Isotopic labeling experiments that elucidate the mechanism of DNA strand cleavage by the hypoxia-selective antitumor agent 1,2,4-benzotriazine 1,4-di-*N*-oxide

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Figure S1

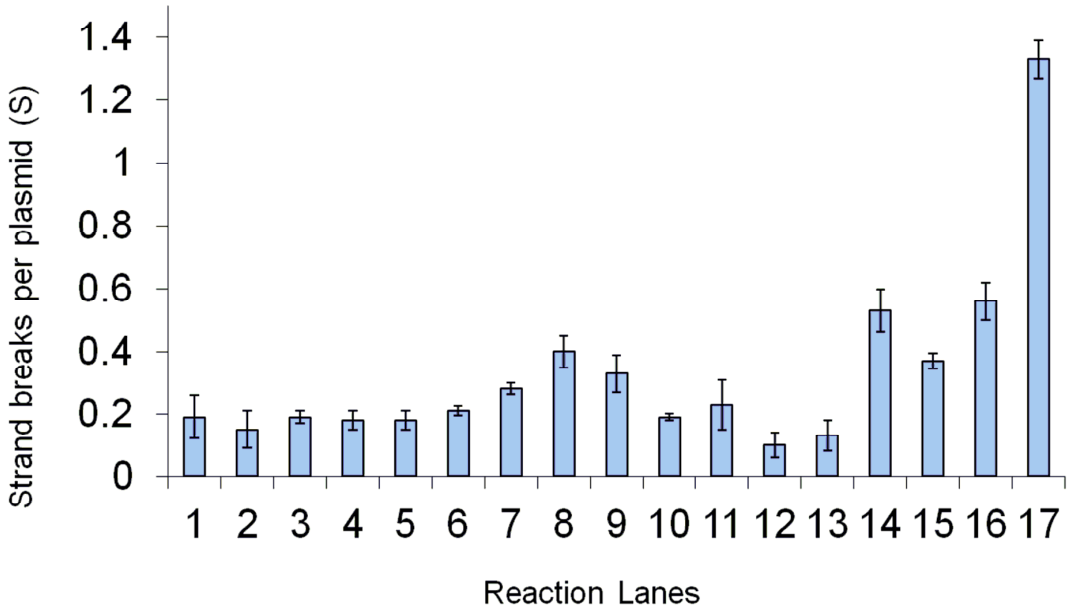


Figure S1. Control reactions relevant to DNA strand cleavage by **1b**. Unless otherwise noted, all reactions contained DNA (33 μg/mL, pGL-2 Basic), **1b** (50 μM), sodium phosphate buffer (in H_2O, 50 mM, pH 7.0), acetonitrile (3.3% v/v), catalase (100 μg/ mL), superoxide dismutase (10 μg/mL), and desferal (1 mM) and were incubated under anaerobic conditions at 25 °C for 4 h, followed by analysis using agarose gel electrophoresis. In reactions containing enzyme, the concentration was (33 mU/mL) and in the reactions containing NADPH the concentration was 500 μM. Lane 1, DNA only (S = 0.19 ± 0.07); lane 2, DNA + cytochrome P450 reductase + NADPH aerobic (S = 0.15 ± 0.06); lane 3, DNA + cytochrome P450 reductase (S = 0.19 ± 0.02); lane 4, DNA + **1b** alone (S = 0.18 ± 0.03); lane 5, DNA + **1b** + cytochrome P450 reductase + NADPH aerobic (S = 0.18 ± 0.03); lane 6, DNA + **1b** + cytochrome P450 reductase (S = 0.21 ± 0.01); lane 7, DNA + **1b** + NADPH aerobic (S = 0.28 ± 0.02); lane 8, DNA + **1c** alone (S = 0.40 ± 0.05); lane 9, DNA + **1c** + cytochrome P450 reductase + NADPH aerobic (S = 0.33 ± 0.06); lane 10, DNA + **1a** alone (S = 0.19 ± 0.01); lane 11, DNA + **1a** + cytochrome P450 reductase + NADPH aerobic (S = 0.23 ± 0.08) ; lane 12, DNA + **3b** alone (S = 0.10 ± 0.04); lane 13, DNA + **7** alone (S = 0.13 ± 0.05); lane 14, DNA + **3b** + cytochrome P450 reductase + NADPH (S = 0.53 ± 0.07); lane 15, DNA + **7** + cytochrome P450 reductase + NADPH (S = 0.37 ± 0.02); lane 16, DNA + **3b** + **7** + cytochrome P450 reductase + NADPH (S = 0.56 ± 0.06); lane 17, DNA + **1b** + **7** + cytochrome P450 reductase + NADPH (S = 1.33 ± 0.06). The results are the average of three or more experiments. The value S represents the mean number of strand breaks per plasmid molecule and is calculated using the equation $S = -\ln f_I$, where f_I is the fraction of plasmid present as form I.

