論文の内容の要旨

論文題目

Development of Bicyclic *N*-Nitrosamines as Small Molecule NOS Mimics: Featuring Controllable NO Release Concentration and High Cellular Retention Ability

（NO 放出濃度の調節可能な細胞内保持性二環性ニトロソアミンの創製）

氏　　名：フィルマン

1. Introduction and Aims (Chapter 1)

Nitric oxide (NO) is a free radical in gas state at room temperature with short half-life but plays important roles as a secondary messenger in living organism. Nitric oxide signaling has been reported to take part in very broad pharmacological systems, from immune system, controlling blood pressure, transmitting nerve signals, and a variety of other signaling processes. It is reported that its role of actions depends on its concentration, ranging from picomolar to micromolar level. And local NO concentration is biologically regulated by three isoforms of nitric oxide synthase (NOSs). Neuronal NOS (nNOS) and endothelial NOS (eNOS) produce low concentration (picomolar to nanomolar) of NO transiently and locally, usually leading to beneficial effects, while inducible NOS (iNOS) produces prolonged high concentration (nanomolar to micromolar) of NO which is related to pathological effects.

It was found that bicyclic *N*-nitrosoamines with a 7-azabenzobicyclo[2.2.1]heptane backbone have a weak N-NO bond due to nitrogen-pyramidalization, which causes the compounds to absorb visible light (420 nm) (**Fig. 1**). We found that the N-NO bond can be cleaved upon visible light irradiation (420 nm) to release NO in cuvette, i.e., under cell-free conditions. Utilizing this property, here I developed small organic molecules that may mimic NOSs functions: since cellular environment is far more complex than in cuvette, NO release from bicyclic nitrosamines upon visible light (420 nm) irradiation in cells need to be confirmed. Besides, cellular retention also will be an issue in the

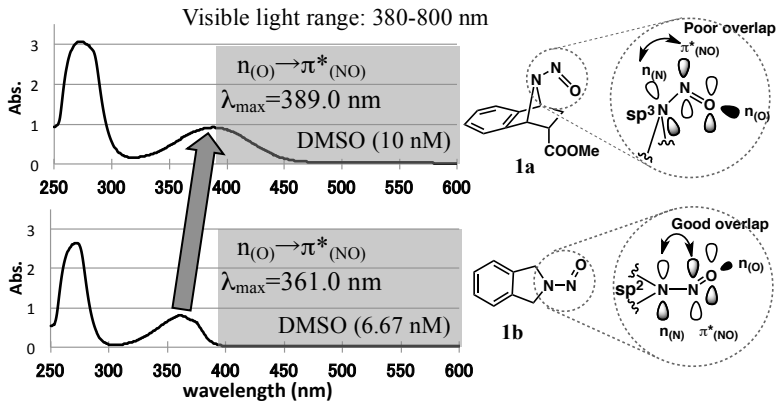


Fig. 1. Bathochromic shift of bicyclic nitrosamine due to *N*-pyramidalization that leads to visible light absorption.

cellular study. The most important thing as artificial NOS mimics is the ability to control the NO release and define the concentration because either beneficial or pathological effects are dependent on its concentration.

2. Cell Applicability Study of Bicyclic Nitrosamines in Piccell (Chapter 2)

Detection of NO release in cells was conducted with Piccell. Piccell is a pig kidney-derived cell (PK-15 cell line) that expresses both of soluble guanylate cyclase (sGC) to trap NO, and Green Fluorescent Protein (GFPs) bound protein kinase G (PKG) as a cyclic guanosine monophosphate (cGMP) receptor. Activated sGC by NO will generate cGMP, which bind to PKG and lead to conformational change, resulting in changes in the interaction of the GFP system.

