Published in final edited form as:

Chemistry. 2012 March 26; 18(13): 3850–3854. doi:10.1002/chem.201200075.

ROS-Inducible DNA Cross-linking Agent as a New Anticancer Prodrug Building Block

Sheng Cao, Yibin Wang, and Xiaohua Peng

Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, 3210 N. Cramer St., Milwaukee, WI 53211, U.S.A.

Keywords

anticancer agents; DNA cross-linking; DNA alkylation; H₂O₂-activation; arylboronates

Some antitumor drugs react with DNA by inducing DNA interstrand cross-links (ICLs) which can block DNA transcription and replication.^[1] ICL-inducing agents, such as nitrogen mustard, mitomycin C, cisplatin, and psoralens have been used in cancer therapy.^[2] However, the major disadvantage of these agents is their poor selectivity for cancer cells. One novel approach to reduce the toxicity of cross-linking agents for normal cells would be the creation of prodrugs that undergo tumor-specific activation. Inducible DNA cross-linking or alkylating agents have been developed by several research groups.^[3–6] However, few of them can induce DNA crosslinks selectively under tumor-specific conditions. One of the exclusive features of cancer cells is the high level of oxidative stress that is associated with the increased amounts of reactive oxygen species (ROS).^[7–10] Therefore, it would be advantageous to develop novel cross-linking agents that can be activated by the high level of ROS in cancer cells.

Among different ROS, such as hydrogen peroxide (H₂O₂), hydroxyl radical (•OH), and superoxide radical anions (O₂^{•−}), H₂O₂ has a pivotal role because it is a stable ROS and generated from nearly all sources of oxygen radicals.^[11] Increased levels of H₂O₂ in cancer cells compared to normal cells have been reported.^[7,12] These factors make H₂O₂ an ideal candidate as a therapeutic target to develop new ROS-inducible prodrugs with high selectivity to cancer cells. Arylboronic esters are particularly suitable to this prodrug approach because H₂O₂ can readily cleave the boronic ester to release the quinone methide (QM).^[13] Quinone methides are important intermediates in a large number of DNA cross-linking and alkylating processes. With simple modifications suggested by Rokita's group,^[4] Freccero's group,^[5] Zhou's group^[6] and others,^[14] QM can induce DNA ICLs through different strategies for initiation including UV irradiation, fluoride ion, heating, or oxidation et.. Meanwhile, Cohen's group reported the activation of Matrix Metalloproteinase inhibitor *in situ* by protecting the hydroxyl group of the zinc-binding group (ZBG) with boronic ester as the H₂O₂-sensitive trigger.^[15] Boronate-based probes have been developed by Chang's group, Lo's group, and others for selective detection and imaging of hydrogen peroxide in cells.^[16] Recently, our group has shown that a prodrug of nitrogen mustard coupled with an arylboronate can be triggered by H₂O₂ to release active drugs that can kill cancer cells.^[17] However, therapeutic utility would require a more efficient trigger that can be coupled with multiple potent effectors to maximize the ROS-inducible cytotoxicity of prodrugs. We expect that the arylboronic ester and biarylboronic ester derivatives **1–3** can be activated by

Correspondence to: Xiaohua Peng.

Fax: (+01)414-2295530, pengx@uwm.edu.

H₂O₂ to release biquinone methide and two amine effectors. This work describes the synthesis and biological studies of **1-3**.

Compound **2** was synthesized starting from commercially available 2,5-dibromo-*p*-xylene **2a** (Scheme 1). Palladium-catalyzed borylation of **2a** provided boronated intermediate **2b**, which reacted with NBS yielding brominated analog **2c**. Compound **2c** was converted to **2** using trimethylamine in CH₃CN. In a similar way, **1** and **3** were synthesized (Supporting Information, Scheme S1 and S2).

