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Hydroxyl radical generation *via* photoreduction of a simple pyridine *N*-oxide by an NADH analogue†

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Received 5th July 2005, Accepted 22nd July 2005
First published as an Advance Article on the web 2nd August 2005

Photoreduction of pyridine *N*-oxide, which has a key structure of antitumor agents for hypoxic solid tumors, by 1-benzyl-1,4-dihydronicotinamide in deaerated aprotic media resulted in generation of hydroxyl radical, leading to the oxidation of salicylic acid to 2,3- and 2,5-dihydroxybenzoic acids, and catechol.

Recently, considerable effort has been made to develop effective drugs against solid tumors, which exist under hypoxic (oxygen-poor) conditions in inefficient vascular systems.[1,2] Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-di-*N*-oxide), which has a heterocyclic *N*-oxide structure, is a clinically promising antitumor agent against hypoxic cells.[3] The DNA damage induced by tirapazamine is proposed to result from the generation of hydroxyl radical (•OH) or the direct oxidation of the deoxyribose backbone of DNA after one-electron reduction of the *N*-oxide to form an activated intermediate.[4–6] However, the actual DNA-damaging species has yet to be clarified. On the other hand, photosensitizers available for photodynamic therapy are advantageous to localize the toxicity to a selected site (tumor cells), thus avoiding toxicity to normal cells.[7] However, since most photosensitizers require O_2 to produce reactive oxygen species, they are not effective toward anaerobic solid tumors. Thus, photoactivated compounds, which generate reactive oxygen species under anaerobic conditions, are certainly required for the development of drugs effective against solid tumors without affecting normal cells.

We report herein •OH generation from a simple unsubstituted pyridine *N*-oxide (PyO), which has a largely negative reduction potential, *via* one-electron reduction by photoexcited 1-benzyl-1,4-dihydronicotinamide (BNAH) used as a model compound of dihydronicotinamide adenine dinucleotide (NADH) in DMF under anaerobic conditions. The effects of the substituent at the C-4 position of pyridine *N*-oxides on the mechanism of photoinduced electron transfer from BNAH to pyridine *N*-oxides as well as the reactivity of the corresponding radical anions are clarified based on the spectral and electrochemical data together with the calculated molecular structures by the density functional method, providing a valuable insight into the development of antitumor agents for hypoxic cells.

Salicylic acid (SA) was employed to detect •OH generated in the photoreaction of pyridine *N*-oxides with BNAH in deaerated DMF. SA reacts with •OH to form 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) as major products and catechol as a minor product.[8–13] These oxidized products of SA are stable and are readily isolated and quantified by a reverse-phase HPLC equipped with an electrochemical detector (HPLC-ECD). SA does not react with $O_2^{\bullet-}$ at an appreciable rate as compared to •OH. Although SA also reacts with singlet oxygen ($^1O_2$), only 2,5-DHBA is formed exclusively, instead of the mixture of 2,3-DHBA, 2,5-DHBA, and catechol.[14]

Aprotic solvents, such as DMF and acetonitrile (MeCN) were used because of the poor solubility of pyridine *N*-oxides toward water, although the reactivity in aqueous media is important for an *in vivo* situation. When a deaerated DMF solution of PyO (5.0×10^{-3} mol dm^{-3}) and BNAH (5.0×10^{-3} mol dm^{-3}) was irradiated with UV light ($\lambda > 290$ nm) in the presence of SA (3.2×10^{-2} mol dm^{-3}), 2,3-DHBA, 2,5-DHBA, and catechol were detected by the HPLC-EC analysis as shown in Fig. 1a. The ratio of the yields of 2,5-DHBA, 2,3-DHBA, and catechol are 48:35:16. Irradiation of PyO or BNAH alone in the presence of SA resulted in no formation of oxidized products of SA (Fig. 1c and d). Under dark conditions, neither DHBA product nor catechol was formed even in the presence of all the components, *i.e.*, PyO, BNAH, and SA (Fig. 1e). These results demonstrate that •OH is generated in the photoreaction of PyO with BNAH in deaerated DMF as shown in Scheme 1. In fact, addition of

† Electronic supplementary information (ESI) available: Transient absorption spectra of the photoreaction between BNAH and PyO (S1), EPR spectrum of $NO_2PyO^{\bullet-}$ (S2), spectral change in the photoreaction between BNAH and NO_2PyO (S3), and DFT minimized structures (S4). See http://dx.doi.org/10.1039/b509447j