Direct application of compound **1a** to Piccell (10 μM, 1 h incubation) did not change the fluorescence ratio in Piccell, but photo-iraddiation induced NO generation (**Fig. 2**). Irradiation at 440 nm or 350 nm for 5 sec was performed after incubation without washing. NOC7, a commercially available NO donor that spontaneously release NO when dissolved in water, was used as a positive control of NO detection (100 nM) by Piccell. Both irradiation with visible light (440 nm) and uv light (350 nm) induced release of NO from the bicyclic nitrosamine **1a**. When the duration of irradiation was changed, Piccell's signal was changed as well, and the signal was increased as the duration time was prolonged. The calibrated concentrations of NO released from **1a** upon irradiation with visible light (440 nm) are 56±3.3 pM and 323±49.5 pM for 1 sec and 5 sec irradiation, respectively. Based on this result, it is confirmed that NO can be uncaged upon irradiation in cellular environment. Concentration of NO can be controlled by duration of irradiation and as the duration is prolonged, concentration of NO released is increased.

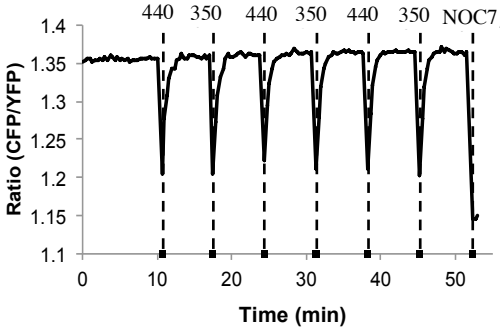


Fig. 2. Detection of NO release from **1a** (10 μM, 1 h incubation) by Piccell upon irradiation (350 nm and 440 nm, both 5 sec).

However when washing was applied, Piccell's signal can be detected upon irradiation of **1a**, but the signal gradually disappeared (within 60 min) (data not shown). This observation suggested that

compound **1a** can penetrate into the cell and can be uncaged upon irradiation with visible light (440 nm). This signal disappearance phenomenon was also induced by leakage of nitrosamine **1a** from the inside of Piccell upon washing. Based on this result, nitrosamine **1a** lacks cell retention ability and **1a** can leak out from cells during washing. To overcome this leakage, the methyl ester substituent was changed to an acetoxymethyl ester (AM-ester). The AM ester can be hydrolyzed by esterase to generate a carboxylate anion in cells that is barely permeable through cell membrane.

Modification of methyl ester to AM ester nitrosamines showed improves of cell retention ability. As the location of AM ester nitrosamines was moved to benzene ring, significant increase of cell retention was observed. Various derivatives (**Fig. 3.**) have been synthesized to study the generality of mono-AM ester nitrosamine compounds but the cell retention profiles were not consistent. Therefore another strategy to obtain highly retainable bicyclic nitrosamine was approached.

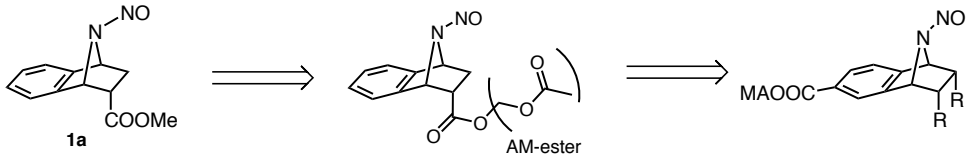


Fig. 3. First attempt to synthesize cell retainable bicyclic nitrosamine.

3. Achieving Highly Retainable Bicyclic Nitrosamines (Chapter 3)

Different strategy was approached in order to obtain highly retainable bicyclic nitrosamines. Different numbers and different positions of AM ester functional group was attached to the right side of the 7-azabenzobicyclo[2.2.1]heptane skeleton, designated as right-wing AM esters, and to the left side of the bicyclic ring at the benzene ring, designated as left-wing AM esters (**Fig. 4.**).