Figure S2

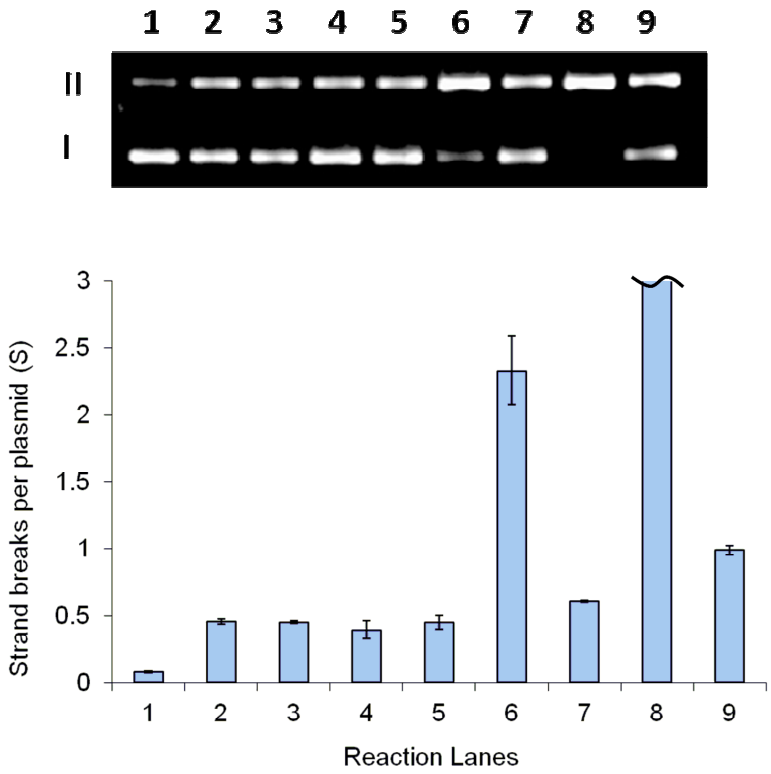


Figure S2 Gel and bar graph showing cleavage of supercoiled plasmid DNA by **1b** (50-100 μM) in the presence of the NADPH:cytochrome P450 reductase enzyme system in phosphate-buffered D_2O/CD_3OD. Uncut, supercoiled (form I) and nicked (form II) DNA are labeled on the gel image. Unless otherwise mentioned, all reactions contained DNA (33 μg/mL, pGL-2 Basic), sodium phosphate buffered-D_2O (50 mM, employing the same buffer ratio as that used for preparation of a pH 7.0 buffer in H_2O), acetonitrile (3.3% v/v), catalase (100 μg/ mL), superoxide dismutase (10 μg/mL), and desferal (1 mM) and were incubated under anaerobic conditions at 25 °C for 4 h, followed by agarose gel electrophoretic analysis. In reactions containing enzyme the concentration was (33 mU/mL) and in the reactions containing NADPH the concentration was 500 μM. Lane 1, DNA in water (S = 0.08 ± 0.01); lane 2, DNA + catalase + desferal + SOD (S = 0.46 ± 0.02); lane 3, same as lane 2 except with 500 mM methanol-d_4 (S = 0.45 ± 0.01); lane 4, DNA + catalase + desferal + SOD + cytochrome P450 reductase + NADPH (but no **1b**) (S = 0.39 ± 0.07); lane 5, DNA + catalase + desferal + SOD + NADPH and 50 μM **1b** (no enzyme) (S = 0.45 ± 0.05); lane 6, DNA + catalase + desferal + SOD + cytochrome P450 reductase + NADPH and 50 μM **1b** (S = 2.33 ± 0.26); lane 7, same as lane 6, except containing methanol-d_4 (500 mM) (S = 0.61 ± 0.01); lane 8, DNA + catalase + desferal + SOD + cytochrome P450 reductase + NADPH and 100 μM **1b** (>99% strand cleavage, S > 4.61); lane 9, same as lane 8, except containing methanol-d_4 (500 mM) (S = 0.99 ± 0.04). The results reflect the average of three experiments. The value S represents the mean number of strand breaks per plasmid molecule and is calculated using the equation $S = -\ln f_I$, where f_I is the fraction of plasmid present as form I.