Initially, the activity of compounds **1-3** towards DNA was investigated using a 49 mer DNA duplex **4** by measuring DNA ICL formation. The reactions of **1-3** with DNA were carried out in phosphate buffer (pH = 8.0) at 37 °C. ICL formation and cross-linking yield were analyzed via denaturing polyacrylamide gel electrophoresis (PAGE) with phosphorimager analysis (Image Quant 5.2). In the absence of H₂O₂, ICLs were not observed with **1-3** (Figure 1, lanes 2-4), which indicated that QM was not formed. In the presence of H₂O₂, compound **2** efficiently induce the ICL formation (Figure 1, lanes 9-11). However, no ICL was observed with **1** and **3** (Figure 1, lanes 6-8 and 12-14). The cross-linking efficiency of **2** was affected by the concentration of the drug, the ratio of drug to H₂O₂, and the pH value of the buffer solution. DNA cross-linking by **2** was observed at a concentration as low as 10 μM (Figure 1, lane 9) and 2 mM of drug **2** led to 24% ICLs (Figure 1, lane 11). The best ratio of drug to H₂O₂ is 2:1 (Supporting Information, Figure S1). Higher cross-linking yield was observed under basic conditions (pH 8 and pH 9) (Supporting Information, Figure S2). This is consistent with the earlier observation that the reaction between arylboronic acid and hydrogen peroxide was pH-dependent.^[13,17,18] The ICL formation induced by **2** displayed first-order kinetics with a rate constant (k_{obs}) of $(4.9 \pm 0.3) \times 10^{-5} \text{ s}^{-1}$ ($t_{1/2} = 4 \text{ h}$) (Figure S3).

The deboronation and activation of **2** is selective for H₂O₂ over other reactive oxygen species (Figure 2). The reaction of **2** with H₂O₂ at 200 μM induced about 8% ICL formation, while the cross-linking yield with *tert*-butylhydroperoxide (TBHP), hydroxyl radical, *tert*-butoxy radical, superoxide, and nitric oxide is less than 1% and less than 5% ICL was observed for hypochlorite.

In order to acquire further insight into the reactivity of **2**, we determined DNA monoalkylations by examining the stability and reactivity of purified ICL products and the reacted single stranded DNA. The stability and alkaline lability of DNA alkylation products depend upon the reaction site. The N7-alkylated products of purines are labile to piperidine treatment, which can result in DNA cleavage.^[3c,19] The ICLs formed from **2** are stable to heating in phosphate buffer (90 °C, pH 7.2, 30 min) and partially stable to 1 M piperidine treatment (90 °C, 30 min) (Supporting Information, Figure S4 lanes 3 and 4). DNA cleavage bands were observed with all dGs and dAs when the purified ICLs were treated with 1 M piperidine (Supporting Information, Figure S4 lanes 4, 11, 18, and 25). Weak cleavage bands were observed with dCs flanked by dAs and/or dGs. These results indicated that the alkylations occurred at N7 of dG and dA, and partially with dC. The stable ICLs observed after piperidine treatment suggested that N7 is not the only alkylation site (Supporting Information, Figure S4 lanes 4, 11, 18, and 25). It is highly likely that the alkylation took place at exocyclic amines. Monoalkylation of all guanine and adenine units was also observed with single-stranded DNA **4a** or **4b** (Supporting Information, Figure S4 lanes 2, 9, 16, and 23). In a control experiment, oligonucleotide **4** was treated with H₂O₂ alone with the

concentrations of 10 μM , 100 μM , and 1.0 mM, weak cleavage bands were observed at the purine and pyrimidine sites (Supporting Information, Figure S5). This indicated that H_2O_2 alone induced trace amount of hydroxyl radical upon heating, which led to the equal cleavages with purine and pyrimidine nucleotides.^[20]

In an effort to identify the interstrand cross-linking site of compound **2**, we used hydroxyl radical cleavage analysis of the purified cross-linking product.^[20] However, a completely cleaved ladder similar to the hydroxyl radical control experiment was observed with stronger cleavage bands at the positions of dGs and dAs (Supporting Information, Figure S4 lanes 5, 12, 19, and 26). This further confirmed that the interstrand cross-linking should occur at these positions. However, the exact interstrand cross-linking site and pattern are still unknown. This is caused by three factors: a. high reactivity of the quinone methide generated from **2** upon H_2O_2 -activation; b. the complexity of the reaction products which include interstrand cross-linking product, intrastrand cross-linking and monoalkylated product, and adducts formed with dG, dA, and dC; c. the cross-linking and alkylated products are labile to piperidine treatment.

