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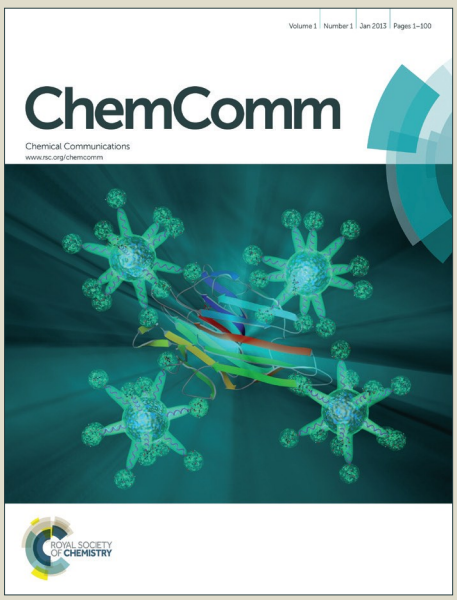

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ARTICLE TYPE

Target-selective photo-degradation of AFP-L3 and selective photo-cytotoxicity against HuH-7 hepatocarcinoma cells using an anthraquinone-PhoSL hybrid

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A purpose-designed anthraquinone–*Pholiota squarrosa* lectin (PhoSL) hybrid effectively degraded α-fetoprotein-L3 (AFP-L3) associated with liver cancer. Degradation was achieved using light irradiation in the absence of any additives and under neutral pH conditions. Moreover, the hybrid effectively exhibited selective photo-cytotoxicity against HuH-7 hepatocarcinoma cells upon photo-irradiation.

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related mortality worldwide and represents more than 90% of primary liver cancers.[1] HCC is diagnosed in over half a million people worldwide each year, is the fifth most common cancer globally and the third most common cause of cancer-related death (after lung and gastric cancer), and thus represents a major worldwide health problem.

Levels of α1-6 fucosylated α-fetoprotein (AFP-L3) are elevated in HCC patients. Core Fucα1-6 oligosaccharide, shown in Fig. 1, has several biological functions, including as a biomarker for HCC.[2] Therefore, an agent able to mediate the selective degradation of AFP-L3 by specific recognition of the core Fucα1-6 oligosaccharide would be a very attractive and one of the useful candidates as an HCC anti-cancer drug. In this context, Kobayashi and Kawagishi *et al*. recently isolated a novel Fucα1-6-specific lectin from the mushroom *Pholiota squarrosa*. The purified lectin, designated as PhoSL (*P. squarrosa* lectin), consists of 40 amino acids (NH_2-APVPVTKLVCDGDTYKCT-AYLDFGDGRWVAQWDTNVF-HTG-COOH).[3] PhoSL is stable at a range of pH values and temperatures and is robust during purification and assay. We previously reported that certain anthraquinone derivatives degrade proteins by emitting reactive oxygen species under photo-irradiation conditions[4] and we thus hypothesized that an anthraquinone hybrid incorporating PhoSL would effectively and selectively degrade AFP-L3 under photo-irradiation conditions, thus selectively exhibiting photo-cytotoxicity against HCC cells overexpressing AFP-L3. We here present the molecular design, chemical synthesis and evaluation of a novel light-activatable anthraquinone-lectin (PhoSL) hybrid that effectively degrades AFP-L3 and exhibits selective cytotoxicity against HCC cells under photo-irradiation.

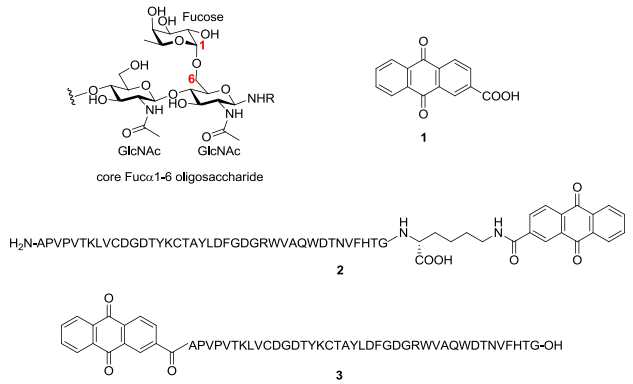

Figure 1. The chemical structures of core Fucα1-6 oligosaccharide, anthraquinone derivative **1**, and anthraquinone-PhoSL hybrids **2** and **3**.