Figure S3

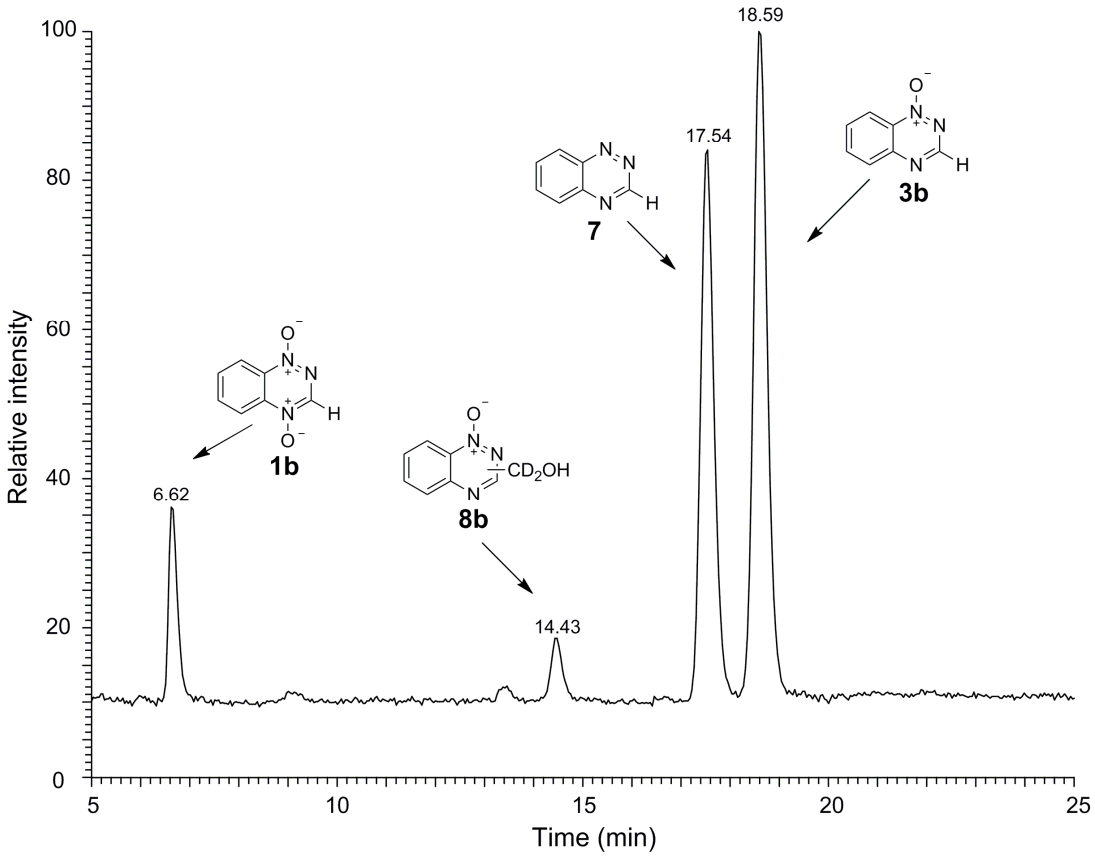


Figure S3. HPLC chromatogram (UV 240 nm) of the products generated by the hypoxic metabolism of **1b** in buffered D_2O/CD_3OD.

Figure S4

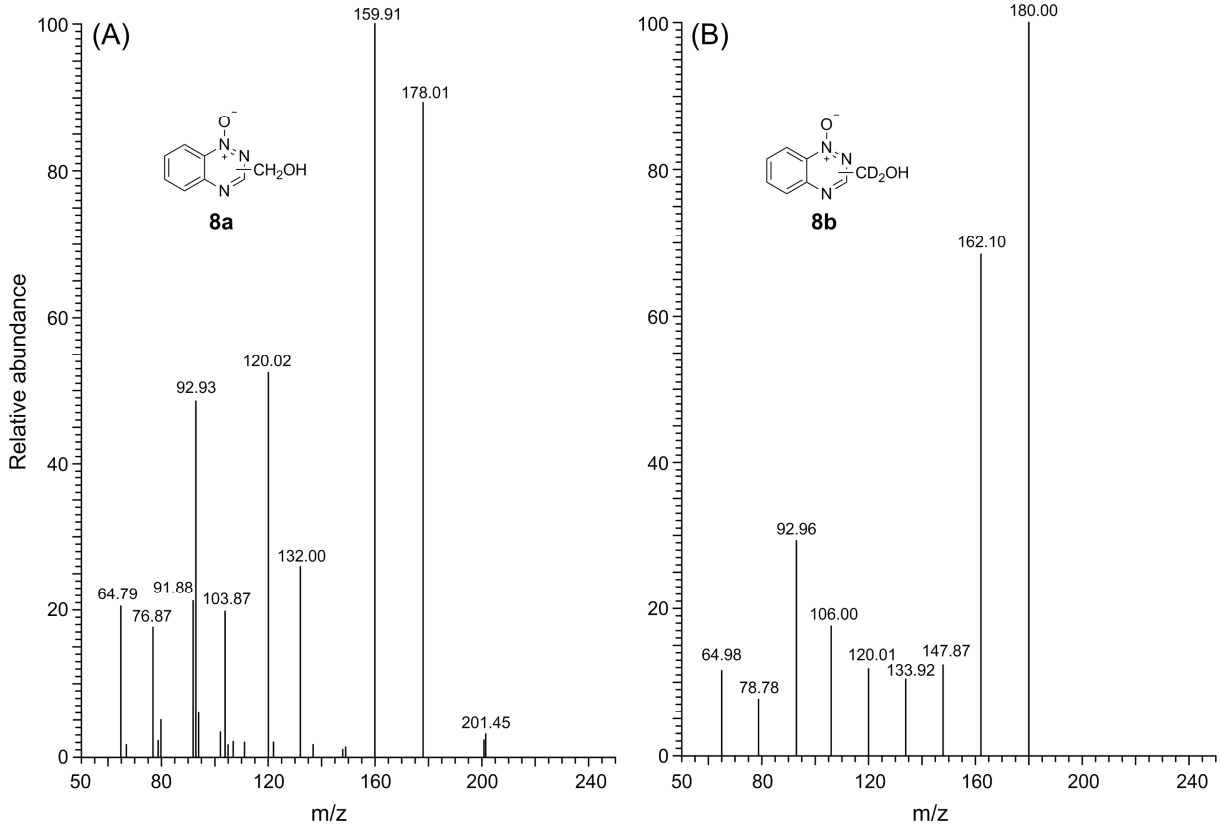


Figure S4. MS/MS spectra of the metabolite **8** generated (A) in the presence of methanol (**8a**) and (B) in the presence of methanol-d_4 (**8b**).