DOI: 10.1039/b509447j

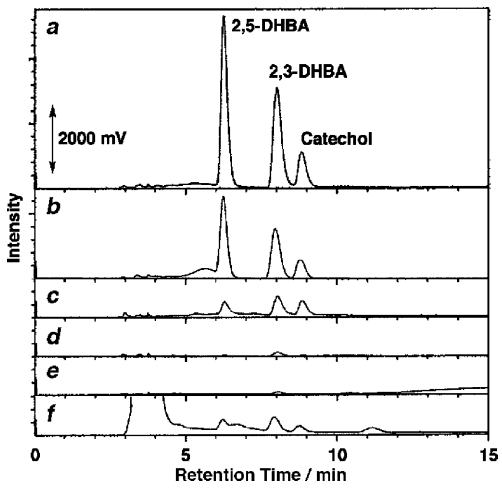
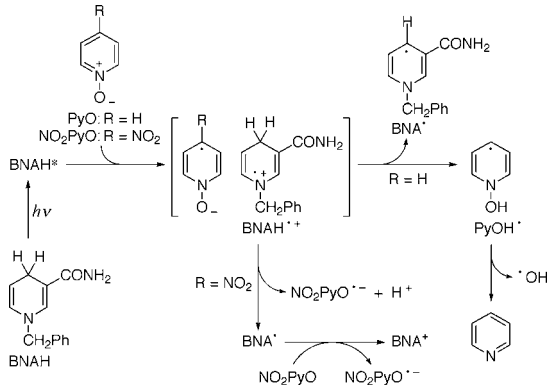


Fig. 1 HPLC-ECD chromatograms of products formed during irradiation (1 h) of (a) PyO (5.0×10^{-3} mol dm^{-3}), BNAH (5.0×10^{-3} mol dm^{-3}), and SA (3.2×10^{-2} mol dm^{-3}); (b) PyO (5.0×10^{-3} mol dm^{-3}), BNAH (5.0×10^{-3} mol dm^{-3}), SA (3.2×10^{-2} mol dm^{-3}), and ethanol (5.7×10^{-1} mol dm^{-3}); (c) PyO (5.0×10^{-3} mol dm^{-3}) and SA (3.2×10^{-2} mol dm^{-3}); (d) BNAH (5.0×10^{-3} mol dm^{-3}) and SA (3.2×10^{-2} mol dm^{-3}); (e) PyO (5.0×10^{-3} mol dm^{-3}), BNAH (5.0×10^{-3} mol dm^{-3}), and SA (3.2×10^{-2} mol dm^{-3}) without irradiation; (f) NO_2PyO (5.0×10^{-3} mol dm^{-3}), BNAH (5.0×10^{-3} mol dm^{-3}), and SA (3.2×10^{-2} mol dm^{-3}) in deaerated DMF at 298 K.



Scheme 1

ethanol, which is a $^{\bullet}$OH scavenger, resulted in a decrease in the yield of three oxidized SA products (Fig. 1b).

Photoirradiation of BNAH is known to give the singlet excited state, BNAH*.[15] The fluorescence of BNAH* is quenched efficiently by the addition of PyO in deaerated MeCN. The quenching rate constant is determined as 1.4×10^{10} mol^{-1} dm^3 s^{-1}, which is close to the diffusion limited value in MeCN. Nanosecond laser excitation of a deaerated MeCN solution of PyO and BNAH results in the formation of PyO$^{\bullet-}$ ($\lambda_{max} = 540$ nm) and BNAH$^{\bullet+}$ ($\lambda_{max} = 380$ nm)[16] (see Electronic Supplementary Information, Fig. S1†).

The reaction mechanism of electron-transfer reduction of PyO by BNAH is shown in Scheme 1. Photoinduced electron transfer from BNAH* to PyO takes place to produce a radical ion pair of BNAH$^{\bullet+}$ and PyO$^{\bullet-}$, since the oxidation potential of BNAH* ($E^0_{ox} = -2.65$ V *vs.* SCE) determined in DMF[17] is more negative than the reported reduction potential of PyO ($E^0_{red} = -2.30$ V) in DMF.[18] Proton transfer from BNAH$^{\bullet+}$, thus produced, to PyO$^{\bullet-}$ gives PyOH$^{\bullet}$ and BNA$^{\bullet}$. The resulting PyOH$^{\bullet}$ then undergoes the N–O bond cleavage to produce $^{\bullet}$OH and pyridine. Judging from the more positive oxidation potential of BNA$^{\bullet}$ ($E^0_{ox} = -1.05$ V)[17] in DMF than the reduction potential of PyO ($E^0_{red} = -2.30$ V),[18] no electron transfer from BNA$^{\bullet}$ to PyO would occur and instead a coupling between two molecules of BNA$^{\bullet}$ may occur to give a dimer $(BNA)_2$.

