Photoactivatable HNO-releasing compounds using the retro-Diels–Alder reaction†

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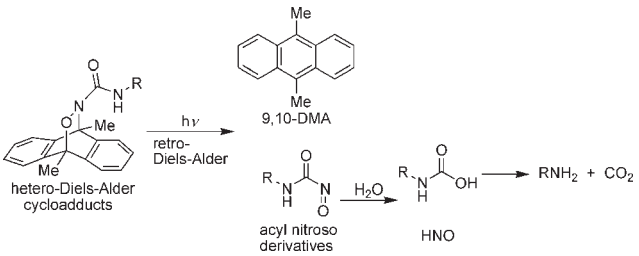
We synthesized hetero-Diels–Alder cycloadducts from acyl nitroso derivatives and 9,10-dimethylanthracene, to be photoinducible HNO-releasing agents and found that introduction of conjugated nitroaromatic groups effectively enhanced the responsiveness of HNO release to UV-A irradiation; we confirmed photoinduced HNO formation by EPR and GCMS analysis.

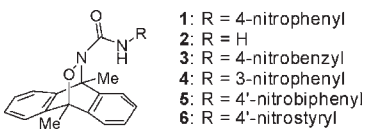
The potential pharmacological activity of nitroxyl (HNO) in relation to nitric oxide (NO) has recently received much attention. While NO is known to act as a vasodilator *via* activation of soluble guanylate cyclase, HNO is reported to induce a variety of physiological effects such as positive inotropy, vasodilation, and cardioprotection through mechanisms different from those of NO.[1,2] Very recently, HNO was also reported as an anticancer agent.[3] In view of these benefits, HNO donors are expected to be potentially useful as therapeutic agents in the treatment of cardiovascular diseases, and also as experimental pharmacological agents. Despite the potential activity of HNO, its detailed mechanisms and applications have scarcely been studied since this compound is difficult to handle due to its chemical instability.[4] Because of this, HNO donors are indispensable for both HNO investigation and the design of therapeutic agents. Some compounds such as Angeli's salt, Piloty's acid and cyanamide (NH_2CN) have been reported to generate HNO under certain conditions.[5–7] However, their release of HNO is dependent on the spontaneous degradation of the donors, which is not a controlled event. In addition, in some cases, the by-products have distinct biological activities of their own. Hence, the application of these compounds to biological systems has been quite restricted. It is clear that a variety of novel HNO donors is needed to aid in the research of HNO and its applications.

It is known that hydrolysis of acyl nitroso compounds generates HNO, but these highly reactive forms must be generated *in situ*.[8] Based on *in situ* acyl nitroso compound formation, King *et al.* developed hetero-Diels–Alder cycloadducts.[9,10] These are known to thermally decompose to release HNO *via* acyl nitroso formation. If HNO release could be regulated by photoirradiation, the controlled release would be very useful for biological applications. We have already developed photoinduced NO donors *via* a unique photochemical reaction.[11] For the purpose of improving the hetero-Diels–Alder cycloadducts formed from acyl nitroso derivatives and 9,10-dimethylanthracene (9,10-DMA), we designed and synthesized compounds bearing conjugated aromatic groups which would readily induce the retro-Diels–Alder (retro-DA) reaction by photoirradiation (Scheme 1). The synthesized compounds were confirmed by spectral analyses, including ^{1}H- and ^{13}C-NMR, IR, and MS, and elemental analysis (see ESI†).



Scheme 1 HNO release through a photoinduced retro-DA reaction.

We first evaluated photoinduced enhancement of HNO release from a known hetero-Diels–Alder cycloadduct (**1**) bearing a 4-nitrophenyl substituent (Chart 1). Direct detection of HNO has not been established as yet due to its complicated chemical properties. In this study, we adopted several indirect methods to estimate photoinduced HNO generation. Firstly, photoinduced retro-DA reactions were examined by measuring absorption spectra and proton nuclear magnetic resonance (^{1}H-NMR) spectra. By monitoring the unique absorption of 9,10-DMA at 398 nm, it was found that the formation of 9,10-DMA from **1** in acetonitrile–water solution (9 : 1) was accelerated by ultraviolet-A (UV-A, 330–380 nm) irradiation (Fig. 1). This acceleration was dependent on the light intensity. It was also clarified by ^{1}H-NMR analysis that photoirradiation converted **1** into 9,10-DMA and 4-nitroaniline (Fig. 2, I). The formation of 4-nitroaniline was suppressed by the



1: R = 4-nitrophenyl
2: R = H
3: R = 4-nitrobenzyl
4: R = 3-nitrophenyl
5: R = 4′-nitrobiphenyl
6: R = 4′-nitrostyryl

Chart 1 Designed and synthesized molecules.