We utilized anthraquinone derivative **1** as a protein photodegrading agent (Fig. 1). The structure of the sugar binding pocket of PhoSL remains unclear; therefore, **2** and **3** were tentatively designed as anthraquinone-PhoSL hybrids. Hybrid **2** comprises anthraquinone **1** linked at the *C*-terminal of PhoSL through a lysine spacer between **1** and PhoSL, while hybrid **3** consists of anthraquinone **1** attached to the *N*-terminal of PhoSL. Designed hybrids **2** and **3** were synthesized using standard solid-phase synthesis (see SI) and their binding ability and specificity for the core Fucα1-6 oligosaccharide were examined by capillary affinity electrophoresis using fluorescently (8-aminopyrene-1,3,6-trisulfonic acid, APTS) labelled oligosaccharides **4**-**8**.[5] PhoSL was used as a positive control for the assay; as shown in Fig. 2-a, only the peak corresponding to the oligosaccharide **8**, which contains the core α1-6 fucoside moiety, shifted to a longer retention time in a dose dependent manner, confirming that PhoSL bound to **8** specifically and exhibited no significant affinity for oligosaccharides **4**-**7** which lack the core α1-6 fucoside structure. Furthermore, hybrid **2** also bound only to **8**,

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similar to PhoSL (Fig. 2-b)), whereas hybrid **3** did not bind oligosaccharides **4**-**8** (see SI: Fig. S2). Interestingly, the binding ability of **2** to **8** was stronger than that of PhoSL.

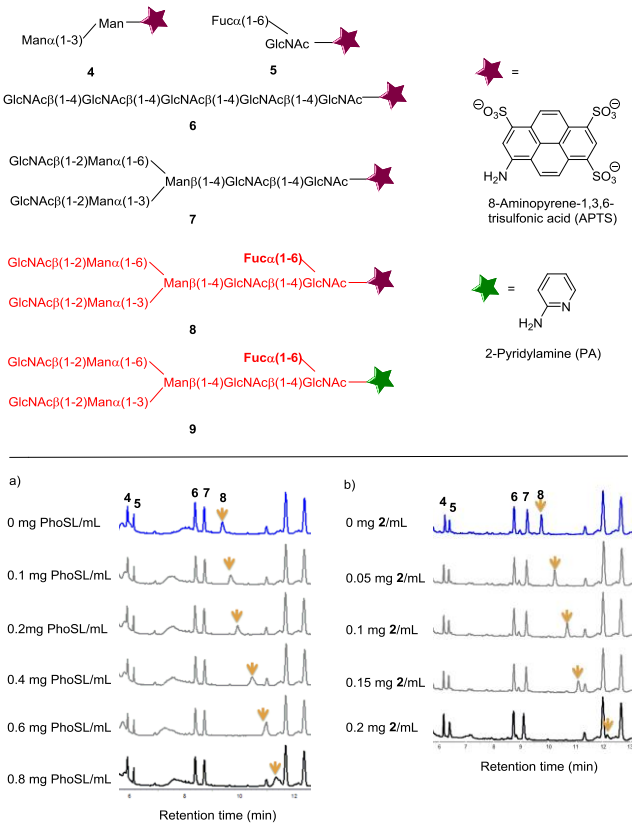

Figure 2. Assay of the interaction of PhoSL and hybrid **2** with the fluorescently (8-aminopyrene-1,3,6-trisulfonic acid, APTS)-labelled oligosaccharides **4**-**8** by capillary affinity electrophoresis. PhoSL and hybrid **2** were present at the concentrations indicated. a) Capillary electrophoresis profile using PhoSL. b) Capillary electrophoresis profile using hybrid **2**.

With these favorable results for hybrid **2** in hand, we next examined the photo-degradation ability of **2** against the target oligosaccharide **9** which possesses 2-pyridylamine (PA)[6] at the reducing terminal as a label for fluorescence detection during HPLC analysis. The results of the photo-degradation experiment (365 nm, 100 W), monitored by HPLC, are shown in Fig. 3. No degradation of **9** occurred under these photo-irradiation conditions, even in the presence of a large excess of **2**. However, **2** degraded under photo-irradiation conditions, strongly suggesting that **2** produced reactive oxygen species which can photo-degrade oligosaccharides[7], proteins,[4] and itself, but not **9** (Fig. 4-a)). This is probably due to the distance between the anthraquinone moiety in **2** and **9**. We expected that the anthraquinone moiety in **2** was located too far from **9** in the sugar-binding pocket in **2** to support the degradation of **9**; however, if the anthraquinone moiety in **2** could be brought sufficiently close to AFP-L3 upon recognition of the core fucα1-6 oligosaccharide, then AFP-L3 would be photo-degraded by **2** (Fig. 4-b)).

