博士論文

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)

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

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By

FIRMAN

Supervisor: Prof. Tomohiko Ohwada

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Firman

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Abbreviations

%	percent
AM	Acetoxymethyl ester
Anal.	Analysis
Ar	Argon
brs	broad singlet
Calcd.	Calculated
$CDCl_3$	*deuterated* chloroform
CFP	Cyan Fluorescent Protein
cGMP	cyclic Guanosine MonoPhosphate
CGY	fusion protein of CFP, PKG 1α, and YFP
cLOGP	calculated LogP
δ	chemical shift in ppm with respect to the standard solvent peak
d	doublet
d	*deuterated*
DAF-2	Diaminofluorescein-2
DAF-2T	Diaminofluorescein-2 Triazole
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCl-DA Cal-AM	Dichlorodiaminocalcein with acetoxymethyl ester
dd	doublet doublet
DIC	*N,N′*-Diisopropylcarbodiimide
DIEA	*N,N′*-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EDCI HCl	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide Hydrochloride
eNOS	endothelial Nitric Oxide Synthase
eq	equivalent
ERK	extracellular signal-regulated kinase
ESI	Electrospray ionization
Fig	Figure
FRET	Fluorescence Resonance Energy Transfer
g	gram

GTP	Guanosine triphosphate
h	hour
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HIF-1α	hypoxia-inducible factor 1α
HOMO	Highest Occupied Molecular Orbital
HRMS	High Resolution Mass Spectroscopy
Hz	Hertz
i.e.	*id est*, that is
IL-1	Interleukin-1
iNOS	inducible Nitric Oxide Synthase
J	Coupling constant
LAH	Lithium Aluminium Hydride
LPS	Lipopolysaccharide
LUMO	Lowest Unoccupied Molecular Orbital
m	multiplet
M	Molar
MEOD	*deuterated* methanol, MeOH-d_4
mg	miligram
MHz	MegaHertz
min	minute
mL	mililiter
μL	microliter
μM	microMolar
mmol	milimole
Mp	Melting point
n-	*normal*-
NBS	*N*-Benzyl Succinimide
nM	nanoMolar
nm	nanometer
NMR	Nuclear Magnetic Resonance
nNOS	neuronal Nitric Oxide Synthase
NO	Nitric Oxide

NOS	Nitric Oxide Synthase
^{o}C	degree Celcius
ox.	oxidation
p	para
PBS	Phosphate Buffered Saline
pH	potential of Hydrogen
PK15	Porcine kidney epithelial cell line
PKG	Protein Kinase G
PKG 1α	Protein Kinase G 1α
pM	picoMolar
ppm	part per million
quint	quintet
rt	room temperature
s	singlet
sec	second
sGC	soluble Guanylate Cyclase
tert-	*tertiery*
td	triplet doublet
TEA	triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TNF-α	Tumor Necrosis Factor α
TOF	Time of Flight
UV	Ultraviolet
Vis	Visible
YFP	Yellow Fluorescent Protein

Chapter 1

Introduction

1.1. Nitric Oxide and Nitric Oxide Synthase (NOS)

Nitric oxide (NO) is an essential messenger in the physiology system with short half-life and high reactivity profile. It is synthesized from L-arginine enzymatically by nitric oxide synthase (NOS). Three different NOS isoforms are present in mammals have been reported i.e. neuronal, endothelial and inducible NOS, which abbreviated as nNOS, eNOS and iNOS, respectively.[1] nNOS and eNOS both are constantly present in resting cells and activated by Ca^{2+} influx, while iNOS is usually not present in resting cells and can be induced by immunostimulatory cytokines, bacterial products or infection. Different from eNOS and nNOS, iNOS generates NO independently of intracellular calcium concentrations.[2] Comparison of nNOS, eNOS, and iNOS is shown in **Table 1.1**.

Once activated, eNOS and nNOS produce NO at low concentration in pulsative pattern for short time (seconds to minutes)[3]. Differently, iNOS once expressed, produces NO for long periods of time (hours to days) continuously[4, 5]. According to calculations, the major differences between iNOS and other NOSs activities do not reside in the concentration of NO generated per enzyme, but rather in the duration of NO produced.[6] In addition, the iNOS protein content in fully activated cells may be higher than the other NOSs content. Thus, regulated pulses of nNOS and eNOS versus constant unregulated NO synthesis of iNOS differentiates between the physiological and the pathological properties of NO.

Table 1.1. Different properties of nNOS, eNOS and iNOS.

	nNOS	eNOS	iNOS
Distribution	Mainly in brain	Mainly in vascular endothelium	Macrophages, astrocytes
Solubility	Soluble	Membrane bound	Soluble
Activation	Ca^{2+}/calmodulin	• Ca^{2+}/calmodulin • Shear stress	• Endotoxin (LPS) • Proinflammatory cytokines (IL-1, TNF-α, IFN-γ)
NO production	Low level (pM~nM) Transient & pulsative	Low level (nM) Transient & pulsative	High level (nM~μM) Prolonged & continuously
Presence	Constantly present in resting cells	Constantly present in resting cells	Not present in resting cells
Main transduction pathway	• sGC-cGMP • Nitrosylation	• sGC-cGMP • Nitrosylation	• Nitrosylation • Tyrosine nitration
Effects	• Cell protection • Antiapoptotic • Chemical transmission	• Vasodilation • Inhibition of platelet aggregation	• Cell toxicity • Apoptosis • Non-specific immune system

Widely known natural target of NO is soluble guanylate cyclase (sGC) in which NO will bind to its heme moiety and activate the enzyme to catalyze the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP)[7, 8]. Increased of cGMP concentration activates cGMP-dependent protein kinase G (PKG), which may affect additional second messenger system, leading to multiple phosphorylation of cellular proteins.[9] Recent literature indicates that sGC is not the only target of the action of NO. Recently an alternate sGC-independent pathway has been reported, which is the *S*-nitrosylation of various proteins in cells.[10] Effects of this *S*-nitrosylation process can either inhibit or upregulate the proteins' activity, e.g., open of Ca^{2+}-activated potassium channel (BK_{Ca} channel) and ryanodine receptor (RyR) channel by *S*-nitrosylation of the enzyme, independent of cGMP.[11]

It has been little addressed about quantitatively how much NO was released in cells and how far and how fast released NO travels inside and outside of the cells. While there have been longstanding arguments about the real physiological NO concentration which is used in cellular events, it is recognized that the concentration of NO, effective enough for various biological communications (**Table 1.2.**), at least ranges from several picomolar up to micromolar level.[12, 13] As a description, concentration dependent of NO functions is shown in **Fig. 1.1**.

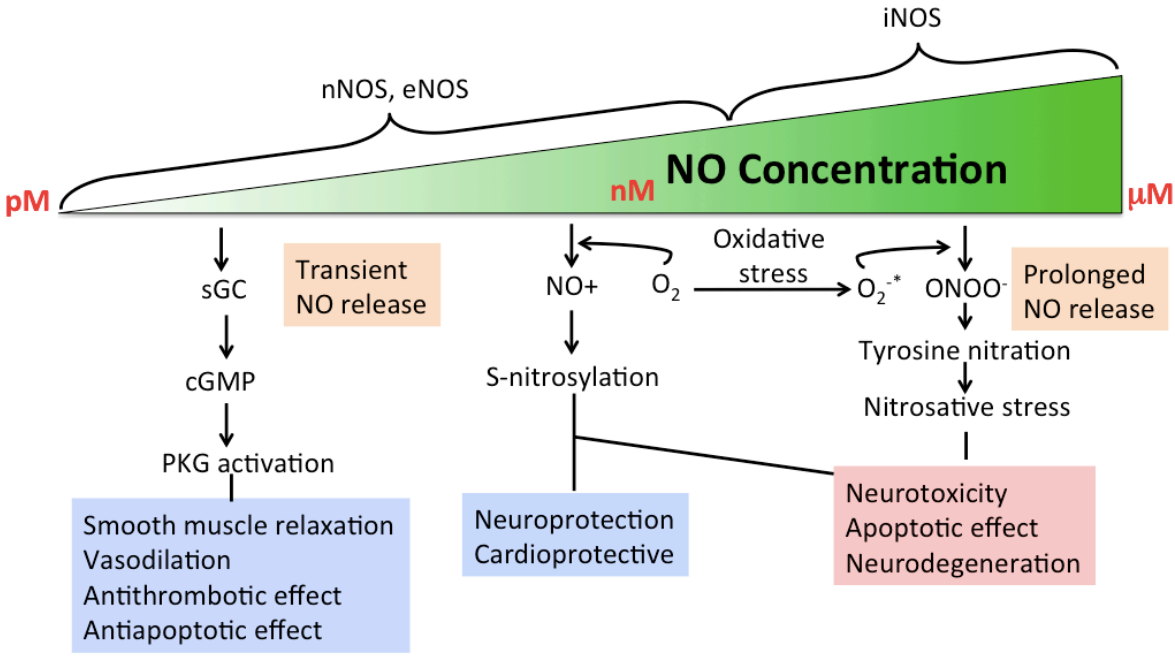


Fig. 1.1. Concentration dependent of NO function in cells.

Table 1.2. Concentration dependence of NO bioactivity.