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† Electronic supplementary information (ESI) available: Details of the synthesis of **1–6** and results of H-NMR, UV/Vis, EPR, and GC analysis. See DOI: 10.1039/b811985f

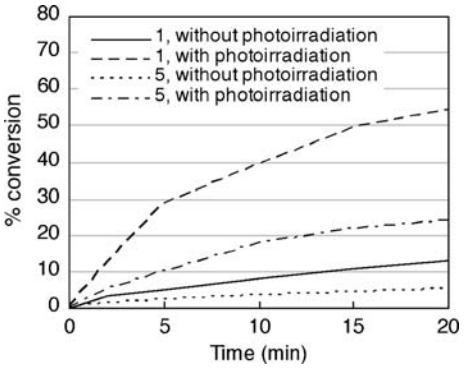

Fig. 1 Conversion of cycloadducts in acetonitrile–water (9 : 1) was calculated from the increase in the absorption of 9,10-DMA at 398 nm.

Table 1 N_2O formation

Compounds	Conditions		N_2O formation[a]/mmol		
	hν	2-ME[b]	10 min	20 min	30 min
Angeli's salt	—	—	0.254	0.424	0.533
1	—	—	0.203	0.373	0.472
	+	—	0.261	0.484	0.594
	+	+	ND[c]	ND[c]	ND[c]
5	—	—	0.081	0.198	0.329
	+	—	0.223	0.364	0.484
	+	+	ND[c]	ND[c]	ND[c]
6	—	—	0.085	0.215	0.346
	+	—	0.122	0.260	0.426
	+	+	ND[c]	ND[c]	ND[c]

[a] Calculation based on Angeli's salt (1.5 μmol) decomposition according to Hughes and Wimbledon.[13] [b] 2-Mercaptoethanol (10 equiv.). [c] N_2O was not detected.

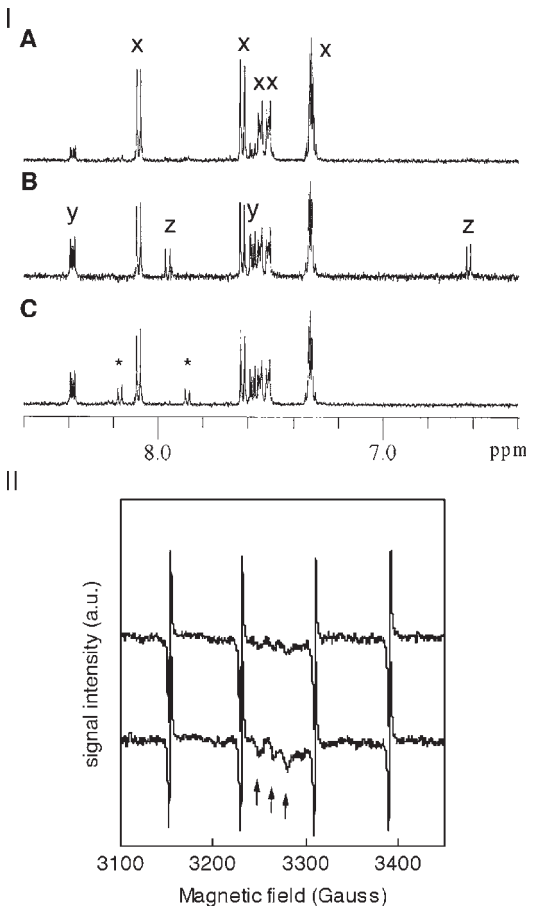

Fig. 2 I. ^{1}H-NMR spectral change by photoinduced conversion of **1** in DMSO–water (9 : 1). Spectrum A was obtained just after preparing the solution, and spectrum B shows the same solution after 10 min of UV-A irradiation. Spectrum C shows the solution under the same conditions as those for B except for the addition of 2-ME. Each peak was labelled with a letter such as x, compound **1**; y, 9,10-DMA; and z, 4-nitroaniline. Assignment of those peaks is shown in the ESI.† Peaks labelled with an asterisk are the reaction product of compound **1** (or acyl nitroso derivative) and 2-ME. II. EPR spectra of **5** in DMSO–water (9 : 1) with hemin under anaerobic conditions (arrowed signals). Spectra were measured either under dark conditions (top) or under UV-A irradiation (bottom) in the presence of manganese as an external standard.

addition of 2-mercaptoethanol (2-ME), probably because of a nucleophilic attack on the carbonyl carbon of an acyl nitroso derivative. These outcomes supported the photoinduced retro-DA reaction of **1**. Secondly, HNO trapping-electron paramagnetic resonance (EPR) spectroscopy and gas chromatographic (GC) analysis were performed to confirm HNO generation.[8–10,12] EPR spectra of a mixture of **1** and hemin in dimethyl sulfoxide (DMSO)–water solution (9 : 1) showed the typical three-line EPR signal of a ferrous nitrosyl complex under anaerobic conditions. The reductive nitrosylation of a ferric heme by HNO provides evidence for the release of HNO from **1**. GC analysis of the reaction headspace under anaerobic conditions showed that photoirradiation of **1** in DMSO–water (9 : 1) gave N_2O formed *via* HNO dimerization and dehydration (Table 1).[4] The amount of the formed N_2O was calculated and expressed as the equivalent amount of Angeli's salt (1.5 μmol). Although we also tried direct calibration of N_2O formation by using authentic N_2O gas injection, it was difficult to obtain reproducible quantitative results due to the water-soluble property of N_2O and the difficulty of the accurate dilution of N_2O gas.