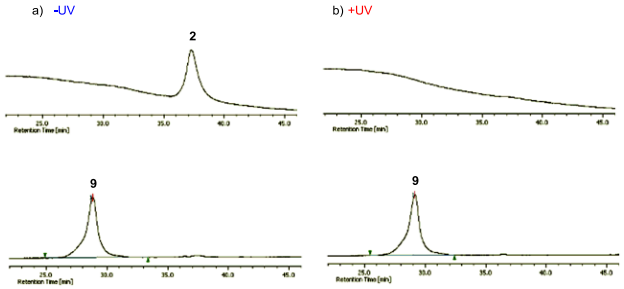

Figure 3. Photo-degradation of PA-labelled oligosaccharide **9** by hybrid **2**. **9** (500 nM) was incubated with **2** (60 μM) in Tris-acetate buffer (100 mM, pH 7.0)/MeCN (7:3) at 25 °C for 2 h during irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the sample and the reaction mixture was analyzed by HPLC. a) HPLC profiles of **2** and **9** without photo-irradiation. b) HPLC profiles of **2** and **9** with photo-irradiation.

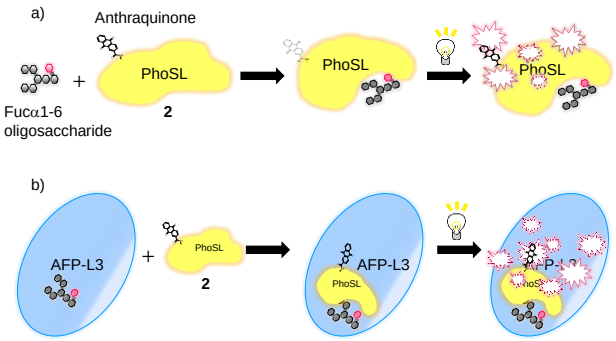

Figure 4. a) Self photo-degradation of **2** without photo-degradation of Fucα1-6 oligosaccharide. b) Photo-degradation of AFP-L3 by **2** following recognition of Fucα1-6 oligosaccharide by **2**.

We therefore conducted electron paramagnetic resonance (EPR) spin trapping experiments using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) to confirm the generation of reactive oxygen species from the reaction of photo-excited **2** with O_2. It was found that photo-irradiation of **2** in the presence of DMPO gave a product with the EPR spectrum characteristics of the DMPO-hydroxyl radical spin adduct DMPO-·OH (Fig. 5-b), which results from the reaction of DMPO with ·OH and/or the decay of the DMPO-superoxide anion spin adduct DMPO-·OOH.[8,9] In addition, it was confirmed that no peaks corresponding to DMPO-·OOH or DMPO-·OH were detected either by treatment of DMPO with **2** without photo-irradiation or by photo-irradiation of DMPO in the absence of **2** (Fig. 5-a). These results indicate that a reactive oxygen species was certainly produced by the reaction of photo-excited **2** with O_2.

We next examined the degradation ability and selectivity of **2** against AFP-L3 upon photo-irradiation (365 nm, 100 W). AFP-L1 has no surface target oligosaccharide and was used as a negative control glycoprotein. The reaction was monitored by SDS-PAGE and the results are shown in Fig. 6. Comparison of lanes 3 and 4 with lane 2 in Fig. 6-a shows that neither photo-irradiation of AFP-L3 in the absence of **2** or treatment of AFP-L3 with **2** in the absence of photo-irradiation resulted in a change in the SDS-PAGE profile. In contrast, lanes 5-7 show the fading or disappearance of the bands corresponding to AFP-L3, indicating that hybrid **2** caused significant degradation of AFP-L3 upon