Figure S5

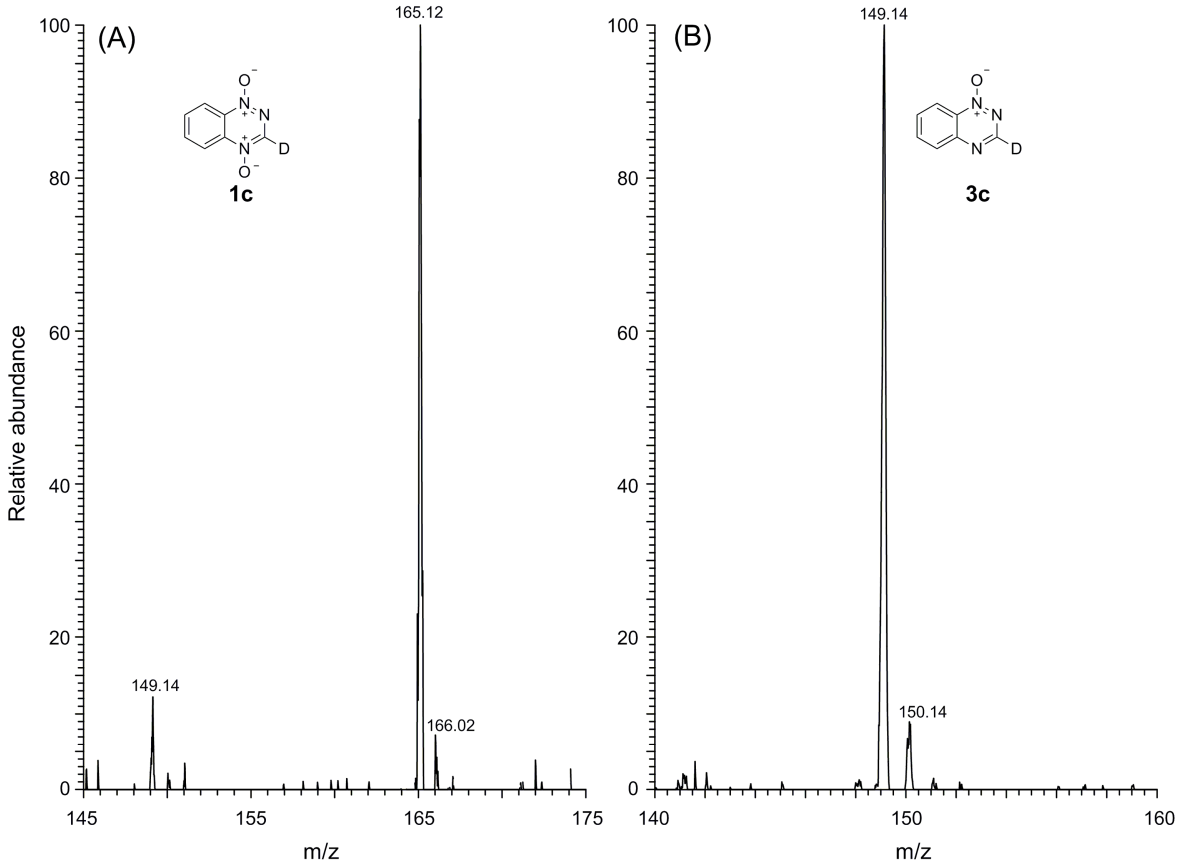


Figure S5. MS of **1c** and **3c** present in the mixture generated by hypoxic metabolism of **1c** by NADPH:cytochrome P450 reductase in buffered H_2O. (A) MS spectra of **1c** (relative peak intensities of m/z 165:166:167 = 100:8.0:1.5) and (B) MS spectra of the metabolite **3c** (relative peak intensities of m/z 149:150:151 = 100:8.5:1.6).

Figure S6. ^{1}H NMR of **1c** (DMSO, 500 MHz)