[NO]	Signal Transduction Mechanism	Physiological Result
<1~30 nM	Phosphorylation of extracellular signal-regulated kinases (ERK)[14, 15]	Mediation of proliferative and protective effects
30~60 nM	Phosphorylation of Akt (protein kinase B)[16, 17, 18]	Protection against apoptosis[19]
100 nM	Stabilization of hypoxia-inducible factor 1α (HIF–1α)[20]	Tissue injury protection
400 nM	Phosphorylation and acetylation of p53[21]	Cytostatic to apoptotic responses, cell cycle arrest
>1 μM	Protein nitrosation (poly-ADP-ribose) polymerase, caspases) [22, 23]	Apoptosis, full cell cycle arrest

1.2. Chemical Features of 7-azabenzobicyclo[2.2.1]heptane Nitrosamines

Nitrosamines with a 7-azabenzobicyclo[2.2.1]heptane backbone has been reported previously[24] that they have a weaker N-NO bond compared to the monocyclic nitrosamines. The reason behind their weak N-NO bonding is laid on their non-planar shape. In the monocyclic nitrosamines, the nitrosamines take planar structures because the rotational barriers of the N-NO bond are of a similar magnitude to those of amides. In the planar nitrosamines, the $n_{(N)}$-$\pi^*_{(NO)}$ orbitals of the nitrosamine is well-overlapped. On the other hand, the bicyclic nitrosamines take a pyramidal shape which lead to poor overlap of $n_{(N)}$-$\pi^*_{(NO)}$ orbitals (**Fig. 1.2.**).

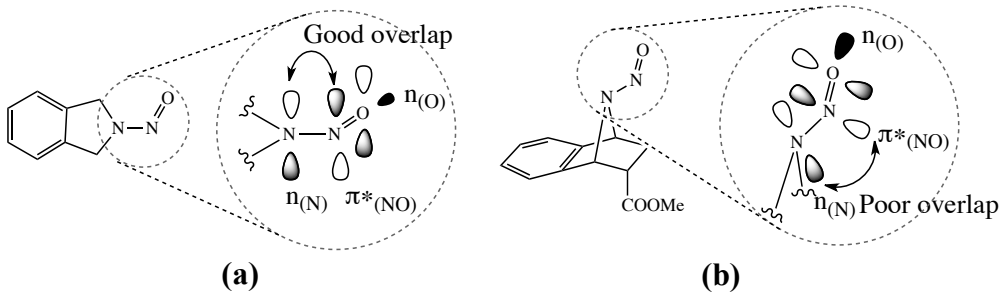


Fig. 1.2. Orbital interactions within the nitrosamine functionality of monocyclic **(a)** and bicyclic **(b)** nitrosamines.

Because of the planarity, good overlap of the $n_{(N)}$-$\pi^*_{(NO)}$ orbitals of monocyclic nitrosamines will lead to the resonance structure that represent the partial double bond character of the N-NO bond in a similar manner to the N-CO bond in amides (**Fig. 1.3.**).

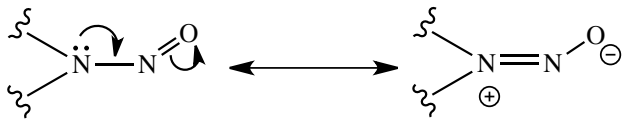


Fig. 1.3. Resonance model of planar nitrogen of *N*-nitrosamines.

Good overlap of the $n_{(N)}$-$\pi^*_{(NO)}$ orbitals will lead to stabilization of the N-NO bond by electron delocalization and the N-NO bond is strengthened. As compared with the monocyclic nitrosamines, bicyclic nitrosamines that have poor $n_{(N)}$-$\pi^*_{(NO)}$ orbital overlap will have a weaker N-NO bond because electron delocalization was interrupted. Interruption of resonance stabilization will lower the $\pi^*_{(NO)}$ orbital in energy, which decrease the HOMO-LUMO energy gap of the bicyclic nitrosamines. Therefore the N-pyramidalization decrease the $n_{(O)} \rightarrow \pi^*_{(NO)}$ excitation energy and reduce the double bond character of the N-NO bond. This phenomenon was supported by bathochromic shift of the $n_{(O)} \rightarrow \pi^*_{(NO)}$ absorption of the bicyclic nitrosamines in UV-Vis spectra[25]. As

photoirradiation excited the electron to LUMO, which is confined out-of-phase of the N-NO bond, and induce cleavage of N-NO bond.

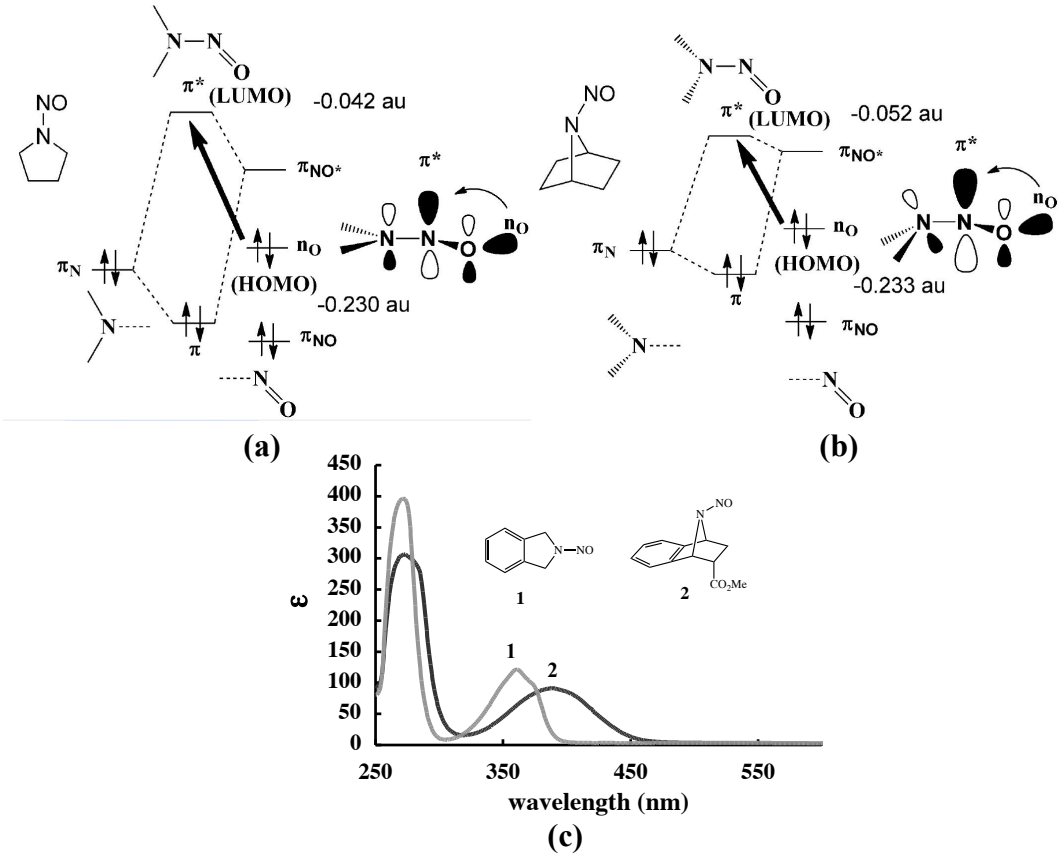
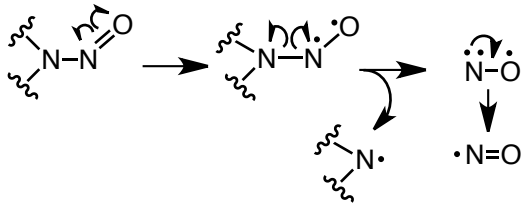


Fig. 1.4. HOMO–LUMO transition of monocyclic **(a)** and bicyclic **(b)** nitrosamines. UV spectra of monocyclic and bicyclic nitrosamines in DMSO **(c)**.

As shown in **Fig. 1.4.** bathochromic shift of bicyclic nitrosamines to longer wavelength and may absorb visible light around 400-450 nm. Using this advantage that was not observed in monocyclic nitrosamines, made bicyclic nitrosamines become potential caged-NO compounds that its NO release can be triggered by visible light irradiation, especially around 400-450 nm.

It was suspected that π bond of N=O bond was radically cleaved due to the irradiation and lead to homolytic cleavage of N-NO bond that release NO radical (**Scheme 1.1.**).



Scheme 1.1. Homolytic cleavage of N-NO bond initiated by irradiation.

1.3. Nitric Oxide Release from 7-azabenzobicyclo[2.2.1]heptane Nitrosamines in Cuvette

Several bicyclic nitrosamines have been synthesized and their NO release was studied with DAF-2[26] as a NO probe (**Fig. 1.5. (a)**). In the detection of NO by DAF-2, oxygen was needed to generate N_2O_3 or NO^+ species that will react with DAF-2 to form DAF-2T and fluorescence can be observed. As for bicyclic nitrosamines in the presence of oxygen, upon irradiation with visible light, fluorescence of DAF-2T was observed. When oxygen was eliminated from the system as anaerobic condition, fluorescence of DAF-2T was not observed after same irradiation was applied. This indicated that visible light irradiation to bicyclic nitrosamine will generate NO radical instead of NO^+ and the N-NO bond was cleaved in homolytic manner. Besides, **Fig. 1.5. (b)** also indicated that without irradiation, bicyclic nitrosamines will not release NO on its own as no fluorescence was detected in the absence of irradiation.

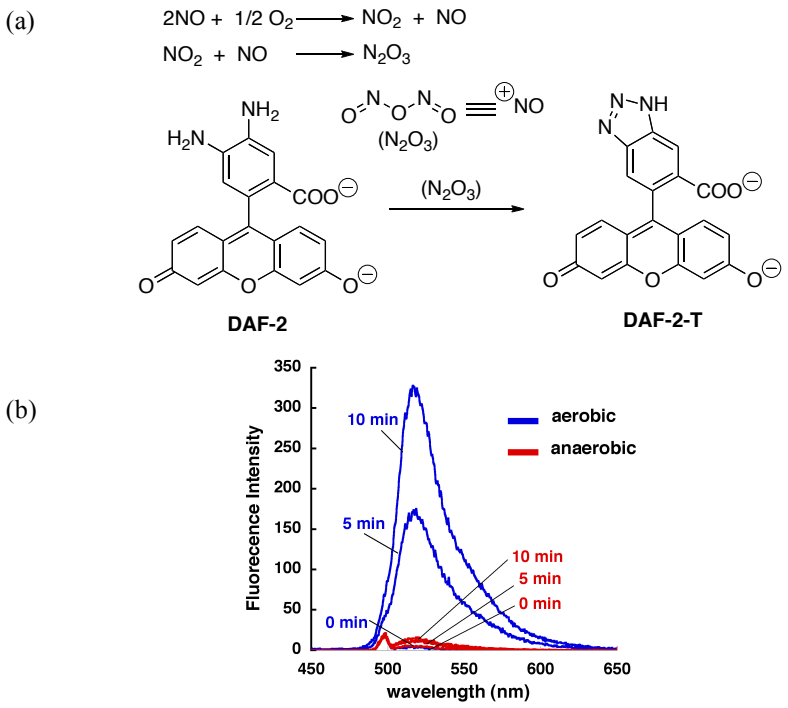


Fig. 1.5. (a). Detection of NO with DAF-2 as the NO probe. **(b)**. DAF-2T fluorescence intensity after irradiation at 420 nm of bicyclic nitrosamine in aerobic condition (blue line) and anaerobic condition (red line).

