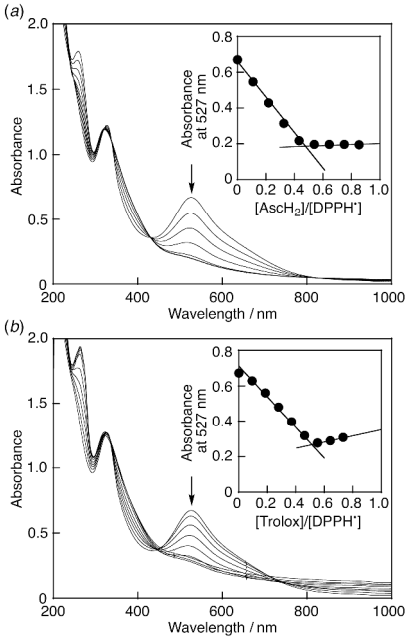


Fig. 5 Spectral changes observed upon addition of (a) $AscH_2$ ($0–5.4 \times 10^{-5}$ M) or (b) Trolox ($0–4.6 \times 10^{-5}$ M) to DPPH$^{\bullet}$/β-CD (6.3×10^{-5} M) in phosphate buffer (0.1 M, pH 7.4). Insets: Plots of the absorbance at 527 nm vs. (a) [$AscH_2$]/[DPPH$^{\bullet}$] and (b) [Trolox]/[DPPH$^{\bullet}$].

$$DPPH^{\bullet}/\beta\text{-CD} + AscH^{-} \longrightarrow DPPH_2/\beta\text{-CD} + Asc^{\bullet-} \quad (1)$$

$$DPPH^{\bullet}/\beta\text{-CD} + Trolox \longrightarrow DPPH_2/\beta\text{-CD} + Trolox(-H)^{\bullet} \quad (2)$$

Spectral titrations (insets of Fig. 3a and 3b) show the same stoichiometry with both antioxidants, the DPPH$^{\bullet}$/antioxidant molar ratio being 2:1.[7] The decay of the absorbance at 527 nm monitored by a stopped-flow technique obeyed pseudo-first-order kinetics, when the concentration of $AscH_2$ ([$AscH_2$]) were maintained at more than a 10-fold excess of DPPH$^{\bullet}$/β-CD concentration (Fig. 6a). The pseudo-first-order rate constant (k_{obs}) linearly increased with increasing [$AscH_2$] (Fig. 7a). From the slope of the linear plot the second-order rate constant (k) for the scavenging of DPPH$^{\bullet}$/β-CD by $AscH_2$ was determined in a phosphate buffer (0.1 M, pH 7.4) to be 7.2×10^3 M^{-1} s^{-1}. The k value for Trolox was also determined in the same manner to be 1.8×10^4 M^{-1} s^{-1} (Fig. 6b and 7b), which is exactly the same as that measured in a 1:1 ethanol–buffer (pH 7.4) solution.[9,14] Thus, β-CD does not inhibit the reaction of DPPH$^{\bullet}$ with the antioxidants, while the stability and reactivity of nitroxyl radicals were reported to be significantly changed by the complexation with cyclodextrins.[15]

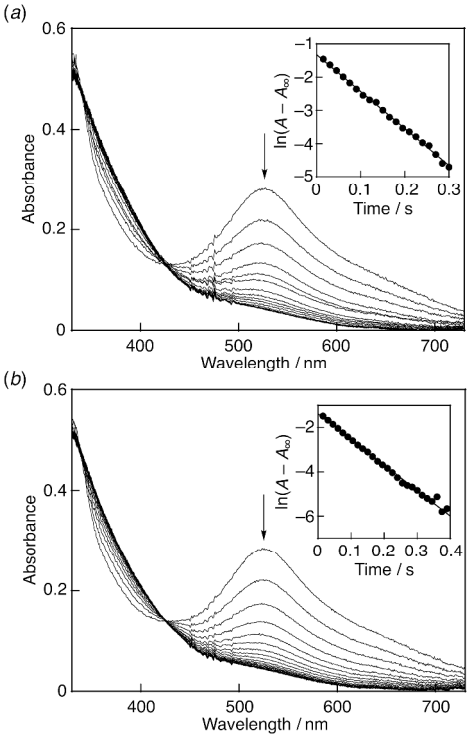


Fig. 6 Spectral changes (interval: 25 ms) observed during reactions of (a) $AscH_2$ (1.4×10^{-3} M) with DPPH$^{\bullet}$/β-CD (2.6×10^{-5} M) and (b) Trolox (6.3×10^{-4} M) with DPPH$^{\bullet}$/β-CD (3.0×10^{-5} M) in phosphate buffer (0.1 M, pH 7.4) at 298 K. Insets: the first-order plots of the absorbance at 527 nm.