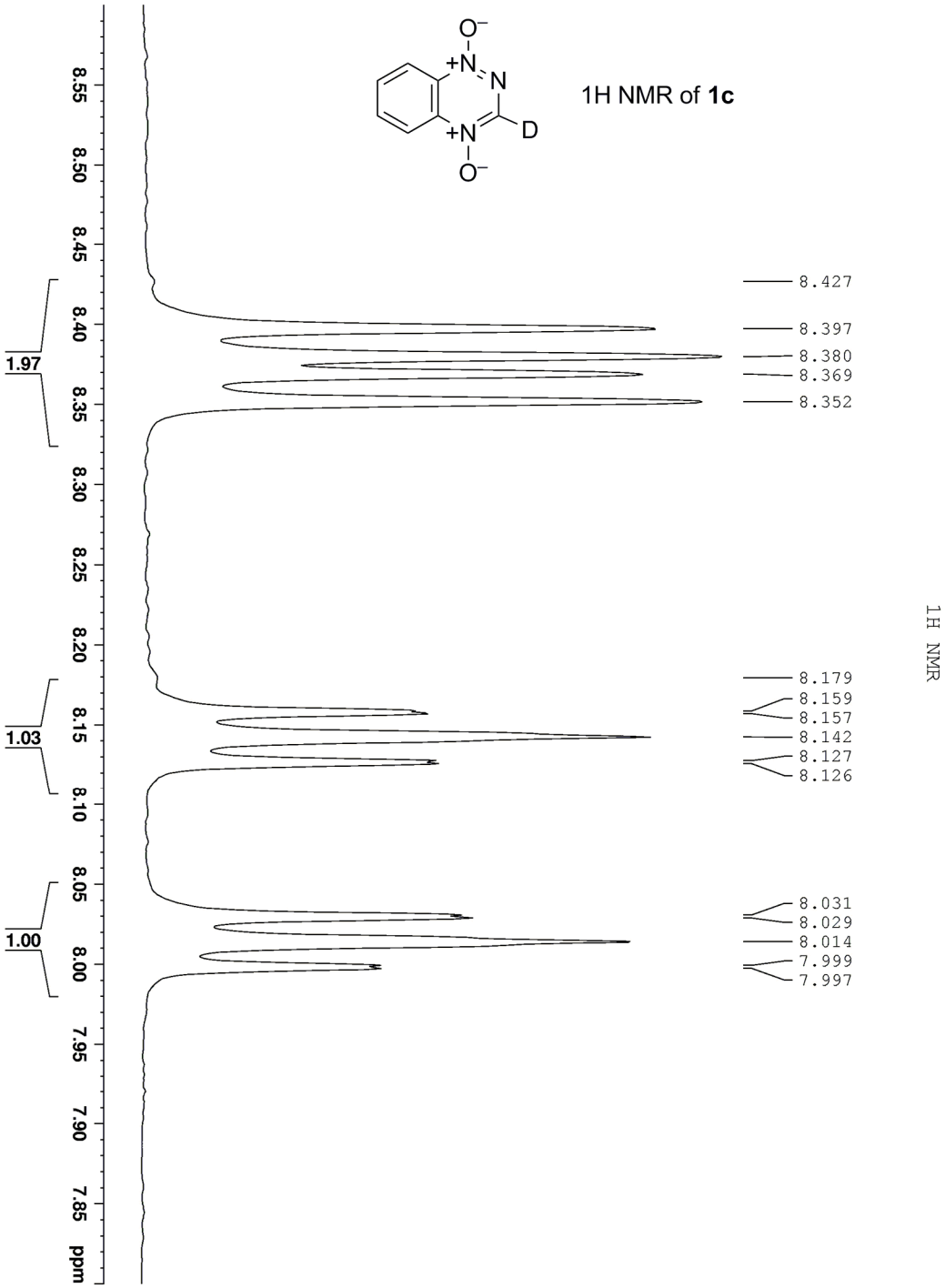


Figure S6 (cont.) ^{13}C NMR of **1c** (DMSO, 125.8 MHz)

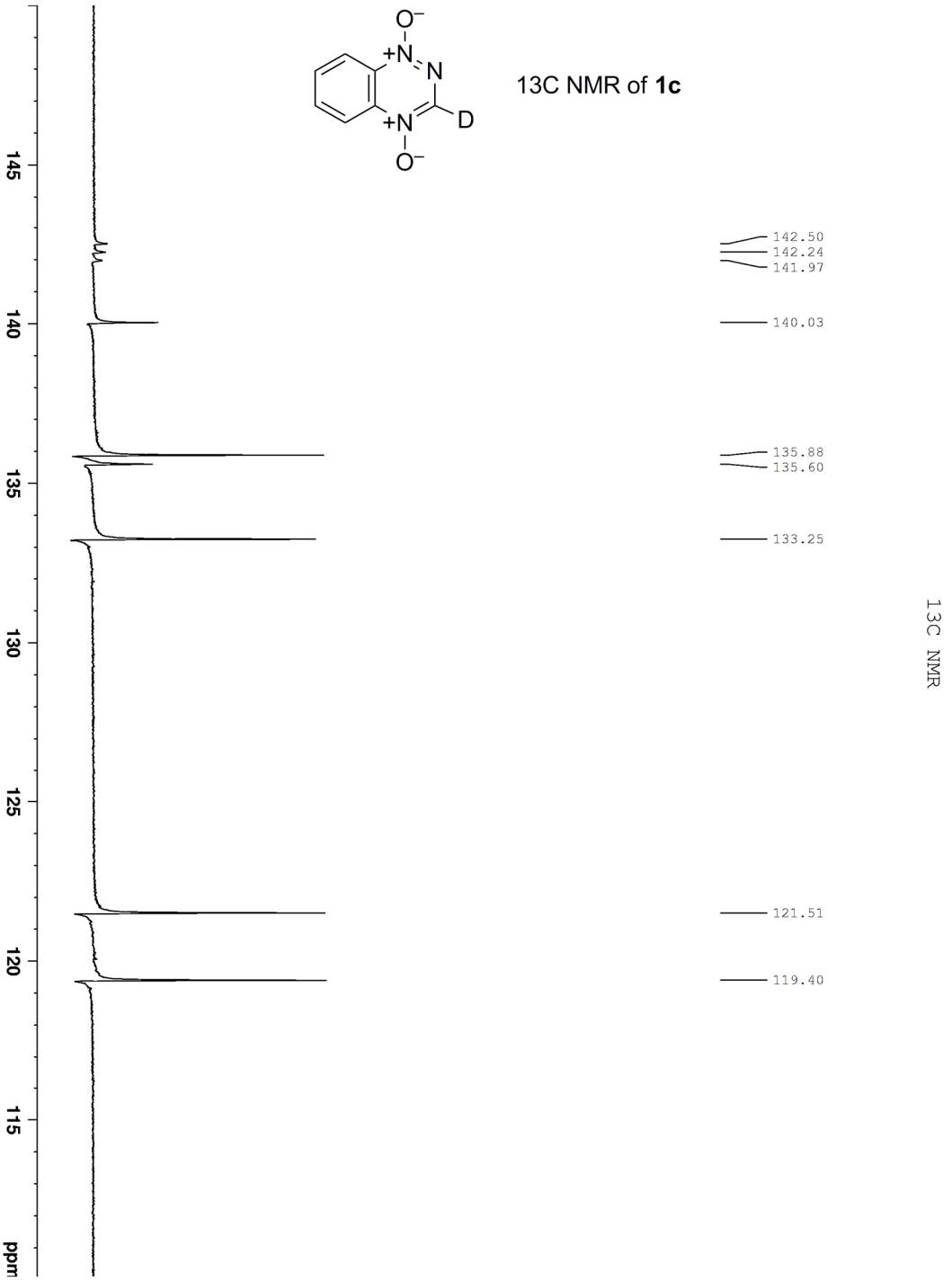


Figure S7. ^{1}H NMR of **7** (DMSO, 500 MHz)