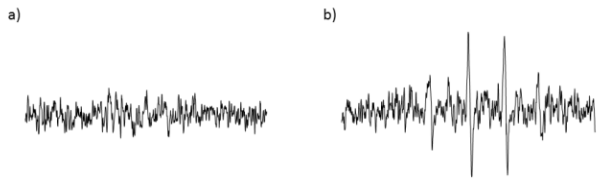

Figure 5. EPR spectrum obtained during photo-irradiation of **2** in the presence of DMPO. **2** (21 μM) and DMPO (500 mM) were incubated in 2%DMSO/PBS (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 1 mM DETAPAC under irradiation with a UV lamp (365 nm, 100 W) placed 40 cm from a flat cell. a) Before irradiation. b) After 10 min irradiation. DETAPAC= diethylenetriaminepentaacetic acid.

photo-irradiation. In addition, it is clear that hybrid **2** exhibited only weak degradation activity towards AFP-L1 when the mixture of AFP-L1 and **2** was irradiated with a long-wavelength UV lamp (Fig. 6-b)). These results clearly demonstrated that interaction between **2**, which possesses the PhoSL moiety, and the target oligosaccharide on AFP-L3, which possesses the core α1-6 fucoside structure, is very important for the effective and selective degradation of AFP-L3 under photo-irradiation. No SDS-PAGE bands corresponding to degraded peptide fragments or aggregated product were observed after photo-irradiation of **2** and AFP-L3, suggesting that the degradation reaction occurred non-site-specifically and that AFP-L3 was degraded into small peptide fragments too small to observe by SDS-PAGE. [4,10,11] In addition, because no degradation of AFP-L3 by hybrid **2** occurred in the absence of light, UV light functioned as a trigger to initiate degradation by the hybrid.

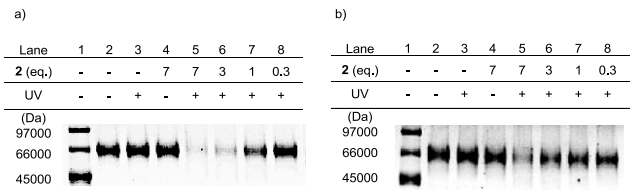

Figure 6. Photo-degradation of a) AFP-L3 and b) AFP-L1. AFP-L3 or AFP-L1 (3.0 μM) was incubated with hybrid **2** in 2%DMSO/PBS (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) at 25 °C for 2 h during irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the sample and the reaction mixes were analyzed by tricine-SDS-PAGE (12% polyacrylamide gel): a) lane 1, size markers; lane 2, AFP-L3 alone; lane 3, AFP-L3 with UV; lane 4, AFP-L3 + **2** (21 μM) without UV; lanes 5-8, the concentrations of **2** were 21, 9, 3.0, and 0.90 μM with UV, respectively. b) lane 1, size markers; lane 2, AFP-L1 alone; lane 3, AFP-L1 with UV; lane 4, AFP-L1 + **2** (21 μM) without UV; lanes 5-8, the concentrations of **2** were 21, 9, 3.0, and 0.90 μM with UV, respectively.

These photo-degradation results were supported by MALDI-TOF MS analysis, as shown in Fig. 7: incubation of AFP-L3 with **2** without photo-irradiation gave the MS peaks corresponding to AFP-L3 and the adduct of AFP-L3 with **2** while incubation of AFP-L3 with **2** under photo-irradiation resulted in disappearance of those MS peaks corresponding to AFP-L3 and the adduct. No MS peaks corresponding to degradation fragments could be detected due to the randomness of the degradation, leading to many fragments present in very small amounts. [4,10,11]