When PyO is replaced by 4-nitropyridine *N*-oxide (NO_2PyO), neither DHBA products nor catechol was produced after irradiation of a deaerated DMF solution containing NO_2PyO, BNAH, and SA (Fig. 1f).

This result demonstrates that the radical anion of NO_2PyO ($NO_2PyO^{\bullet-}$) generated in photoinduced electron transfer from BNAH to NO_2PyO is relatively stable and does not undergo the N–O bond cleavage. In fact, an EPR spectrum of $NO_2PyO^{\bullet-}$, which has a *g* value of 2.0054, was observed after irradiation of a deaerated MeCN solution of NO_2PyO and BNAH (Fig. S2a†). The hyperfine coupling constants (hfc) of the observed EPR spectrum of $NO_2PyO^{\bullet-}$ were determined by comparison of the observed spectrum with the computer-simulated spectrum (Fig. S2b†) and they were assigned based on the reported hfc values for $NO_2PyO^{\bullet-}$ in DMF (Fig. S2†).[18]

The UV-vis spectral titration (Fig. S3†) indicates that BNAH acts as a two-electron donor to reduce 2 equiv. of NO_2PyO to $NO_2PyO^{\bullet-}$. Since the E^0_{ox} value of BNAH* (−2.65 V) is more negative than the E^0_{red} value of NO_2PyO (−0.77 V),[18] photoinduced electron transfer from BNAH* to NO_2PyO occurs to give a radical ion pair of $NO_2PyO^{\bullet-}$ and BNAH$^{\bullet+}$. BNAH$^{\bullet+}$ undergoes deprotonation to give BNA$^{\bullet}$. The delocalization of an electron on the $NO_2PyO^{\bullet-}$ molecule due to the electron-withdrawing NO_2 group may preclude the protonation of $NO_2PyO^{\bullet-}$. In fact, no hyperfine structure due to the N–OH proton was observed in the EPR spectrum of $NO_2PyO^{\bullet-}$. The subsequent electron transfer from BNA$^{\bullet}$ to NO_2PyO may also occur rapidly, judging from the E^0_{ox} value of BNA$^{\bullet}$ (−1.05 V), which is lower than the E^0_{red} value of NO_2PyO (−0.77 V).[18] Thus, once photoinduced electron transfer from BNAH to NO_2PyO occurs, 2 equiv of $NO_2PyO^{\bullet-}$ are produced.

DFT calculations using B3LYP/6-31G* basis set for PyOH$^{\bullet}$ and $NO_2PyOH^{\bullet}$ were carried out to investigate the difference in the reactivity of radical species of pyridine *N*-oxides depending on a substituent at the C-4 position (Fig. S4†). The calculated N–O bond length in PyOH$^{\bullet}$ (1.50 Å) is significantly longer than that in $NO_2PyOH^{\bullet}$ (1.40 Å). This suggests that the N–O bond cleavage in PyOH$^{\bullet}$ may occur much easier than that in $NO_2PyOH^{\bullet}$. Furthermore, PyOH$^{\bullet}$ is significantly bent as indicated by the out-of-plane N–O bond bending angle (α) of 152°, whereas $NO_2PyOH^{\bullet}$ is relatively flat ($\alpha = 169°$). Similar results have been reported for the N–O bond fragmentation in *N*-methoxy-substituted aromatic compounds.[19] The N–O bond cleavage requires mixing of π* and σ* orbitals, which is achieved by bending the N–O bond out of the plane of the aromatic ring.

In conclusion, photoreduction of a simple pyridine *N*-oxide by BNAH in deaerated aprotic medium resulted in generation of $^{\bullet}$OH, which can oxidize SA to 2,3-DHBA, 2,5-DHBA, and catechol. The electron-withdrawing group such as NO_2 on the aromatic ring can significantly stabilize the radical anion of pyridine *N*-oxide, precluding the subsequent $^{\bullet}$OH release. We are currently investigating the detailed effects of various substituents on the photoreactivities of pyridine *N*-oxides in the presence of various reducing agents.

Acknowledgements

This work was partially supported by Grant-in-Aids for Scientific Research (A) (No. 16205020) and for Young Scientist (B) (No. 17790044) from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by the Budget for Nuclear Research of the Ministry of Education, Culture, Sports, Science and Technology, Japan based on the screening and counselling Atomic Energy Commission.

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