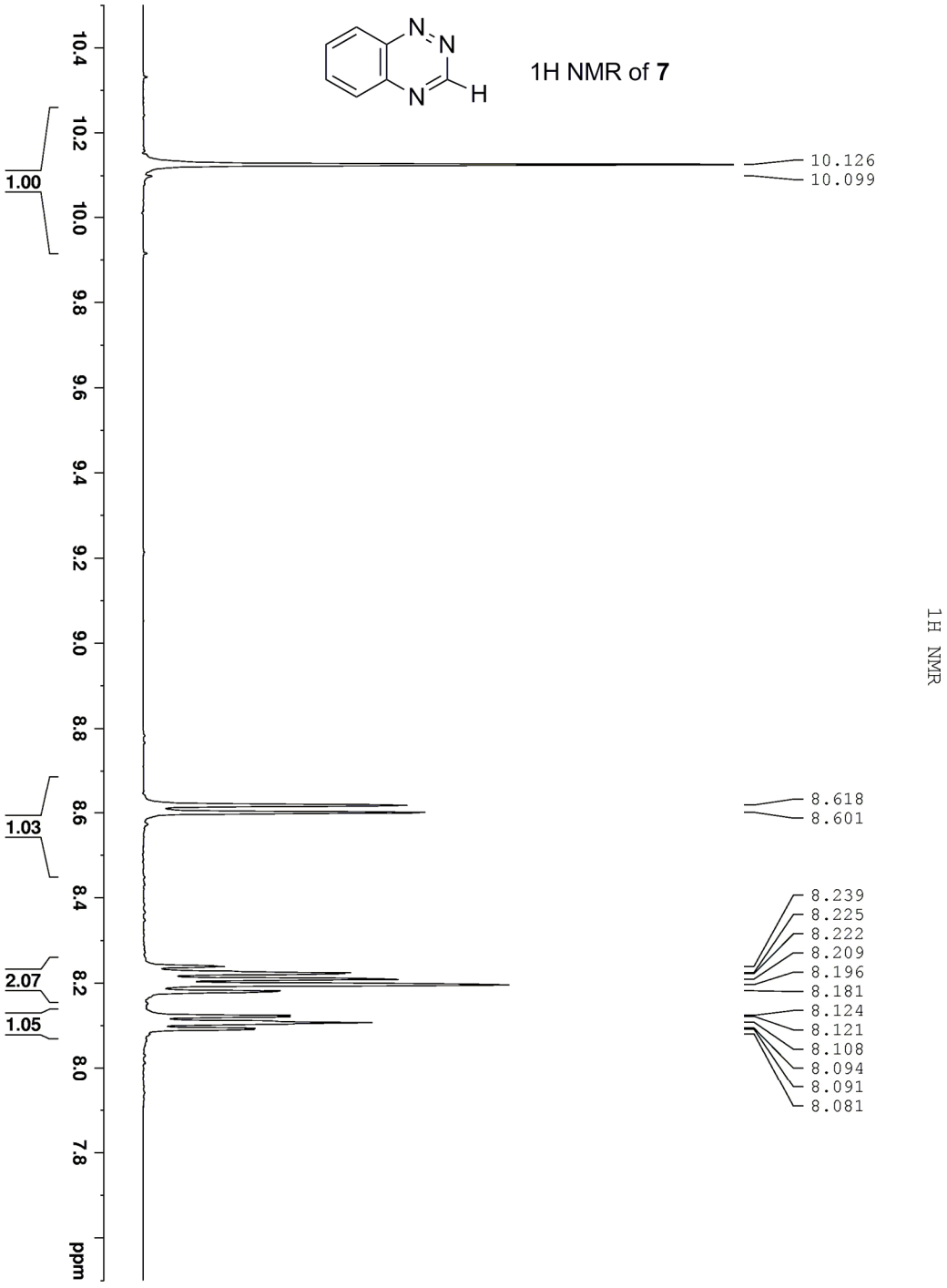
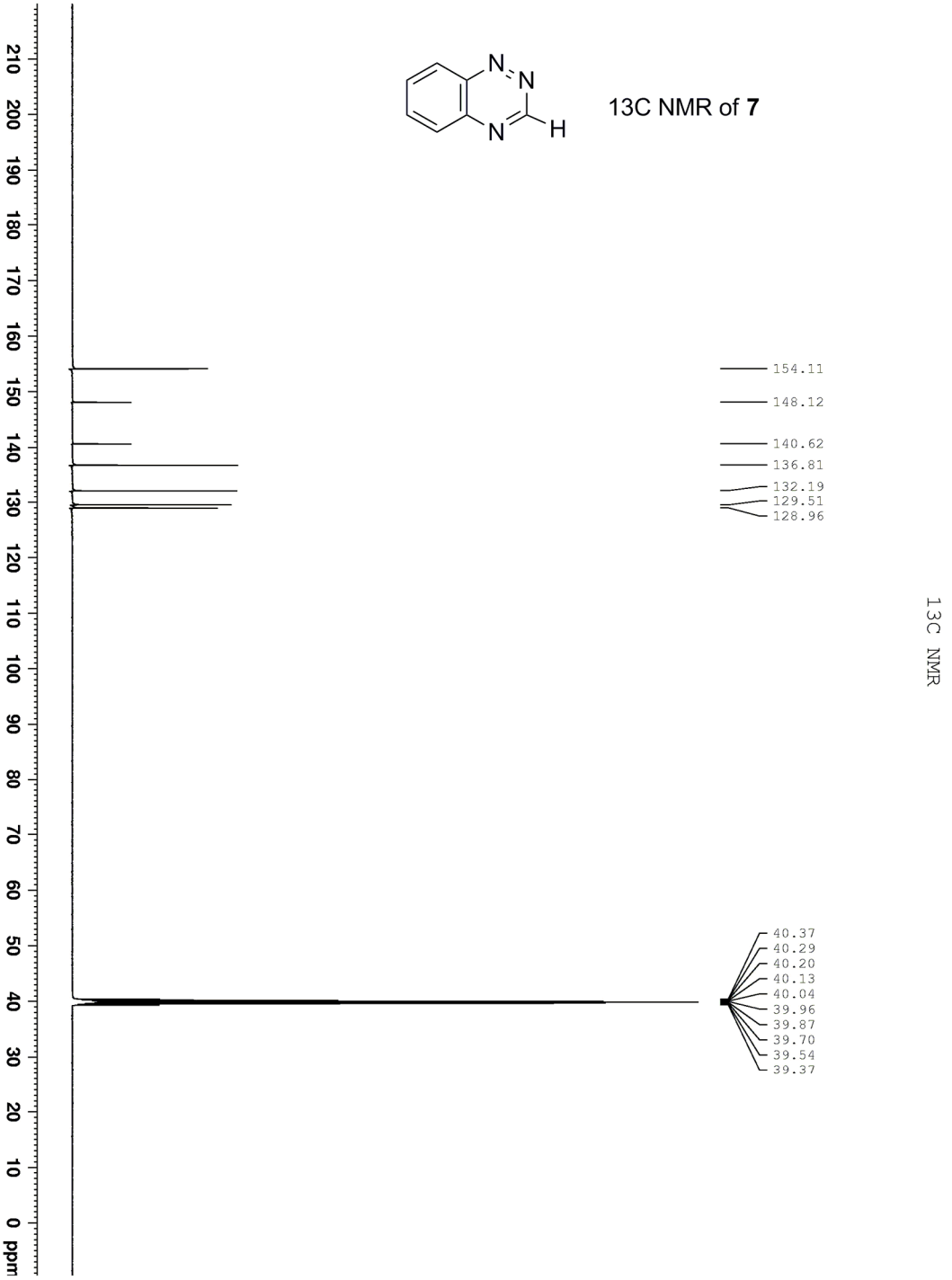