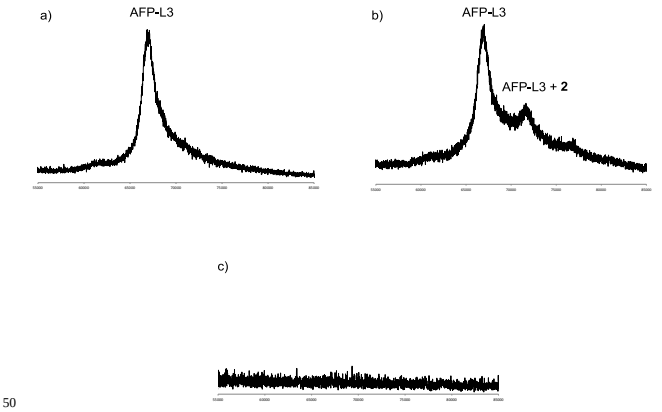

Figure 7. MALDI-TOF MS profile of AFP-L3 obtained following incubation with hybrid 2. AFP-L3 (1.5 eq.) was incubated without a) or with b) and c) 2 (21 μM) in 2%DMSO/PBS (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) at 25 °C for 2 h without a) and b) or with c) photo-irradiation with a UV lamp (365 nm, 100 W) placed 10 cm from the mixture. The resulting products were analysed by MALDI-TOF MS (matrix: 3,5-dimethoxy-4-hydroxycinnamic acid). Peaks were observed at m/z 67000 in a), indicating AFP-L3; at m/z 67000 and 72000 in b), indicating AFP-L3 and AFP-L3+2; those peaks were not observed in c).

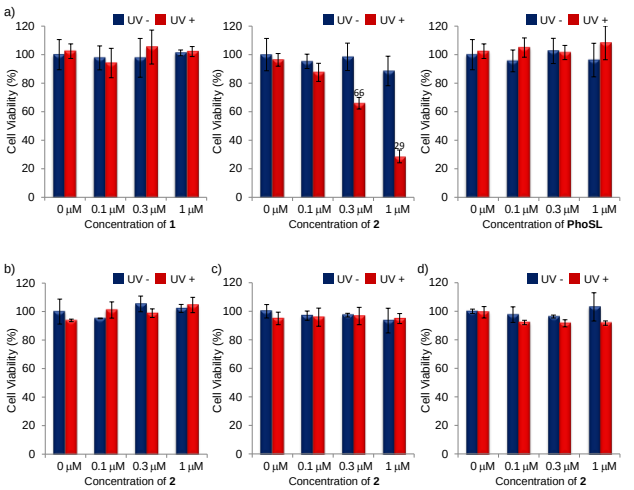

Figure 8. Effects of **1**, **2**, and PhoSL on a) HuH-7 cell, b) A549 cell, c) HeLa cell, or d) HLE cell proliferation. Cells were seeded into 96-well plates (2.00 x10^3 cells per well). After 24 h, compounds were added at the indicated concentrations and the cells were incubated for 24 h at 37 °C in 5% CO_2 in air. Cells were irradiated for 30 min with a UV lamp (365 nm, 30 W) placed 20 cm from the plate. Cells were then kept for 72 h at 37 °C in 5% CO_2 in air, then MTT reagent was added to each well and the cells were incubated for up to 3 additional hours. The absorbance at 540 nm was read using a plate reader.

Finally, we examined the inhibitory activity of **2** on the cell growth of HuH-7, A549, HeLa, and HLE cells using the MTT assay (Figure 8).[12] HLE cells are a hepatocellular carcinoma cell line derived from liver cancer and do not display overexpressed AFP-L3 on the surface, whereas HuH-7 cells are a hepatocellular carcinoma cell line derived from liver cancer which overexpress AFP-L3. A549 and HeLa cells are a human adeno carcinoma cell line derived from lung cancer and a human epithelial carcinoma cell line derived from cervical cancer, respectively, both of which do not display overexpressed AFP-L3 on the surfaces. As

expected, hybrid **2** significantly inhibited the growth of HuH-7 cells upon light irradiation, whereas no inhibition was observed when the anthraquinone derivative **1** without PhoSL, or PhoSL alone, was used instead of **2** (Figure 8-a)). In addition, **2** did not show any cytotoxicity against HuH-7 cells without photo-irradiation. Furthermore, **2** exhibited no inhibitory activity towards the growth of HLE, A549, and HeLa cells, even with photo-irradiation (Figure 8-b-d)). These results clearly indicate that the anthraquinone-PhoSL hybrid **2** exhibits effective and selective photo-cytotoxicity against HuH-7 cells which display overexpressed AFP-L3 containing the core fucα1-6 oligosaccharide. In addition, although we have not yet obtained direct evidence of photo-degradation of the target glycoprotein, AFP-L3, on HuH-7 cells, these results strongly suggest that selective photo-degradation of the target glycoprotein on the cancer cells by hybrid **2** took place and induced significant photo-cytotoxicity

In conclusion, anthraquinone-PhoSL hybrid **2** selectively and effectively degraded AFP-L3 upon irradiation with long-wavelength UV light in the absence of any additives and under neutral pH conditions. Furthermore, **2** showed significant and selective inhibitory activity toward HuH-7 cell growth upon photo-irradiation. These results showed that selective photo-degradation of a target protein can be realized by specific recognition of a surface oligosaccharide and a light activatable molecule, anthraquinone, and that the hybrid molecule can inhibit select cancer cell growth. The results presented here will contribute to the molecular design of novel glycoprotein photo-degrading agents by the selective recognition of specific oligosaccharide structures, thus providing agents for controlling the functions of proteins involved in diseases.