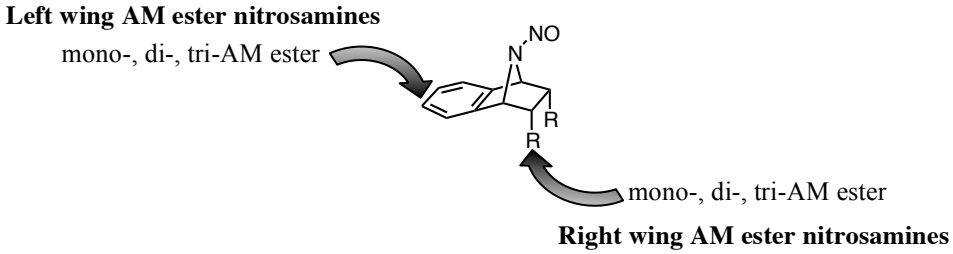


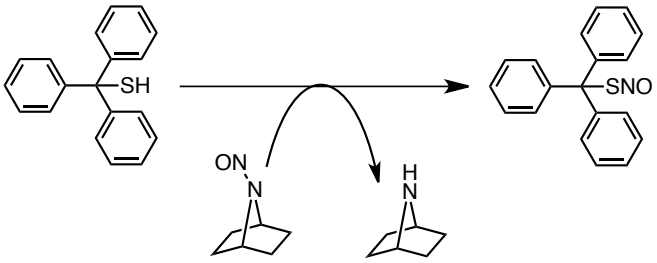
Fig. 4. Strategy to achieve highly retainable bicyclic nitrosamines.

Study of these right-wing and left-wing AM ester nitrosamines in Piccell showed that as the number of AM ester increase, cell retention ability was improved. Detection of NO release from left wing and right wing AM ester nitrosamines by Piccell after 1 hour incubation. In this experiment, multiple washing (7 times) was applied every time before measurements during 10 hours.

Mono-AM ester nitrosamines exhibit cell retention right after incubation with rapid decline of detected NO release after one hour. This phenomena can be explained that the nitrosamines bearing

mono-carboxylate can leak out from the cells and can be washed out from the cells, which lead to low concentration of the nitrosamines in cells for the next uncaging. For the di-AM ester nitrosamines, reduction of NO concentration was still observed, but to less extent as compared with the mono-AM ester nitrosamines. Finally, for tri-AM ester nitrosamines, relatively stable NO release concentration was observed up to 10 hours even though multiple washing was applied before measurements. This suggests that tri-carboxylate groups both at the left wing and right wing positions can prevent the nitrosamines leaked out from the cells up to several hours. The NO release efficiencies are different between right-wing and left-wing tri-AM ester nitrosamines. Release concentration of NO from these tri-AM ester nitrosamines is 90±19.8 pM and 18±1.4 pM for the right-wing and left-wing tri-AM ester nitrosamines, respectively.

To eliminate the possibility whether tri-AM ester nitrosamines could spontaneously transfer NO to thiol in the present of thiol, transnitrosylation study in cuvette was performed.



Scheme 1. Trans-nitrosylation model study in cuvette.

Without irradiation, no trans-nitrosylation was observed for 6 hours at 37 ^{o}C. However when irradiation was applied to induce photo-cleavage of N-NO bond, S-nitrosylation occurred and characteristic band of RSNO can be observed due to the generation of NO by irradiation. Therefore, there is a high possibility that these left and right wing AM ester nitrosamines will not undergo trans-nitrosylation in cells and the NO release can be fully controlled by irradiation only.

4. Conclusion

It is found that bicyclic nitrosamines can be used as potential NO donors and it is also confirmed that these compounds can release NO upon visible light irradiation in cells. Further development of these compounds by installing AM esters improved their cell retention ability and at least three AM esters at an optimal position was needed for this bicyclic scaffold. The released NO in cells also can be quantified. Thus, these highly retainable nitrosamines can be used to mimic or probe NOS functions. Since its NO release was triggered by visible light irradiation, and the range of released NO concentration (100 picomolar level) matches the NO concentration generated in the cells, especially by nNOS and eNOS. Therefore, these nitrosamines are applicable to cells.