Figure S7 (cont.) ^{13}C NMR of **7** (DMSO, 125.8 MHz)



Could traces of CD_3OH present in the LC-MS isotope incorporation assays represent a confounding factor in interpretation of the data? It is established that bioreductive activation of 1,2,4-benzotriazine 1,4-dioxides gives rise to a highly reactive DNA-damaging radical (Refs 21, 24-28, 38-40 of the article). The ultimate DNA-damaging species generated upon reductive activation of 1,2,4-benzotriazine 1,4-dioxides displays reactivity very similar to hydroxyl radical – indeed, may *be* hydroxyl radical (Scheme 1, in the article, Refs 21, 24-28). DNA strand cleavage by activated **1b** was inhibited by either CH_3OH or CD_3OD (Figure S2). Thus, it is clear that both CH_3OH and CD_3OD can serve as atom donors that quench the reactive intermediate generated upon bioreductive activation of **1b**. The H or D on the *hydroxyl* group of methanol is expected to exchange rapidly with other exchangeable protons in solution (such as those on H_2O), while the H and D atoms on the methyl group of methanol do not exchange. For this reason we conducted the CD_3OD assays in buffered D_2O (99.96% D content) to maintain most of the added methanol in the CD_3O**D** form. It is important to recognize, however, that small amounts of exchangeable protons are present in the assay mixtures in the DNA damage and hypoxic metabolism experiments. For example, in some experiments, the enzyme NADPH:cytochrome P450 reductase was introduced into the assay mixtures in buffered H_2O (0.5 μL of enzyme in buffered H_2O in a final volume of 300 μL). Some exchangeable protium was also introduced via the NADPH stock solutions. In total, these sources of exchangeable protons generated a final mixture of water containing approximately 99.5% D content. The deuterium content of the hydroxyl group in methanol is expected to mirror the deuterium content of the water. Therefore, in the deuterium-containing assays, an approximate 99.5:0.5 ratio of CD_3O**D**:CD_3O**H** was expected (that is, 0.5% of the atom donor contained a proton on the alcohol group). It is important to consider whether this small amount of CD_3OH could affect the outcome of the deuterium labeling experiment described in this work. That is, we must ask: is it possible that an atom-abstracting drug radical such as **6** was, in fact, generated in the CD_3OD/D_2O assays – but that this species incorporated no deuterium label because the drug radical selectively abstracted hydrogen atoms from the hydroxyl position of the very small amount (0.5%) of CD_3O**H** present in the reaction mixture? Such a scenario seems very unlikely for several reasons. Bond enthalpies significantly favor atom abstraction from the methyl group of methanol rather than the hydroxyl group.[1] Indeed, one study reported that, in the case of the highly reactive

radical HO•, atom abstraction from the hydroxyl group of methanol *does not occur* at room temperature.[2] Another group offered a conservative estimate based upon a survey of the literature concluding that 10% of atom abstraction might occur at the hydroxyl group.[1] Of course, a very large isotope effect could skew atom abstraction away from the methyl group and toward the hydroxyl group in CD_3OH; however, at least in the case of a highly reactive radical such as hydroxyl radical, the measured deuterium isotope effect was modest, in the range of 2-5.[2,3] More importantly, we presented *experimental* evidence that abstraction in this system occurs at the methyl group of CH_3OH and CD_3OD/H! Specifically, the metabolites **8** clearly indicate that significant amounts of $\bullet CD_2OH$/D and $\bullet CH_2OH$ were generated (Figures 3, S3, and S4). There was no evidence for products arising from the generation of $CH_3O\bullet$ or $CD_3O\bullet$. Thus, the reactive intermediate generated following bioreductive activation of **1b** clearly carried out atom abstraction from the *methyl* group of methanol in both the D_2O/CD_3OD and H_2O/CH_3OH assays – yet, in the D_2O/CD_3OD assay, *no* detectable incorporation of deuterium into the metabolite **3** was observed. The evidence for generation of significant amounts of $\bullet CD_2OD$ radicals *without* simultaneous incorporation of deuterium into the metabolite **3**, argues strongly against atom abstraction by **6** (or any related aryl radical) as a predominant reaction channel for bioreductively-activated **1b**. Also relevant to this issue are our observations that the deuterium label in **1c** was completely retained during metabolic conversion to **3**. These results provided further evidence against the formation of the aryl radical **6**.