To understand the difference for ICL formation between compounds **2** and **1** or **3**, their reaction mechanism was investigated by NMR analysis of the monomer reactions. The reaction of **1-3** with H_2O_2 was performed in 10 mM deuterated potassium phosphate buffer (pH 8.0) as well as in DMSO/ D_2O . When phosphate buffer was used as a solvent, the reaction was too fast to observe any intermediates. Compounds **1-3** were completely consumed within 5 min and converted to the phenol derivatives **6**, **8**, and **10** (Scheme 2 and Supporting Information, Figure S6B, S7B, & S9B). The formation of **6**, **8**, and **10** was confirmed by ^1H NMR, ^{13}C NMR, and HRMS-ESI analysis of the isolated materials (Supporting Information Figure S21-S27).

When the reaction of **2** with H_2O_2 was carried out in a mixture of DMSO/ D_2O , we were able to observe all intermediates formed (Scheme 3 and Figure 3A-D). The integral change of protons $\text{C}_3\text{-H}$, $\text{C}_6\text{-H}$, $\text{C}_2'\text{-H}$ and $\text{C}_5'\text{-H}$ characterized the kinetic transformation of compound **2** into the phenol derivative **6**. Analysis 3 h after addition of H_2O_2 revealed that compound **2** was completely consumed (Figure 3C), as evidenced by the absence of diagnostic resonances at δ 7.99 (singlet) and δ 4.86 (singlet) corresponding to the vinyl and methylene protons of **2**, respectively. The resonances corresponding to **2** were replaced by those that were indicative of molecule **5**. Evidence for these intermediates was obtained from the appearance of two different aromatic protons ($\sim \delta$ 7.85, 7.11) (Figure 3B,C), and two different methylene protons ($\sim \delta$ 4.72, 4.46). After incubating the sample at room temperature for 24 h (Figure 3D), the resonances corresponding to **5** were replaced by those of **6** ($\sim \delta$ 6.95, 4.37). Similarly, the activation of **1** and **3** with H_2O_2 followed by the formation of phenol derivatives were observed by NMR analysis (Supporting Information, Figure S8 & S10 and Scheme S4 & S5).

The NMR analysis of monomer reaction indicated that compound **2** can be efficiently activated by H_2O_2 to give the intermediate **6**. The formation of **6** was triggered by the stepwise oxidation of the carbon-boron bond initiated by a nucleophilic attack by H_2O_2 (**2** $\rightarrow$ **2d** and **5** $\rightarrow$ **2e**), followed by deboronation (**2d** $\rightarrow$ **5** and **2e** $\rightarrow$ **6**) (Scheme 3). We believed that compound **6** directly generated α -QMs (**2f** and **2h**) under physiological conditions that alkylate and cross-link DNA. By comparison with compounds **8** and **10**, an additional hydroxyl group in **6** leads to an electron rich aromatic ring which is an important factor for the efficient ICL formation induced by **2**. Rokita *et al.* have shown that the electron-donating group favors QM generation and regeneration, while electron-withdrawing group hinders the formation of QM.^[4d,4e,5b] This could explain the obvious difference between compounds **1/3** and **2** regarding the cross-linking properties.

In order to determine whether QM was generated from the oxidation process or from the intermediate **6**, QM trapping experiments with a large excess of ethyl vinyl ether (EVE) were performed. When **2** was incubated at 37 °C for 3 h in the presence of H₂O₂ and EVE, no detectable QM-EVE adduct (**11**) was observed and **2** was completely converted to **6** (Scheme 4). However, **11** was detected if **6** was incubated at the same temperature for 48 h. Compound **11** was confirmed by ¹H and ¹³C NMR analysis. The generation of QM via **6** was further supported by the cross-linking capability of **6** (Supporting Information, Figure S11).

In conclusion, we have developed three H₂O₂-inducible DNA bisalkylating and/or cross-linking agents (**1-3**) which contain boronic ester. Among those compounds, the non-toxic compound **2** can be selectively activated by H₂O₂ to generate a powerful and reversible DNA alkylating agent **6**, which directly produces QMs under physiological conditions and release the leaving group trimethyl amine. The mechanism of H₂O₂-activation, ICL formation, and DNA alkylation was determined by NMR analysis as well as by a QM trapping experiment. Although the ICL yield by **2** is moderate, the alkylating and cross-linking potency can be improved by introducing an alkylating, DNA binding, or intercalating agent in the position of trimethyl amine. Therefore, an effective strategy has been developed to design and synthesize novel potent anticancer prodrugs that can be activated under tumor-specific conditions (high level of ROS) to release multiple active species by using compound **2** as a building block. Such model will also be equally applicable to the development of prodrugs for the treatment of other diseases that are associated with H₂O₂. The synthesis of boronate-based prodrugs with different functional leaving groups is underway in our lab.