Study of NO release from previously synthesized bicyclic nitrosamines was shown in **Fig. 1.6.** As the substituent was changed, the NO release efficiency was affected. Generally, bicyclic nitrosamines with benzene ring showed higher efficiency of NO release

than nitrosamines with bicyclic ring only. Therefore, for further development of bicyclic nitrosamines, presence of the benzene ring was considered.

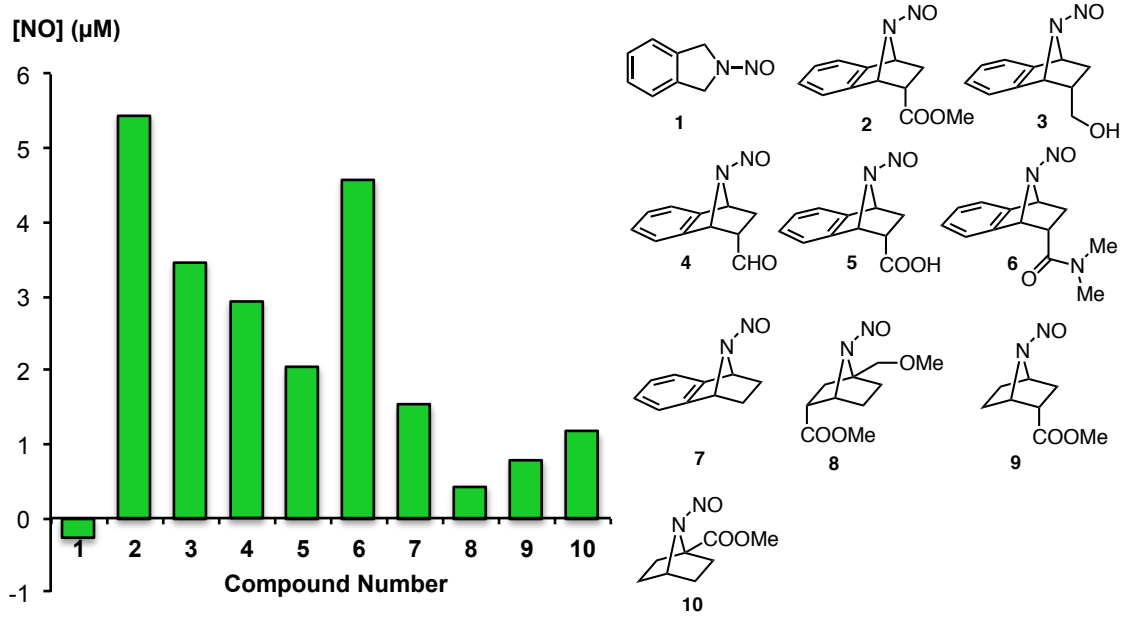
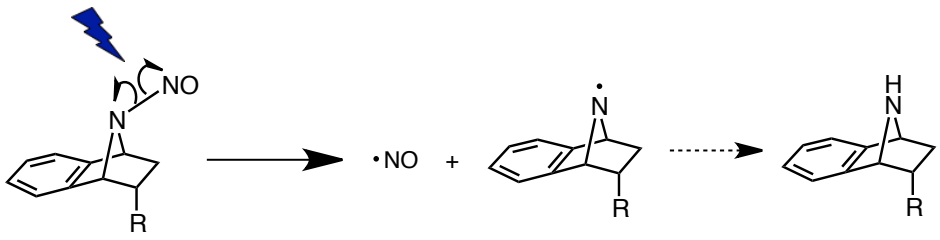


Fig. 1.6. Comparison of NO release efficiency from various nitrosamines with DAF-2 as the NO probe after irradiation at 420 nm for 10 minutes.

After irradiation with visible light, homolytic cleavage of N-NO bond was occurred and NO radical was generated from the bicyclic nitrosamine. Beside NO radical, amine radical was also generated, which it may abstract an electron from another species to form an amine (**Scheme 1.2.**).



Scheme 1.2. Homolytic cleavage of N-NO bond induced by photoirradiation.

An NMR study has been performed to detect the formation of amine from bicyclic nitrosamine after irradiated with visible light. After irradiated for 24 hours, amine formation was detected by NMR and it can be confirmed with the authentic related amine compound (**Fig. 1.7.**).

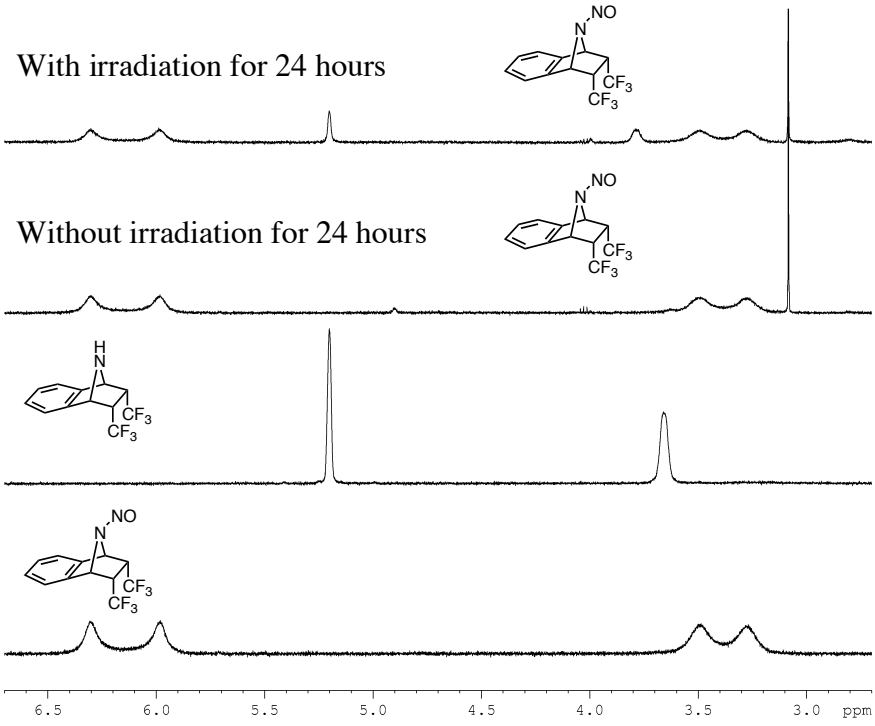


Fig. 1.7. Detection of amine by NMR (in $CDCl_3$) from bicyclic nitrosamine after irradiated with visible light.

1.4. Aims of This Study

In the previous works, development of bicyclic nitrosamines as photo-induced caged-NO was conducted in cell-free environment to study their properties and factors that may affect its ability for NO release, such as effect of functional groups. However their cell applicability has not been studied and their behaviors in living cells were unknown. For the sake of modeling biological events by using small-molecular caged NO, i.e., artificial NOS mimics, it will be important to control and define the concentration of NO released in cells under temporal control.

Most of NO-donors such as NONOates and the latest stimulus-dependent NO donors have been used for short-duration exposure of NO to the cells. However the concentration of NO and timing of exposure to NO are strictly controlled in cells, as different concentration of NO will trigger different biological events. In these aspects, exposure of NO with controlled amount and timing for long-duration experiment may have a physiological relevance. Herein, development of second-generation visible light triggered caged-NO based on non-planar bicylic *N*-nitrosamines with attention to cell-retention property featuring controllable NO release in cells was described. More explicitly, I aimed the following four aspects.

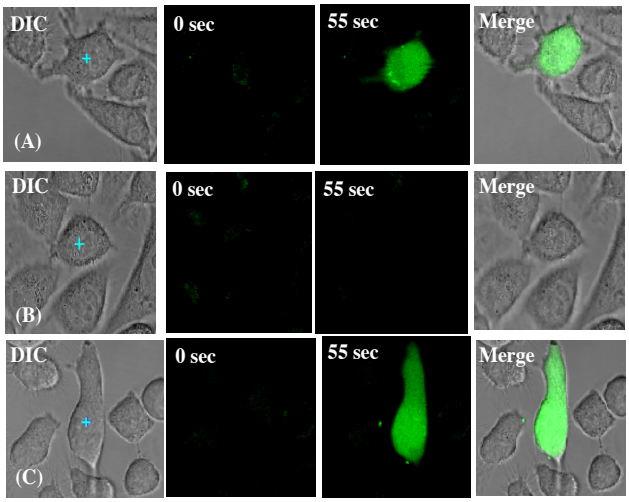
1) Confirmation of NO release from bicyclic nitrosamines in cells upon visible light irradiation with Piccell as model study.
2) The possibility to control the NO release from bicyclic nitrosamine by controlling the duration of irradiation.
3) Effect of numbers and/or position of acetoxymethyl ester (AM ester) functional group in cell retention ability.
4) The potential of bicyclic nitrosamines to be used as artificial NOS mimic with controllable NO release and high cellular retention profile.