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Notes and references

1 a) H. B. El-Serag, *Gastroenterology* 2012, **142**, 1264; b) R. Lencioni, *Hepatology* 2010, **52**, 762; c) H. Nishikawa, Y. Osaki, *Int. J. Oncol*. 2013, **43**, 1333; d) J. M. Llovet, *J. Gastroenterology* 2005, **40**, 225; e) J. M. Llovet, S. Ricci, V. Mazzaferro, *et al*., *N. Engl. J. Med*. 2008, **359**, 378; f) H. B. El-Serag, *N. Engl. J. Med*. 2011, **365**, 1118.

2 H. Tateno, S. Nakamura-Tsuruta, J. Hirabayashi, *Glycobiology* 2009, **19**, 527.

3 Y. Kobayashi, H. Tateno, H. Dohra, K. Moriwaki, E. Miyoshi, J. Hirabayashi, H. Kawagishi, *J. Biol. Chem*. 2012, **287**, 33973.

4 a) Y. Aoki, S. Tanimoto, D. Takahashi, K. Toshima, *Chem. Commun*. 2013, **49**, 1169; b) A. Suzuki, M. Hasegawa, M. Ishii, S. Matsumura, K. Toshima, *Bioorg. Med. Chem. Lett*. 2005, **15**, 4624; c) K. Toshima, *Mol. Biosyst*. 2013, **9**, 834; d) S. Tanimoto, D. Takahashi, K. Toshima, *Chem. Commun*. 2012, **48**, 7659.

5 K. Nakajima, M. Kinoshita, Y. Oda, T. Masuko, H. Kaku, N. Shibuya, K. Kakehi, *Glycobiology* 2004, **14**, 793.

6 H. Hase, T. Ikenaka, Y. Matsushima, *Biochem. Biophys. Res. Commun*. 1978, **85**, 257.

7 a) D. Takahashi, T. Miura, K. Toshima, *Chem. Commun*. 2012, **48**, 7595; b) Y. Imai, S. Hirono, H. Matsuba, T. Suzuki, Y. Kobayashi, H. Kawagishi, D. Takahashi, K. Toshima, *Chem. Asian J*. 2012, **7**, 97; c) D. Takahashi, S. Hirono, C. Hayashi, M. Igarashi, Y. Nishimura, K. Toshima, *Angew. Chem. Int. Ed*. 2010, **49**, 10096; d) M. Ishii, S. Matsumura, K. Toshima, *Angew. Chem. Int. Ed*. 2007, **46**, 8396; e) D. Takahashi, K. Toshima, *Chem. Commun*. 2012, **48**, 4397.

8 E. Wertz, B. Bolton *in Electron Spin Resonance*, McGraw-Hill, New York, 1972.

9 H. M. Swartz, J. R. Bolton, D. C. Borg in *Biological Application of Electron Spin Resonance*, Wiley-Interscience, New York, 1972.

10 a) G. B. Jones, J. M. Wright, G. Hynd, J. K. Wyatt, M. Yancisin, M. A. Brown, *Org. Lett*. 2000, **2**, 1863; b) T. Furuta, M. Sakai, H. Hayashi, T. Asakawa, F. Kataoka, S. Fujii, T. Suzuki, Y. Suzuki, K. Tanaka, N. Fishkin, K. Nskanishi, *Chem. Commun*. 2005, **41**, 4575.

11 a) R. T. Dean, S. Fu, R. Stocker, M. J. Davies, *Biochem. J*. 1997, **324**, 1; b) M. J. Davies, *Biochem. Biophis. Res. Commun*. 2003, **305**, 761; c) M. Gracanin, C. L. Hawkins, D. I. Pattison, M. J. Davies, *Free Rad. Bio. Med*. 2009, **47**, 92.

12 T. Mosmann, *J. Immunol. Methods* 1983, **65**, 55.