Experimental Section

1,4-Dimethyl-2,5-di(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl)phenyl (**2b**)

2,5-dibromo-p-xylene **2a** (1.06 g, 4 mmol), bis(pinacolato)diboron (3.05 g, 12 mmol), KOAc (2.36 g, 24 mmol), and PdCl₂(dppf) (196 mg, 0.24 mmol) were dissolved in DMF (40 mL) under argon atmosphere. The mixture was heated at 85 °C for 48 h, cooled and then water (100 mL) was added, the mixture was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layer was washed with water and brine, and then dried over Na₂SO₄, and the solvent was evaporated. The crude product was purified through column chromatography with 0-50% EtOAc in hexane to provide compound **2b** as white solid (1.28 g, 90%). ¹H NMR (300 MHz, CDCl₃) δ 1.39 (s, 24H), 2.51 (s, 6H), 7.57 (s, 2H); ¹³C NMR (500 MHz, CDCl₃) δ 140.58, 136.94, 83.43, 24.91, 21.52.

1,4-Dibromomethyl-2,5-di(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl)phenyl (**2c**)

Compound **2b** (1.08 g, 3 mmol) was dissolved in CH₃CN (45 mL), and NBS (1.34 g, 7.5 mmol) and AIBN (52.4 mg) were added. The mixture was refluxed at 90 °C for 6 h. Then the mixture was concentrated and dissolved in DCM (100 mL). The organic phase was washed with H₂O (3 × 50 mL) and dried with anhydrous Na₂SO₄. The solution was evaporated and the residue was subject to column chromatography on silica gel with 0-50% DCM in hexane to give the desired product **2c** as a white solid (0.71 g, 60 %). ¹H NMR (300 MHz, CDCl₃) δ 1.38 (s, 24H), 4.88 (s, 4H), 7.79 (s, 2H); ¹³C NMR (500 MHz, CDCl₃) δ 143.36, 137.85, 84.13, 33.43, 24.90.

1,4-di(trimethylammonium bromide-methyl)-2,5-di(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2'-yl)phenyl (**2**)

Compound **2c** (0.1 g, 0.19 mmol) was suspended in CH₃CN (5 mL), and 4.2 M trimethylamine (0.14 mL, 0.57 mmol) in ethanol was added dropwise with stirring. The

reaction mixture was concentrated after 12 h at rt and gave **2** as white solid (0.12 g, 95%). ¹H NMR (300 MHz, DMSO) δ 1.35 (s, 24H), 3.05 (s, 18H), 4.86 (s, 4H), 8.02 (s, 2H). ¹³C NMR (500 MHz, DMSO) δ 141.57, 134.66, 84.76, 65.84, 52.10, 24.49; HRMS (ESI) *m/z* Calcd for C₂₆H₄₈B₂Br₂N₂O₄ [(M-2Br)/2]⁺ 237.1900, found 237.1862.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for financial support of this research from the National Cancer Institute (1R15CA152914-01) and UWM start-up funds. We thank Dr. Michael Seidman for helpful comments.