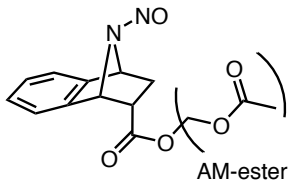
Chapter 2

Cell Applicability Study of Bicyclic Nitrosamines

2.1. Previous Study of 7-azabenzobicyclo[2.2.1]heptane Nitrosamines in Cells

Bicyclic nitrosamines with 7-azabenzobicyclo[2.2.1]heptane scaffold have demonstrated its potential used as caged-NO that its NO release can be triggered by irradiation in cuvette.[25] To bring the applicability of these bicyclic nitrosamines to the next level, study of NO release in cells was conducted. Initial study of NO release from bicyclic nitrosamines in cells was performed in HeLa cells. Our previous result showed that bicyclic nitrosamine **2** with methyl ester could penetrate through cell membrane into the cell. In the presence of fluorescent NO probe, DCl-DA Cal-AM, fluorescence was observed after irradiation, as shown in **Fig. 2.1. (c)**.[27] However nitrosamine **2** is lack of cell retention, which after incubation and wash, no fluorescence was observed even after irradiation was applied (**Fig. 2.1. (b)**).





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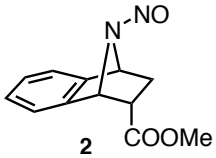

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Fig. 2.1. Application of compound **11** (with washing) **(a),** compound **2** (with washing) **(b),** and compound **2** (without washing) **(c)** to HeLa cells in the presence of fluorescent NO probe, DCl-DA Cal-AM. **(a)** 30 min incubation of **11** and DCl-DA Cal-AM, followed by washing with PBS buffer. **(b)** 30 min incubation of **2** and DCl-DA Cal-AM, followed by washing with PBS buffer. **(c)** 30 min incubation of DCl-DA Cal-AM, followed by washing with PBS buffer. Then, **2** was added and fluorescence was measured without further washing. Differential interference contrast (DIC), fluorescence image before (0 sec) and after (55 sec) irradiation at 458 nm (argon laser) at + mark, and merged image.

In order to study the cell retention, methyl ester nitrosamine **2** was modified to acetoxy methyl (AM) ester as compound **11**. Application of compound **11** to HeLa cells in the presence of NO probe, DCl-DA Cal-AM, NO release was detected after irradiation.

These results demonstrated that bicyclic nitrosamines with AM ester functional group have cell permeability and cell retention ability. AM ester functional group was reported and used by Tsien[28] to facilitate Ca^{2+} ion probe (BAPTA) that has cell permeability problem. By protecting the carboxylic acids' functional group as AM ester, it will increase the cell permeability. Inside cells, AM ester is recognized by esterase as substrate therefore it will be hydrolyzed to carboxylic anion and its capability to permeate through the highly hydrophobic lipid bilayer was lost. Therefore the compound will be retained in cells. Applying this strategy to bicyclic nitrosamines, these compounds may retain in cells to facilitate constant NO release for longer period of time (**Fig. 2.2.**).

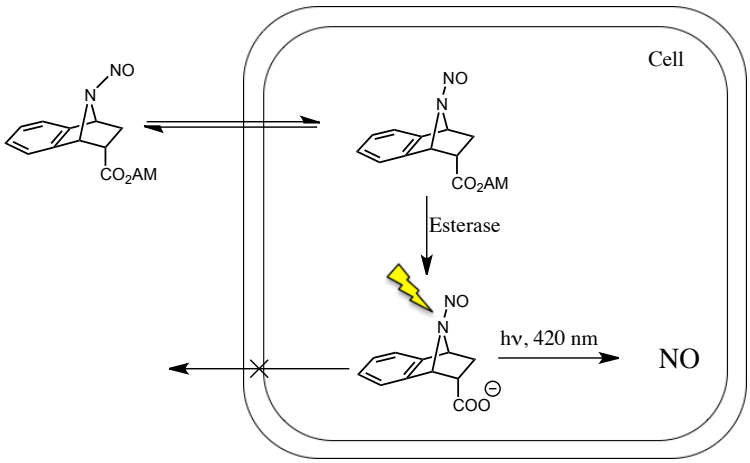


Fig. 2.2. *N*-nitrosoamines cell retention mechanism by acetoxy methyl ester substituent.

However, there are some disadvantages of using this fluorescein derivatives probe to detect NO generation. It is reported that the limit detection of NO with this probe is 5 nM[26] which, is relatively high concentration compared to NO concentration generated by eNOS and nNOS. Even though this probe is highly selective for NO, the reaction between DAF-2 and NO itself was not very efficient and non-reversible. Karaki has reported the reaction yield between DAF-2 and NO was only 2-4% after irradiation.[27] Besides, after DAF-2T was formed, it is an irreversible reaction and decrease of NO concentration cannot be observed. Because of this limitation, another method for NO generation, which is highly selective, low NO concentration detection and reversible, was preferred.

2.2. Piccell, Cell-based of NO Probe

Sato, *et al.*[29] invented Piccell as a biomolecular NO detector. Piccell is a mutant derived from pig kidney-derived cell line (PK15) that is modified to express two-chimera protein, the α– and β–subunits of soluble guanylate cyclase (sGC) that both of them are connected with a fluorescent cGMP indicator protein named CGY.[30] The CGY protein contains cGMP-depedent protein kinase 1α (PKG 1α), as receptor for cGMP, fused with blue- and red-shifted green fluorescent protein (GFP), the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) as donor and acceptor fluorophores for fluorescent resonance energy transfer (FRET).

After NO was bound to sGC, the cyclase activity at both sGCα and sGCβ will be stimulated and increased up to 400-fold, resulting in the repeated conversion of GTP to cGMP. When cGMP was present and bound to CGY protein, the conformation of PKG 1α will change and the central chromophore of GFPs are drawn close to each other and FRET can take place. When dissociation of cGMP with CGY protein was happened, the decrease of FRET was also detected (**Fig. 2.3.**).

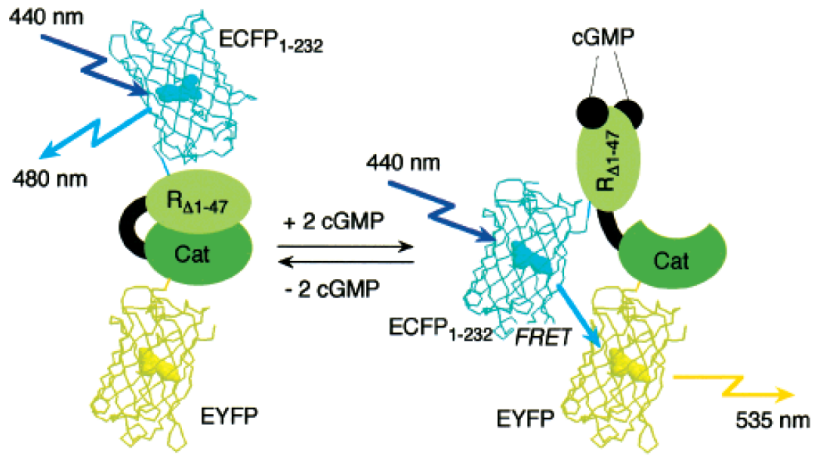


Fig. 2.3. FRET process of green fluorescent protein in Piccel when cGMP bind to PKG-1α.

Because Piccell expresses green fluorescence proteins, after irradiated with visible light at 440 nm in free state without NO, it will show red color. If NO was presence, due to the FRET it will turn green, as shown in **Fig 2.4**.

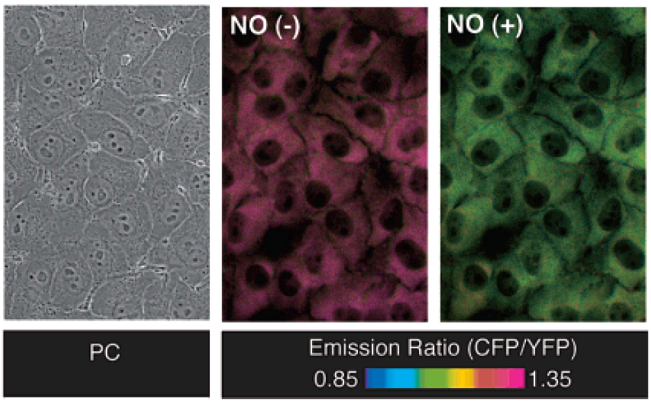


Fig. 2.4. Piccel without (middle) and with (right) the presence of NO.

They also reported that NO can be detected selectively at picomolar concentration by piccell in cell media. Limit detection of NO by piccell is 20 pM and they use sGC to trap NO in cells. sGC is natural receptor of NO that selectively recognize NO in cells. Therefore this NO detection is highly selective with better reaction yield compared to NO-DAF-2. Because of its low limit detection and high selectivity, Piccell was chosen to study the applicability of bicyclic nitrosamines in cells. In addition, Piccell itself also is a living cell, which it can be used directly as a model to study NO release in cells.

It is reported that activated sGC by NO may generate 50-100 molecules of cGMP/sec.[29] Because of this reason, visualization of NO in piccell was amplified and may detect NO at very low concentration. Utilizing CGY protein's ability to detect cGMP and was combined with NO-activated sGC protein (**Fig. 2.5.**), made piccell is a very sensitive, selective, reversible and reproducible indicator for NO.