Finally, we also carried out the hypoxic metabolism of **1b** in a buffered-D_2O/CD_3OD solution where additional steps were taken to remove all exchangeable protium sources. In this experiment, a sample of the NADPH:cytochrome P450 reductase enzyme in deuterated buffer was prepared by passing the stock enzyme solution through a Sephadex G-25 (Sigma-Aldrich) spin column that had been equilibrated in deuterated buffer. The phosphate buffer salts were dissolved in D_2O, lyophilized to remove residual exchangeable protium, and redissolved in D_2O. Similarly, the stock solution of NADPH was prepared in D_2O, lyophilized to remove residual H_2O, and redissolved in D_2O. Additives such as desferal that might bring exchangeable protium to the assay mixture were omitted. The hypoxic metabolism of **1b** under these conditions generated the same metabolites shown in Figure 3 of the paper and the LC/MS spectra of the mono-*N*-oxide metabolite **3** and the "no-oxide" metabolite **7** revealed no significant levels of deuterium incorporation (Figure S8).

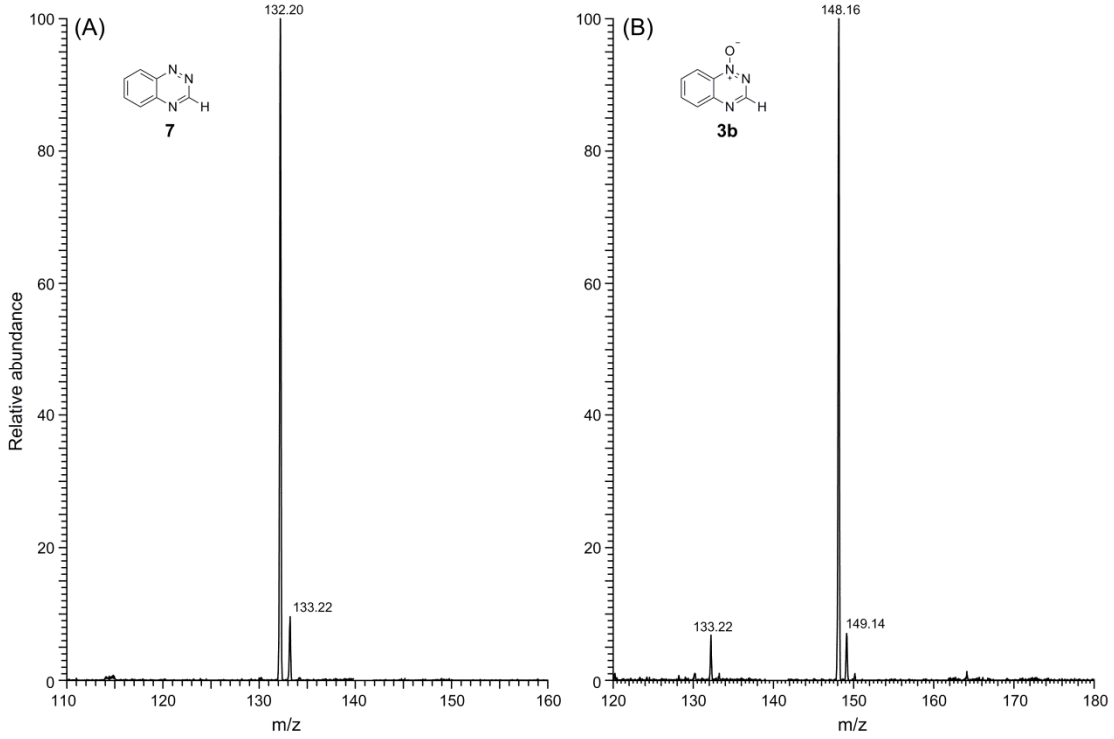


Figure S8. LC-MS of metabolites **7** and **3** generated by hypoxic metabolism of **1b** by NADPH:cytochrome P450 reductase in "completely deuterated" D_2O/CD_3OD buffer described above. (A) MS spectra of **7** (relative peak intensities of m/z 132:133:134 = 100:9.2:0.8) and (B) MS spectra of the metabolite **3b** (relative peak intensities of m/z 148:149:150 = 100:8.8:1.0). The ratio of M+H, M+H+1, and M+H+2 are very near to those expected to arise from natural isotope abundances in the molecular formulas $C_7H_6N_3$ (100:8.8:0.3) and $C_7H_6N_3O$ (100:8.8:0.5).

Literature citations

(1) Jodkowski, J. T., Rayez, M.-T., Rayez, J.-C., Bérces, T., Dóbé, S. (1999) Theoretical study of the kinetics of the hydrogen abstraction from methanol. 3. Reaction of methanol with hydrogen atom, methyl, and hydroxyl radicals. *J. Phys. Chem. A 103*, 3750-3765.
(2) Hess, W. P., Tully, F. P. (1989) Hydrogen-atom abstraction from methanol by hydroxyl radical. *J. Phys. Chem. 93*, 1944-1947.
(3) McCaulley, J. A., Kelly, N., Golde, M. F., Kaufman, F. (1989) Kinetic studies of the reactions of atomic fluorine and hydroxyl radical with methanol. *J. Phys. Chem. 93*, 1014-1018.