References

1. a Noll DM, Mason TM, Miller PS. *Chem. Rev.* 2006; 106:277–301. [PubMed: 16464006] b Dronkert MLG, Kanaar R. *Mutat. Res.* 2001; 486:217–247. [PubMed: 11516927]
2. a Rink SM, Solomon MS, Taylor MJ, Rajur SB, McLaughlin LW, Hopkins PB. *J. Am. Chem. Soc.* 1993; 115:2551–2557. b Tomasz M. *Chemistry & Biology.* 1995; 2:575–579. [PubMed: 9383461] c Sherman SE, Lippard SJ. *Chem. Rev.* 1987; 87:1153–1181. d Cimino GD, Gamper HB, Isaacs ST, Hearst TE. *Annu. Rev. Biochem.* 1985; 54:1151–1193. [PubMed: 2411210]
3. a Hong IS, Greenberg MM. *J. Am. Chem. Soc.* 2005; 127:10510–10511. [PubMed: 16045337] b Hong IS, Ding H, Greenberg MM. *J. Am. Chem. Soc.* 2006; 128:485–491. [PubMed: 16402835] c Peng X, Hong IS, Li H, Seidman MM, Greenberg MM. *J. Am. Chem. Soc.* 2008; 130:10299–10306. [PubMed: 18620398]
4. a Zeng Q, Rokita SE. *J. Org. Chem.* 1996; 61:9080–9081. b Veldhuyzen WF, Pande P, Rokita SE. *J. Am. Chem. Soc.* 2003; 125:14005–14013. [PubMed: 14611237] c Wang H, Rokita SE. *Angew. Chem.* 2010; 122:6093–6096. *Angew. Chem. Int. Ed.* 2010; 49:5957–5960. d Weinert EE, Dondi R, Colloredo-Melz S, Frankenfield KN, Mitchell CH, Freccero M, Rokita SE. *J. Am. Chem. Soc.* 2006; 128:11940–11947. [PubMed: 16953635] e Wang H, Wahi MS, Rokita SE. *Angew. Chem.* 2008; 120:1311–1313. *Angew. Chem., Int. Ed.* 2008; 47:1291–1293.
5. a Richter SN, Maggi S, Mels SC, Palumbo M, Freccero M. *J. Am. Chem. Soc.* 2004; 126:13973–13979. [PubMed: 15506758] b Antonio MD, Doria F, Richter SN, Bertipaglia C, Mella M, Sissi C, Palumbo M, Freccero M. *J. Am. Chem. Soc.* 2009; 131:13132–13141. [PubMed: 19694465] c Verga D, Nadai M, Doria F, Perciballe C, Antonio MD, Palumbo M, Richter SN, Freccero M. *J. Am. Chem. Soc.* 2010; 132:14625–14637. [PubMed: 20863115] d Modica E, Zanaletti R, Freccero M, Mella M. *J. Org. Chem.* 2001; 66:41–52. [PubMed: 11429928]
6. a Wang P, Liu R, Wu X, Ma H, Cao X, Zhou P, Zhang J, Weng X, Zhang X, Qi J, Zhou X, Weng L. *J. Am. Chem. Soc.* 2003; 125:1116–1117. [PubMed: 12553783] b Weng X, Ren L, Weng L, Huang J, Zhu S, Zhou X, Weng L. *Angew. Chem.* 2007; 119:8166–8169. *Angew. Chem. Int. Ed.* 2007; 46:8020–8023.
7. Szatrowski TP, Nathan CF. *Cancer Res.* 1991; 51:794–798. [PubMed: 1846317]
8. Toyokuni S, Okamoto K, Yodoi J, Hiai H. *FEBS Lett.* 1995; 358:1–3. [PubMed: 7821417]
9. Kawanishi S, Hiraku Y, Pinlaor S, Ma N. *Biol. Chem.* 2006; 387:365–372. [PubMed: 16606333]
10. a Pelicano H, Carney D, Huang P. *Drug Resist. Update.* 2004; 7:97–110. b Trachootham D, Alexandre J, Huang P. *Nature Rev.* 2009; 8:579–591. c Lopez-Lazaro M. *Cancer Lett.* 2007; 252:1–8. [PubMed: 17150302]
11. a Lee YJ, Shacter E. *Free Radic. Biol. Med.* 2000; 29:684–692. [PubMed: 11033421] b Apopa PL, He X, Ma Q. *J. Biochem. Mol. Toxicol.* 2008; 22:63–76. [PubMed: 18273910]
12. a Zieba M, Suwalski M, Kwiatkowska S, Piasecka G, Grzelewska-Rzymowska I, Stolarek R, Nowak D. *Respir. Med.* 2000; 94:800–805. [PubMed: 10955757] b Lim SD, Sun C, Lambeth JD, Marshall F, Amin M, Chung L, Petros JA, Arnold RS. *Prostate.* 2005; 62:200–207. [PubMed: 15389790]