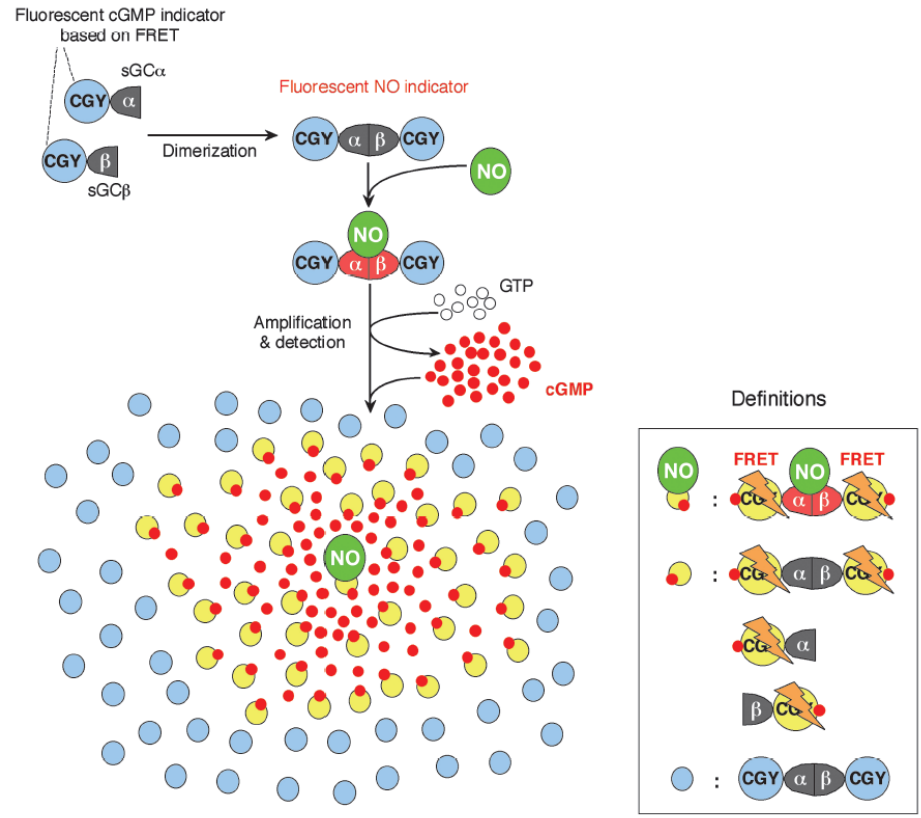


Fig. 2.5. NO detection principal with Piccell.

As the increase of FRET takes place, the emission ratio of CFP/YFP was decrease. After some times, phosphodiesterase will immediately hydrolyze cGMP after the removal of NO, which lead to decrease of FRET and increase the emission ratio of CFP/YFP. As the result, responses of the Piccell are recorded as shown in the **Fig. 2.6.**[31]

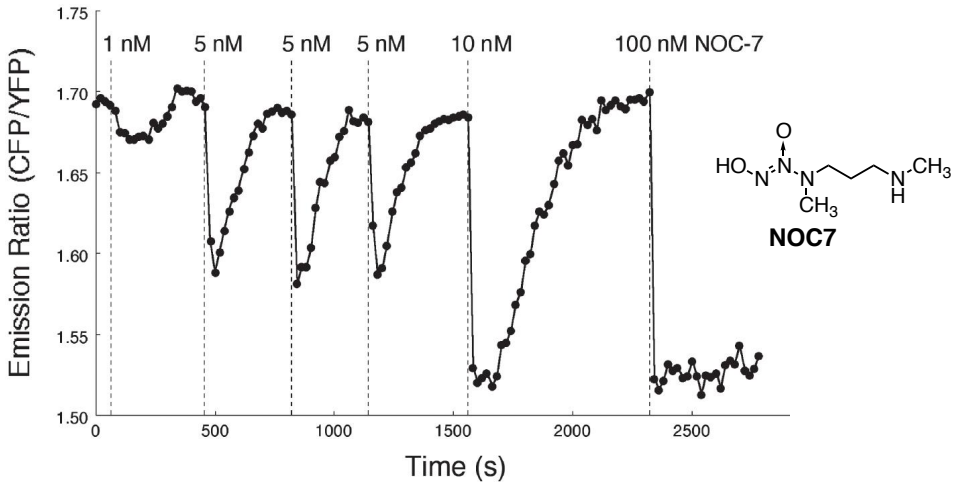


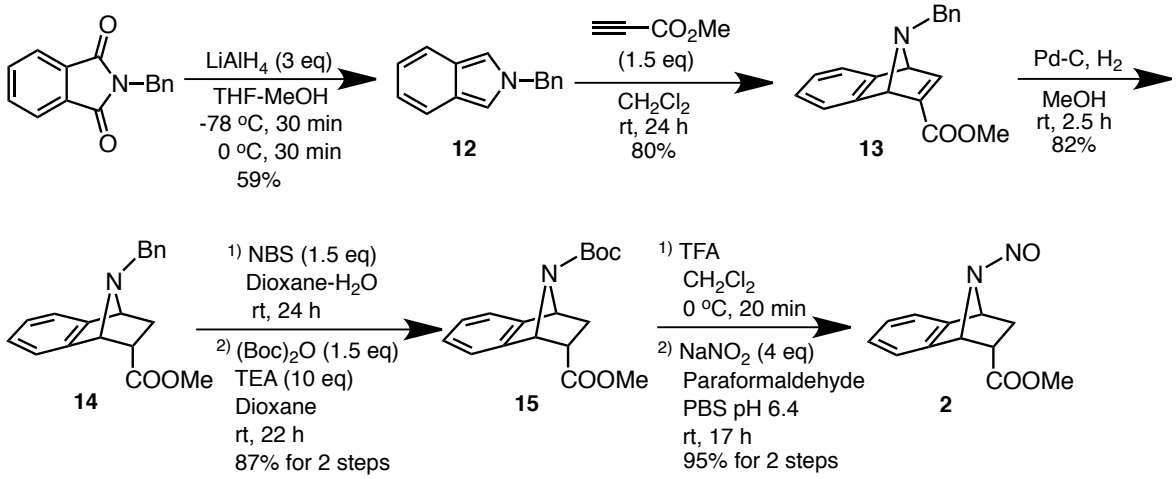
Fig. 2.6. Signal of NO detection from NOC7 by Piccell.

When NO was depleted in Piccell, its signal will return to baseline. If additional NO was added into Piccell, its signal will rise again. With this reversibility, Piccell can be used not only to detect NO generation but also NO depletion in cells.

2.3. Nitric Oxide (NO) Controlled Release Detection in Piccell

2.3.1. Synthesis of First Generation Bicyclic Nitrosamine

As it has been mentioned before, the methyl ester bicyclic nitrosamine **2** is the first generation of bicyclic nitrosamine. This nitrosamine can be synthesized according to the **Scheme 2.1**. Bicyclic ring was constructed by Diels-Alder reaction of isoindole **12** and methyl propiolate. Palladium/carbon catalyzed hydrogenation of **13** will give *endo*-**14**, which after changing the *N*-benzyl group to *N*-Boc followed by TFA deprotection and Keefer's nitrosation[32] reaction, will give nitrosamine **2**.



Scheme 2.1. Synthesis of nitrosamine **2**.

2.3.2. NO Release Detection from First Generation Bicyclic Nitrosamine in Piccell

Study of NO release from nitrosamine **2** in Piccell was shown in **Fig. 2.7. (a)**. After incubation in Piccell for 1 hour, direct irradiation with both visible and UV light without washing indicated NO release in Piccell. NO release triggered by irradiation from bicyclic nitrosamine showed relatively sharp signal in Piccell than NOC7's signals due to very small amount of NO was generated, followed by rapid hydrolysis of cGMP from PKG-1α. In the meanwhile, relatively broad signal of NOC7 in Piccell was caused by instant release of NO and its concentration cannot be controlled. Therefore, much more NO was released and it needs longer time for complete hydrolysis of cGMP from PKG-1α compared to this bicyclic nitrosamine.

Besides, NO release from bicyclic nitrosamine in Piccell also showed relatively stable NO release within one hour and demonstrated Piccell's reversibility to detect both NO generation and NO depletion in cells. If the duration of irradiation time to uncage NO from bicyclic nitrosamine was changed, the NO release concentration would have changed as well. It was demonstrated that when nitrosamine **2** was irradiated with visible light (440 nm) for 1 sec, 56 ± 3.3 pM of NO was released. If the duration of irradiation was increased to 5 sec, the NO release concentration was also increased to 323 ± 49 pM (**Fig. 2.7. (b)**). Concentration of NO release in Piccell was determined based on calibration curve of NO in Piccell, as has been reported by Sato, *et. al*.[29] Based on these results, NO release triggered by irradiation can be controlled and the concentration can be adjusted to the needs by controlling the irradiation time.

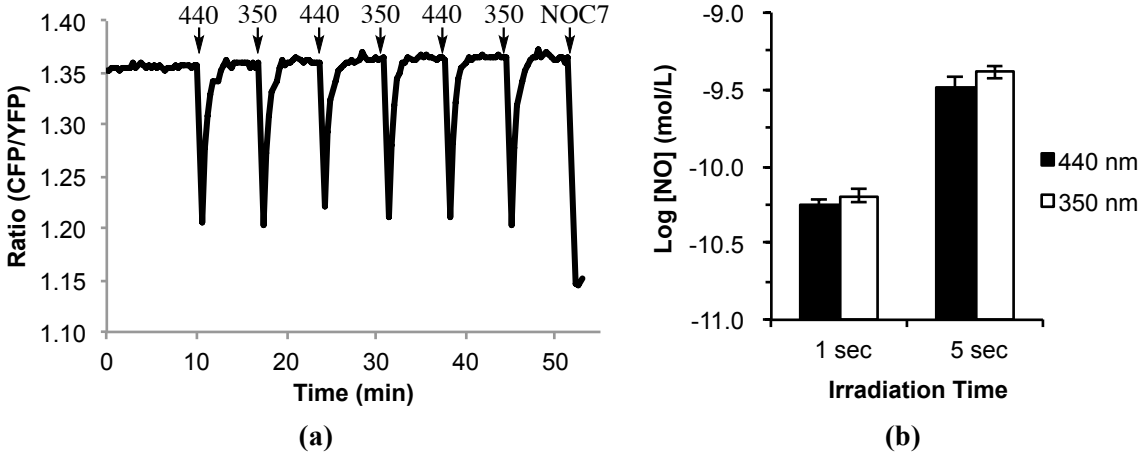


Fig. 2.7. (a). Detection of NO release from **2** (10 μM, 1 h incubation) in Piccell without washing and irradiated with visible light (440 nm) and uv light (350 nm) for 5 sec. At the end of measurement NOC7 (100 nM) was used as positive control. **(b).** Time dependent of NO release concentration from **2** (10 μM, 1 h incubation) upon irradiation (440 nm and 350 nm) for 1 and 5 sec.