The N_2O formation was enhanced by photoirradiation and was significantly reduced by the addition of 2-ME as an HNO scavenging agent. Thus, it was revealed that **1** was able to undergo a photoinduced retro-DA reaction to yield HNO. However, **1** was unfortunately not stable even under dark conditions at ambient temperature, and the photo-enhancement effect of HNO release was small compared with the amount of thermal release.

To improve the photo-enhancement effect, we evaluated the substituent effect on the stability of cycloadducts under dark conditions as well as photoinduced conversion by synthesizing **2–4**. Although the UV-A irradiation might not be directly applicable to cardiovascular disorders, this evaluation would be helpful to confirm the efficiency of our strategy, and to obtain basic data for improving the compounds to be activated by longer wavelengths. Nonetheless, these compounds would be applicable to *in vitro* or cellular experiments, or hopefully to skin cancer.

Compounds **2** and **3** were more thermally stable than **1** and were not activated by UV-A irradiation. As for **4**, its tendency to thermally degrade was the same as that of **1**, but its

promotion of HNO release by UV-A irradiation was significantly lower (Fig. S1†). These results indicate that stability largely depends on the steric hindrance of the substituent. Introduction of conjugated aryl groups as substituents was found to be necessary for the photoinduced retro-DA reaction. Based on these findings, other sterically unhindered conjugated groups, such as nitrobiphenyl and nitrostyryl substituents, were introduced into cycloadducts **5** and **6**. From absorption studies, it was revealed that **5** was more stable than **1** under dark conditions and that the retro-DA reaction of **5** was facilitated by UV-A irradiation (Fig. 1). The change in the absorption spectrum of **6** was too complicated to determine its conversion into 9,10-DMA and the corresponding amine. EPR spectra provided evidence for HNO release from **5** and **6** (Fig. 2, II, and Fig. S3†). GC analysis indicated that the stabilities of **5** and **6** were improved over **1** under dark conditions and that they were significantly prompted to produce N_2O by UV-A irradiation (Table 1). In particular, photoinduced N_2O formation of **5** by a 10 min irradiation was 3-fold greater than that under dark conditions, whereas N_2O induction by photoirradiation of **1** was only 1.3-fold greater. Moreover, the HNO-releasing ability of **5** under photoirradiating conditions was nearly equal to that of Angeli's salt.

For 20 and 30 min irradiation, the photo-enhancement effect became not apparent. This means that photoactivation of the compound effectively occurred within the first 10 min, and N_2O formation through HNO was enhanced in this duration. Photoinduced conversion of **5** into 9,10-DMA was also nearly saturated after 10 min irradiation (Fig. S1†). Improvement of the stability was assumed to cause an increase of the energy of the photoinduced retro-DA reaction and a decrease in HNO release rate. Because HNO dimerization to form N_2O is a second order reaction, N_2O formation is highly dependent on the HNO concentration. That is, the enhancement effect on N_2O formation was less apparent than that on the conversion to 9,10-DMA. Factors affecting the conversion efficiency by photoirradiation are under investigation.

Nonetheless, these results demonstrated that aromatic-group-substituted cycloadducts **1** and **4–6** were capable of releasing HNO and that the release of HNO was accelerated by UV-A irradiation. Among the cycloadducts, the HNO generation of **5** was the most facilitated. These differences probably reflect their respective absorption ranges and steric hindrances. The local maximum absorption wavelength of **5** was 337 nm, while those of **1** and **6** were 324 and 397 nm, respectively. Since the wavelength of the utilized UV-A ranged from 330 to 380 nm, **5** was able to effectively absorb UV-A energy. The maximum wavelength of **4** was similar to that of **5**, but the absorption coefficient was too low. Accordingly, the 4′-nitrobiphenyl functional group was shown to be the most suitable in view of its steric and electronic factors. To date, there have been no observations published about photoactivatable HNO donors or donors whose HNO-releasing ability can be arbitrarily controlled. In this study, we assessed the steric and electronic effects of conjugated aromatic groups in terms of improvement of their stability under dark conditions and their reactivity upon photoirradiation, and our findings showed **5** to be a photoactivatable HNO-releasing agent under UV-A irradiation. These results should contribute to the development of more sophisticated HNO donors. As for use in biological systems, the poor water solubility of the synthesized compounds is one of the key problems to be overcome, and further thermal stability is another one. We are now working to improve the solubility and enhance the stability of these compounds for future biological applications.

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