13. Kuivila HG, Armour AG. *J. Am. Chem. Soc.* 1957; 79:5659–5662.
14. Nakatani K, Higashida N, Saito I. *Tetrahedron Lett.* 1997; 38:5005–5008.
15. Major Jourden JL, Cohen SM. *Angew. Chem.* 2010; 122:6947–6949. *Angew. Chem. Int. Ed.* 2010; 49:6795–6797.
16. a Miller EW, Albers AE, Pralle A, Isacoff EY, Chang CJ. *J. Am. Chem. Soc.* 2005; 127:16652–16659. [PubMed: 16305254] b Dickinson BC, Chang CJA. *J. Am. Chem. Soc.* 2008; 130:9638–9639. [PubMed: 18605728] c Miller EW, Tulyathan O, Isacoff EY, Chang CJ. *Nat. Chem. Biol.* 2007; 3:263–267. [PubMed: 17401379] d Lo L-C, Chu C-Y. *Chem. Commun.* 2003; 39:2728–2729. e Karton-Lifshin N, Segal E, Omer L, Portnoy M, Satchi-Fainaro R, Shabat D. *J. Am. Chem. Soc.* 2011; 133:10960–10965. [PubMed: 21631116]
17. Kuang Y, Balakrishnan K, Gandhi V, Peng X. *J. Am. Chem. Soc.* 2011; 133:19278–19281. [PubMed: 22035519]
18. Kuivila HG. *J. Am. Chem. Soc.* 1954; 76:870–874.
19. Haraguchi K, Delaney MO, Wiederholt CJ, Sambandam A, Hantosi Z, Greenberg MM. *J. Am. Chem. Soc.* 2002; 124:3263–3269. [PubMed: 11916409]
20. a Price MA, Tullius TD. *Methods Enzymol.* 1992; 212:194–219. [PubMed: 1325598] b Maxam AM, Gilbert W. *PNAS.* 1977; 74:560–564. [PubMed: 265521] c Peng X, Pigli YZ, Rice PA, Greenberg MM. *J. Am. Chem. Soc.* 2008; 130:12890–12891. [PubMed: 18778053]

Scheme 1.
Synthesis of compound **2**.

Scheme 2.

The structure of compounds **6**, **8**, and **10** generated from the reaction of **2**, **1**, and **3** with H_2O_2 .

Scheme 3.

Tandem QM generation and ICL formation induced by **2** upon H_2O_2 -activation.

Scheme 4.
Trapping reactions in the presence of EVE.

Figure 1.

Concentration dependence of compounds **1-3** for DNA cross-link formation upon H₂O₂ activation. Lane 1: DNA only (cross-linking yield 0%); lane 2: DNA with **1** (2 mM) only (cross-linking yield 0%); lane 3: DNA with **2** (2 mM) only (cross-linking yield 0%); lane 4: DNA with **3** (2 mM) only (cross-linking yield 0%); lane 5: DNA with H₂O₂ (10 μM) only (cross-linking yield 0%); lane 6: 10 μM **1** + 5 μM H₂O₂ (0%); lane 7: 100 μM **1** + 50 μM H₂O₂ (0%); lane 8: 2 mM **1** + 1 mM H₂O₂ (0%); lane 9: 10 μM **2** + 5 μM H₂O₂ (2%); lane 10: 100 μM **2** + 50 μM H₂O₂ (8%); lane 11: 2 mM **2** + 1 mM H₂O₂ (24%); lane 12: 10 μM **3** + 5 μM H₂O₂ (0%); lanes 13: 100 μM **3** + 50 μM H₂O₂ (0%); lane 14: 2 mM **3** + 1 mM H₂O₂ (0%).

Figure 2. Selectivity of **2** (400 μ M) with various reactive oxygen species (ROS) at 200 μ M. Data were acquired at 25 $^{\circ}$ C in 10 mM phosphate buffer, pH 8, after 48 h.

Figure 3.

^1H NMR kinetic analysis of **2** in mixture of deuterated DMSO (550 μL) and D_2O (96 μL) with 57.7 mM H_2O_2 and 23.1 mM **2**; (B) 30 min after addition of H_2O_2 ; (C) 3 h after addition of H_2O_2 ; (D) 24 h after addition of H_2O_2 . In addition, (A) sample **2** in deuterated DMSO only as a reference.