However, when washing was applied after incubation, concentration of NO release in Piccell was decreased from time to time. The different results between without washing and washing in Piccell was due to change of nitrosamines concentration in cells upon irradiation. Without washing, concentrations of nitrosamine inside and outside of Piccell were in steady state. If the concentration inside Piccell didn't change, even after 40 minutes Piccell would give stable NO release signals (**Fig. 2.7. (a)**).

When washing was applied after incubation, gradient concentration was occurred between inside and outside of cells. Since it was suspected that nitrosamine **2** could permeate through cell's membrane easily, it must have leaked out from cells, which lead to reduction of nitrosamine concentration in Piccell. As the result, as time was increased, amount of leaked out nitrosamine was increased and if irradiation was applied at this moment, Piccell's signal will decline from time to time as shown in **Fig. 2.8**.

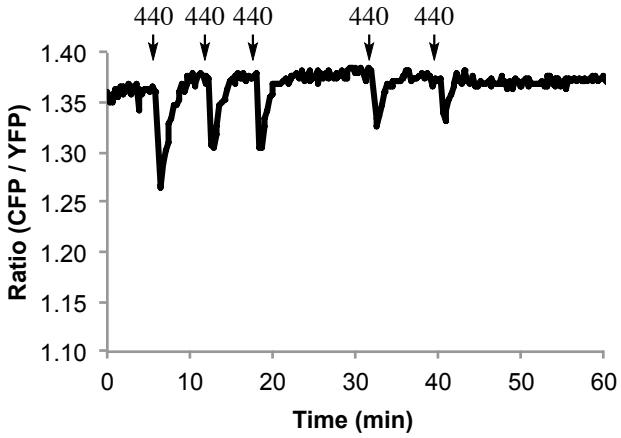
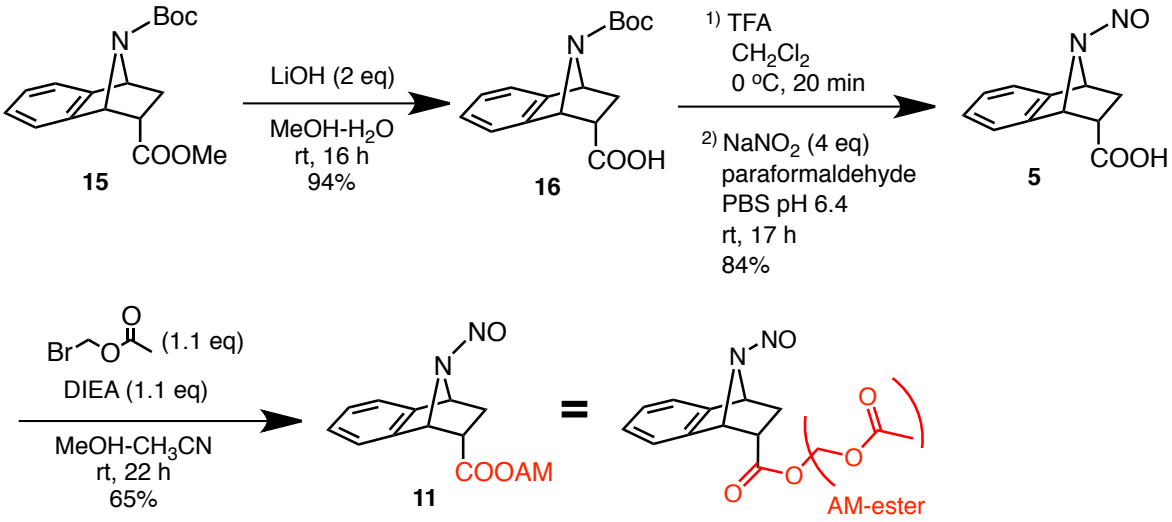


Fig. 2.8. Decline of NO release from nitrosamine **2** (10 μM, 1 h incubation) in Piccell after irradiated with visible light (440 nm, 5 sec) due to gradient concentration of nitrosamine **2** after washing.

2.3.3. Synthesis of First Generation AM-ester Nitrosamine

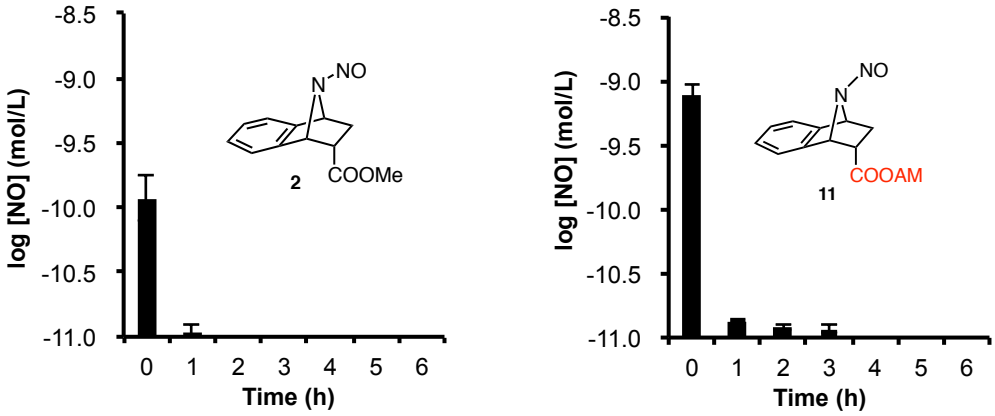
To overcome leakage of compounds from cells, Tsien[28] has reported the potential of acetoxy methyl ester (AM ester) functional group as cell retention functional group. Our group previously also has demonstrated the potential of this AM-ester functional group to improve nitrosamines' cell retention ability (**Fig. 2.1. (a)**).[27] Synthesis of previously used AM ester nitrosamine **11** was shown in **Scheme 2.2**. Synthesis of **11** was performed from intermediate **15**. After hydrolysis with LiOH, *N*-Boc deprotection by TFA and Keefer's nitrosation reaction, related carboxylic acid nitrosamine **5** was obtained. At last esterification with bromomethylacetate gave desired AM-ester nitrosamine **11**.



Scheme 2.2. Synthesis of first generation AM-ester nitrosamine **11**.

2.3.4. Study of NO Release from First Generation AM ester Nitrosamine in Piccell

When the synthesis of nitrosamine **11** was completed, study of NO release in Piccell was performed. As the results showed in **Fig. 2.9.**, one hour since incubation was stopped (t=1 h), nitrosamine **2** with methyl ester functional group showed almost no signal of NO generation in Piccell. Different with this result, nitrosamine **11**, at the same time showed weak signal of NO. This results suggest that washing after incubation and before measurement may wash out nitrosamine **2** from Piccell (same result shown in **Fig. 2.1. (b)**). In the meanwhile, for nitrosamine **11**, even washing was applied both after incubation and before each measurement; small amount of nitrosamine was remained in Piccell and the signal still can be detected (**Fig. 2.9. (b)**). Previous study of this compound in HeLa cells (**Fig. 2.1. (a)**) also showed the same result that demonstrated the potential of this AM-ester functional group.



(a) **(b)**

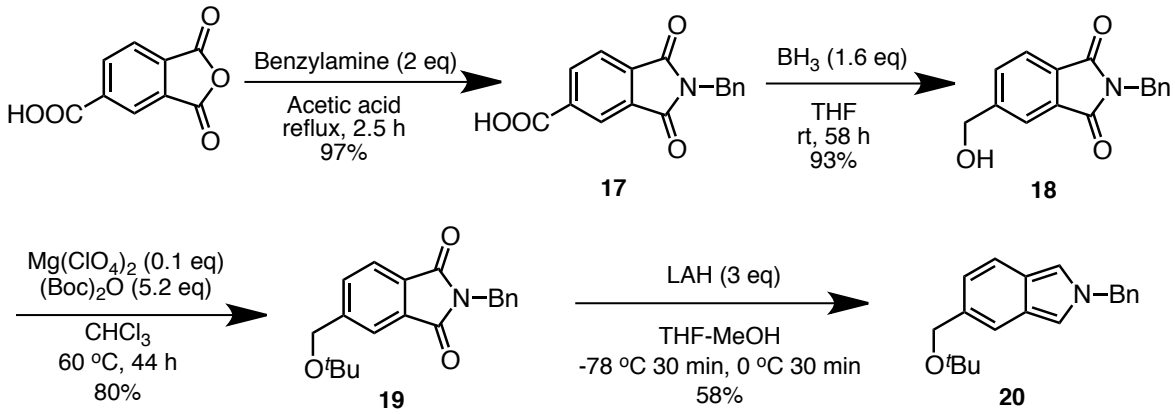
Fig. 2.9. NO release of **(a)** nitrosamine **2** (10 μM, 1 h incubation) and **(b)** nitrosamine **11** (10 μM, 1 h incubation) upon irradiated with visible light (440 nm) for 5 sec after washing (7 times) were applied before each measurement.

2.4. Development of Second Generation of Mono-AM ester Nitrosamines

2.4.1. Synthesis of Benzene ring Substituted Mono-AM ester Nitrosamines

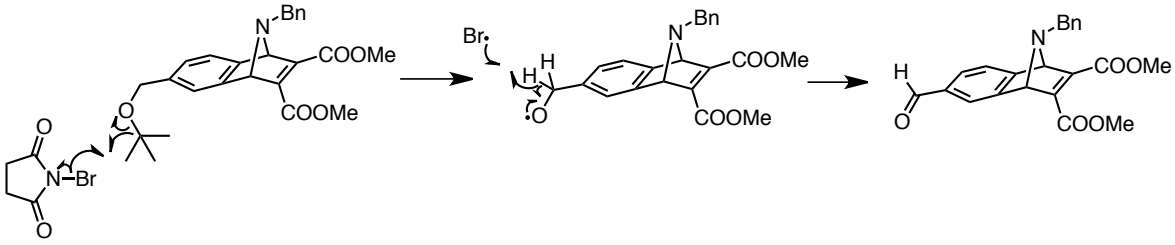
Further study of mono-AM ester nitrosamine **11** was conducted by changing its position from the bicyclic ring's right side to the benzene ring position at the left side of the bicyclic system. Since substitution at the benzene ring was needed, the isoindole to construct bicyclic ring scaffold was also modified to benzene ring substituted isoindole **20**.