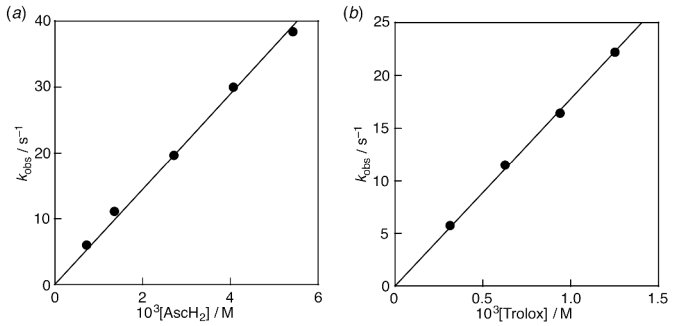


Fig. 7 Plots of k_{obs} *vs.* (a) [$AscH_2$] and (b) [Trolox].

In summary, β-CD-solubilised DPPH$^{\bullet}$ to water has been demonstrated to be a powerful tool to evaluate the antioxidative activity of antioxidants in aqueous media, especially in highly concentrated buffer solutions without precipitation of buffer salts.

This work was partially supported by Grant-in-Aid (Nos. 26460056 to I.N., 26620154 and 266288037 to K.O.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan and ALCA and SENTAN projects from JST.

Notes and references

[a] Radio-Redox-Response Research Team, Advanced Particle Radiation Biology Research Program, Research Center for Charged Particle Therapy, National Institute of Radiological Sciences (NIRS), Inage-ku, Chiba 263-8555, Japan. E-mail: nakanis@nirs.go.jp; Fax: +81-43-255-6819; Tel: +81-43-206-3131

[b] Department of Material and Life Science, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan.

[c] ALCA and SENTAN, Japan Science and Technology Agency (JST), Suita, Osaka 565-0871, Japan.

[d] School of Pharmacy, Showa University, Shinagawa-ku, Tokyo 142-8555, Japan.

[e] Pharmaceutical Manufacturing Chemistry, Kyoto Pharmaceutical University, Kyoto 607-8414, Japan.

[f] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Toho University, Funabashi, Chiba 274-8510, Japan.

[g] Division of Oxidative Stress Research, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan.

[h] Department of Bioinspired Science, Ewha Womans University, Seoul 120-750, Korea.

[f] Faculty of Science and Technology, Meijo University, Shiogamaguchi, Tempaku, Nagoya, Aichi 468-8502, Japan.

† Electronic Supplementary Information (ESI) available: Experimental details and EPR spectra at room temperature (Fig. S1). See DOI: 10.1039/c000000x/

1 M. S. Blois, *Nature*, 1958, **181**, 1199.

2 (*a*) K. U. Ingold and D. A. Pratt, *Chem. Rev.*, 2014, **114**, 9022; (*b*) G. Litwinienko and K. U. Ingold, *Acc. Chem. Res.*, 2007, **40**, 222;

3 (*a*) E. van Wenum, R. Jurczakowski and G. Litwinienko, *J. Org. Chem.*, 2013, **78**, 9102; (*b*) M. Musialik, R. Kuzmicz, T. S. Pawłowski and G. Litwinienko, *J. Org. Chem.*, 2009, **74**, 2699; (*c*) P. Mulder, G. Litwinienko, S. Lin, P. D. MacLean, L. R. Barclay and K. U. Ingold, *Chem. Res. Toxicol.*, 2006, **19**, 79; (d) G. Litwinienko and K. U. Ingold, *J. Org. Chem.*, 2005, **70**, 8982; (e) M.

Musialik and G. Litwinienko, *Org. Lett.*, 2005, **7**, 495; (f) G. Litwinienko and K. U. Ingold, *J. Org. Chem.*, 2004, **69**, 5888; (g) G. Litwinienko and K. U. Ingold, *J. Org. Chem.*, 2003, **68**, 3433.

4 (*a*) R. Amorati, S. Menichetti, C. Viglianisi and M. C. Foti, *Chem. Commun.*, 2012, **48**, 11904; (*b*) M. C. Foti, *Int. J. Chem. Kinet.*, 2012, **44**, 524; (*c*) V. D. Kancheva, L. Saso, S. E. Angelova, M. C. Foti, A. Slavova-Kasakova, C. Daquino, V. Enchev, O. Firuzi and J. Nechev, *Biochemie*, 2012, **94**, 403; (*d*) M. C. Foti, C. Daquino, G. A. DiLabio, K. U. Ingold, *Org. Lett.*, 2011, **13**, 4826; (*e*) M. C. Foti, R. Amorati, G. F. Pedulli, C. Daquino, D. A. Pratt and K. U. Ingold, *J. Org. Chem.*, 2011, **75**, 4434; (*f*) M. C. Foti, C. Daquino, I. D. Mackie, G. A. DiLabio and K. U. Ingold, *J. Org. Chem.*, 2008, **73**, 9270; (*g*) M. C. Foti and C. Daquino, *Chem. Commun.*, 2006, 3252; (*h*) M. C. Foti, C. Daquino and C. Geraci, *J. Org. Chem.*, 2004, **69**, 2309; (*i*) M. C. Foti, L. R. Barlay and K. U. Ingold, *J. Am. Chem. Soc.*, 2002, **124**, 12881.