Synthetic route to isoindole **20** was shown in **Scheme 2.3**. The synthesis start from trimellitic anhydride to create the imide **17** followed by reduction of benzoic acid with borane and protection of the alcohol as *tert*-butyl ether **19**. At last, reduction of imide with LAH will give desired substituted isoindole **20**.



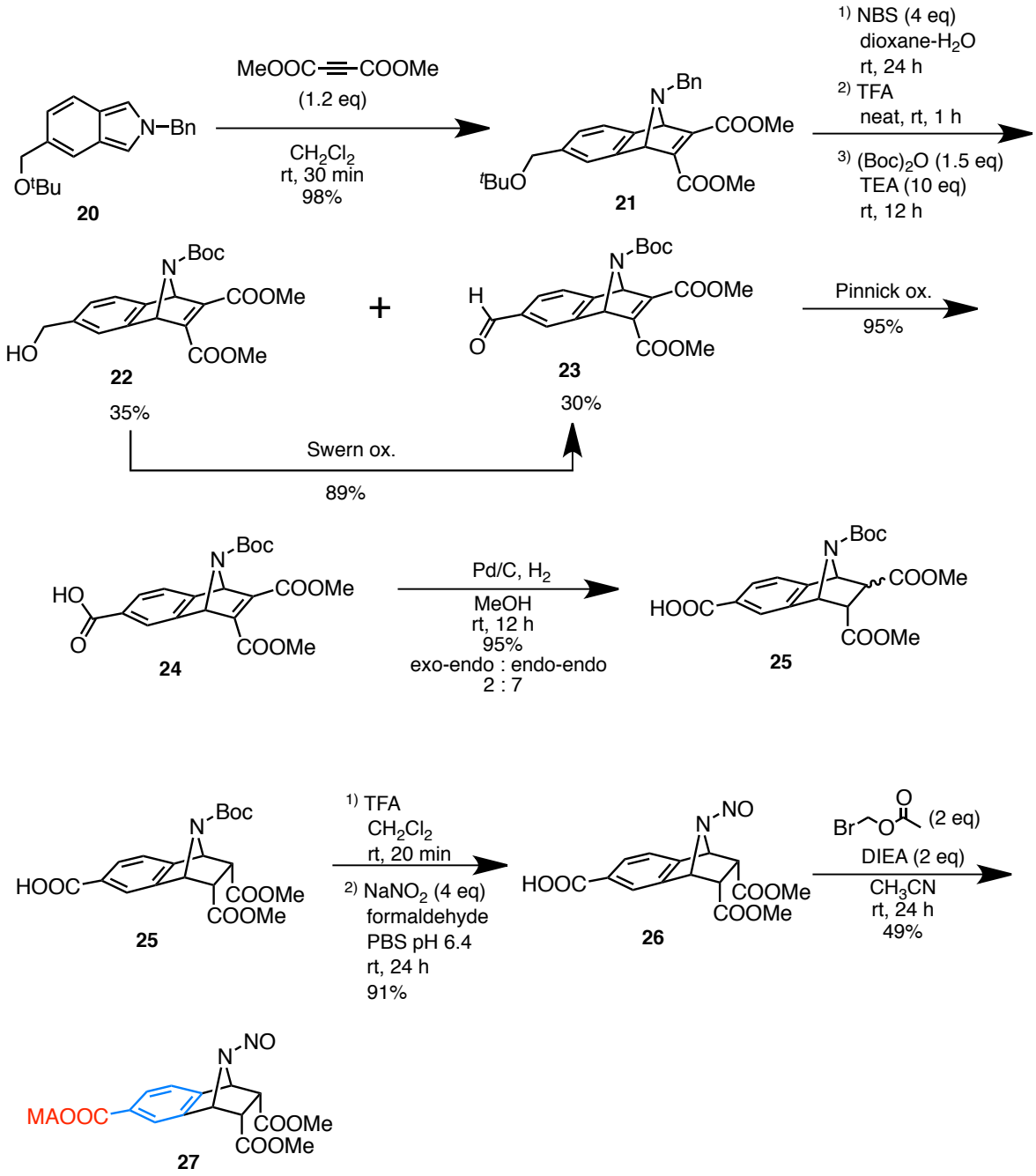
Scheme 2.3. Synthesis of benzene ring substituted isoindole **20**.

With this benzene ring substituted isoindole **20** in hand, nitrosamine **27** can be prepared through Diels-Alder reaction of isoindole **20** with dimethyl acetylenedicarboxylate as the dienophile to give bicyclic compound **21**. Debenzylation of *N*-benzyl protected **21** with NBS followed by *O*-^{t}butyl deprotection with TFA and protection of the amine as *N*-Boc will give free alcohol **22**. During this process, excess of NBS reacted with **21** and gave aldehyde compound **23**. It is proposed that oxidation to aldehyde occurred through radical oxidation by NBS.



Scheme 2.4. Proposed mechanism of radical oxidation by NBS.

The free alcohol **22** was oxidized to aldehyde with Swern oxidation, followed by Pinnick oxidation to give desired carboxylic acid **24**. Palladium/carbon hydrogenation of this compound gave mixture of *endo-endo* and *exo-endo* **25**. After separation only the *endo-endo* **25** was used for the next reaction. Deprotection of *N*-Boc with TFA, followed by Keefer's nitrosation and esterification, finally AM-ester **27** can be obtained.



Scheme 2.5. Synthetic route of benzene ring substituted nitrosamine **27**.

2.4.2. Cell Retention Difference between First and Second Generation of Mono-AM ester Nitrosamines.

Comparison of NO release between **27** and **11** in Piccell showed different profile even though both of the compounds have only one AM ester that differs in the location. Lack of cell retention ability was observed from **11** but as the AM ester was moved to the left side at the benzene position, relatively stable NO release was observed (**Fig. 2.10.**).

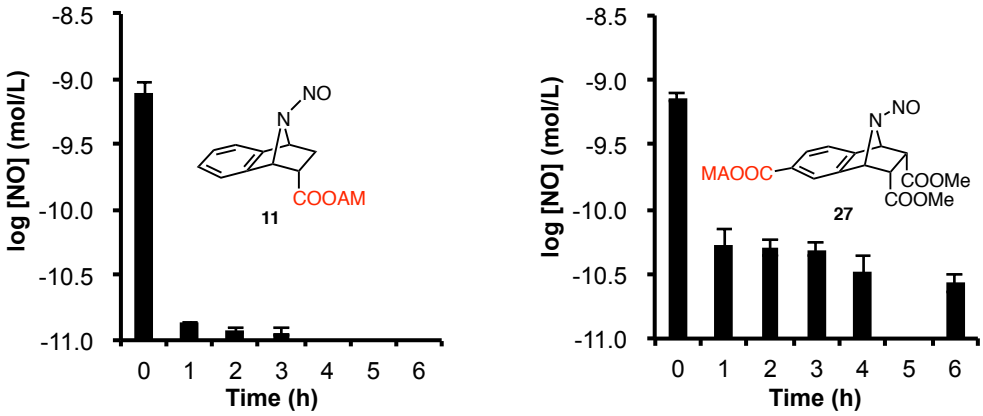
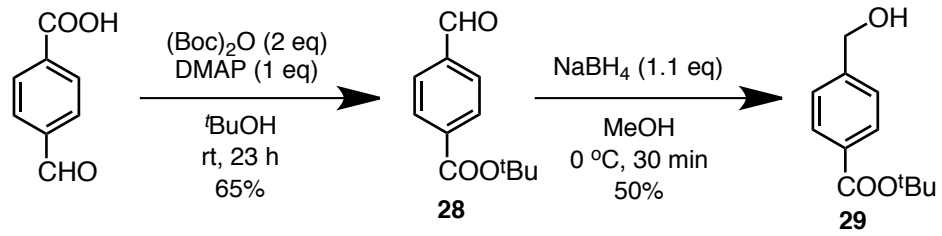


Fig. 2.10. Different profile of NO release from nitrosamine **11** and **27** (10 μM, 1 h incubation) in Piccell upon irradiation with visible light (440 nm, 5 sec) after washing (7 times) were applied before each measurement.

One hour since incubation (t=1 h), rapid decrease of NO release was still observed in nitrosamine **27**, but it was not as fast as **11**. Several factors may affect this, but there is an improvement in cell retention ability, which can be utilized to design more retainable nitrosamines. Therefore, various derivatives of benzene ring substituted AM ester nitrosamines will be synthesized and discussed to study its generality.