5 (*a*) T. Waki, S. Kobayashi, K. Matsumoto, T. Ozawa, T. Kamada and I. Nakanishi, *Chem. Commun.*, 2013, **49**, 9842; (*b*) H. P. Indo, I. Nakanishi, K. Ohkubo, H. Yen, M. Nyui, S. Manda, K. Matsumoto, K. Fukuhara, K. Anzai, N. Ikota, H. Matsui, Y. Minamiyama, A. Nakajima, H. Ichikawa, S. Fukuzumi, T. Ozawa, C. Mukai and H. J. Majima, *RSC Adv.*, 2013, **3**, 4535; (*c*) T. Kawashima, S. Manda, Y. Uto, K. Ohkubo, H. Hori, K. Matsumoto, K. Fukuhara, N. Ikota, S. Fukuzumi, T. Ozawa, K. Anzai and I. Nakanishi, *Bull. Chem. Soc. Jpn.*, 2012, **85**, 877; (*d*) T. Waki, I. Nakanishi, K. Matsumoto, J. Kitajima, T. Chikuma and S. Kobayashi, *Chem. Pharm. Bull.*, 2012, **60**, 37; (*e*) S. Kobayashi, T. Waki, I. Nakanishi, K. Matsumoto and K. Anzai, *Chem. Pharm. Bull.*, 2010, **58**, 1442; (*f*) I. Nakanishi, T. Kawashima, K. Ohkubo, H. Kanazawa, K. Inami, M. Mochizuki, K. Fukuhara, H. Okuda, T. Ozawa, S. Itoh, S. Fukuzumi and N. Ikota, *Org. Biomol. Chem.*, 2005, **3**, 626.

6 S. B. Kedare, R. P. Singh, *J. Food Sci. Technol.*, 2011, **48**, 412 and references cited therein.

7 M. B. Arnao, *Trends Food Sci. Technol.*, 2000, **11**, 419.

8 D.-W. Li, F.-F. Tian, Y.-S. Ge, X.-L. Ding, J.-H. Li, Z.-Q. Xu, M.-F. Zhang, X.-L. Han, R. Li, F.-L. Jiang and Y. Liu, *Chem. Commun.*, 2011, **47**, 10713.

9 O. Friaa and D. Brault, *Org. Biomol. Chem.*, 2006, **4**, 2417.

10 G. Crini, *Chem. Rev.*, 2014, **114**, 10940 and references cited therein.

11 I. Nakanishi, S. Fukuzumi, T. Konishi, K. Ohkubo, M. Fujitsuka, O. Ito and N. Miyata, *J. Phys. Chem. B*, 2002, **106**, 2372.

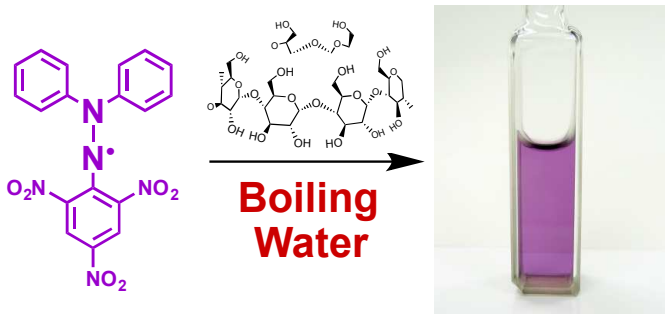
12 (*a*) C. Creutz, *Inorg. Chem.*, 1981, **20**, 4449; (*b*) N. H. Williams and J. K. Yandell, *Aust. J. Chem.*, 1982, **35**, 1133.

13 Clear isosbestic points such as Fig. 3a were not observed due to relatively poor solubility of Trolox to phosphate buffer as compared to $AscH_2$.

14 For 2:1 and 3:1 ethanol–water mixtures, the k values were reported to be 8.6×10^3 and 7.0×10^3 M^{-1} s^{-1}, respectively. See ref. 9.

15 (*a*) M. Spulber and S. Schlick, *J. Phys. Chem. A*, 2010, **114**, 6217; (*b*) D. Bardelang, L. Charles, J.-P. Finet, L. Jicsinszky, H. Karoui, S. R. A. Marque, V. Monnier, A. Rockenbauer, R. Rosas and P. Tordo, *Chem.–Eur. J.*, 2007, **13**, 9344; (*c*) H. Karoui and P. Tordo, *Tetrahedron Lett.*, 2004, **45**, 1043; (*d*) H. Karoui, A. Rockenbauer, S. Pietri and P. Tordo, *Chem. Commun.*, 2002, 3030; (*e*) C. Ebel, K. U. Ingold, J. Michon and A. Rassat, *Tetrahedron Lett.*, 1985, **26**, 741.

Graphical abstract image



2,2-Diphenyl-1-picrylhydrazyl radical was solubilised to water by β-cyclodextrin.