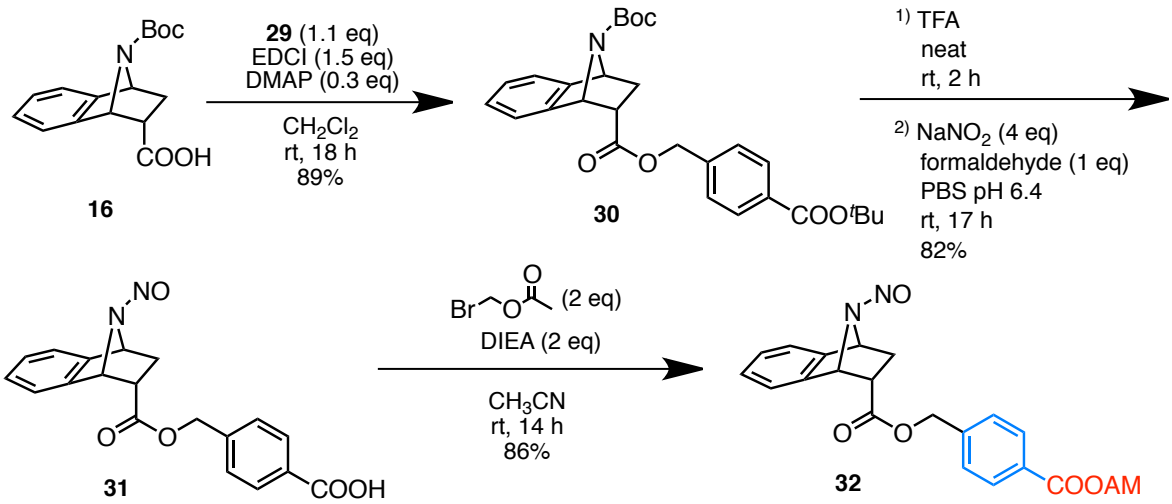
2.4.3. Synthesis of Benzene Ring Substituted Mono-AM ester Derivatives

To study this benzene ring substituted AM ester to a greater extent, various benzene ring substituted AM ester nitrosamines were synthesized. The first compound is bicyclic ring non-fused benzene ring substituted AM ester compound, which phenolic AM ester was linked to the bicyclic system through ester linkage. Synthesis of the phenolic AM ester intermediate was shown in **Scheme 2.6**. The intermediate was prepared from 4-formylbenzoic acid. Protection of the acid as *tert*-butyl ester followed by reduction of aldehyde to alcohol will give desired intermediate **29**.



Scheme 2.6. Synthesis of intermediate **29**.

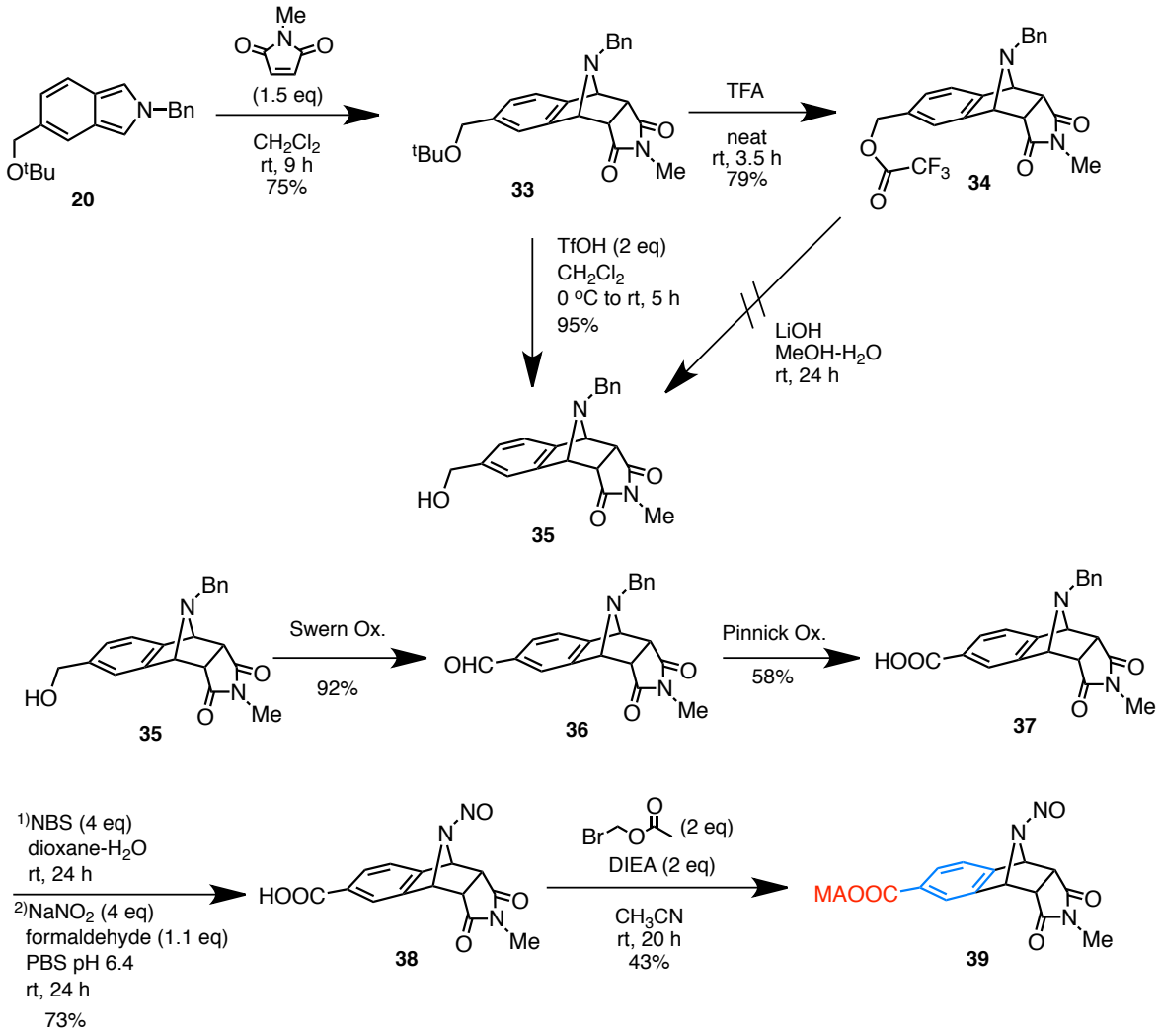
Coupling of intermediate **29** with **17** followed by TFA deprotection, Keefer's nitrosation and AM ester esterification will give nitrosamine **32** (**Scheme 2.7.**).



Scheme 2.7. Synthesis of nitrosamine **32**.

The second benzene ring substituted AM ester nitrosamine was prepared as shown in **Scheme 2.8**. Benzene ring substituted isoindole **20** was used as starting material because bicyclic ring fused benzene ring substituted AM ester nitrosamine is the desired compound. Diels-Alder reaction of isoindole **20** with *N*-methyl maleimide was performed

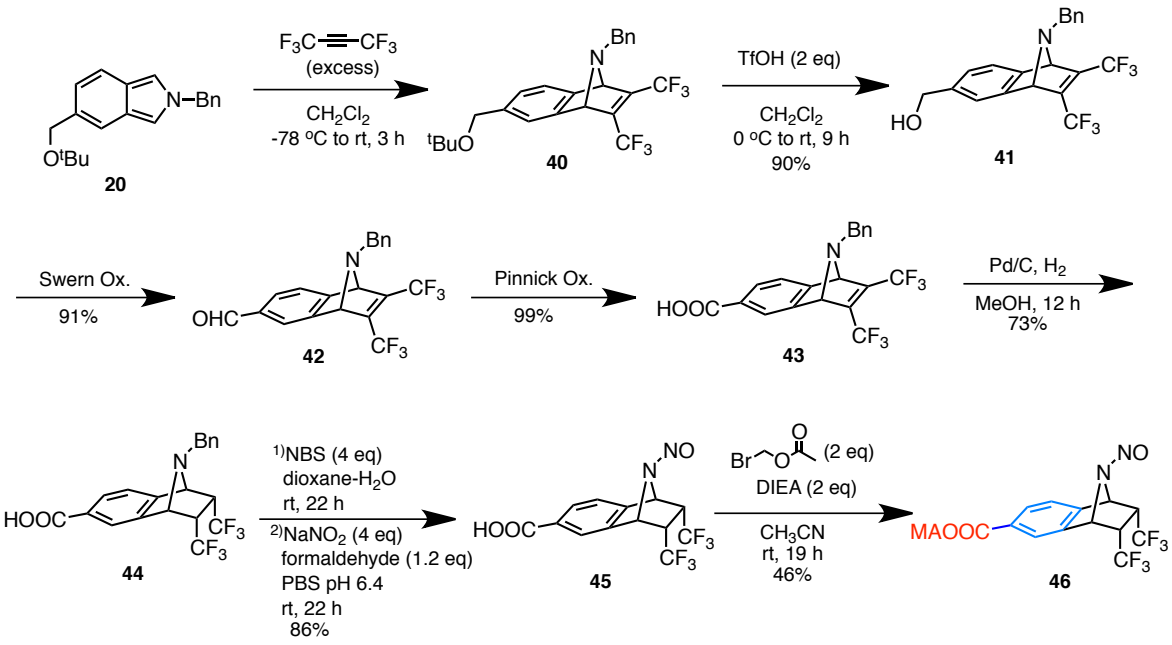
to give compound **33**. TFA deprotection of *tert*-butyl ether in neat condition was conducted but trifluoro acetylation was occurred, which cannot be hydrolyzed under alkaline condition. Therefore the acid for deprotection of *tert*-butyl ether was change to TfOH that even trifluoromethanesulfonic ester was formed, it can be easily decomposed by water during the work up to give free alcohol **35**. Oxidation of the alcohol by Swern oxidation and Pinnick oxidation to carboxylic acid followed by NBS debenzylation, Keefer's nitrosation and AM ester synthesis will give the desired nitrosamine **39**.



Scheme 2.8. Synthesis of nitrosamine **39**.

The third benzene ring substituted AM ester nitrosamine's preparation is shown in **Scheme 2.9**. The same isoindole **20** was used as the diene, but dienophile was change to hexafluoro-2-butyne. Using the same strategy, TfOH was used for *tert*-butyl ether deprotection after Diels-Alder reaction was completed, followed by oxidation to carboxylic

acid and Palladium-carbon hydrogenation. At last NBS debenzylation, Keefer's nitrosation and AM ester synthesis will give nitrosamine **46.**



Scheme 2.9. Synthesis of nitrosamine **46**.

2.4.4. Piccell Study of Benzene Ring Substituted Mono-AM ester Nitrosamines

With these benzene ring substituted AM ester nitrosamines, study of the AM ester retention ability was conducted in Piccell. Bicyclic ring non-fused benzene ring substituted AM ester nitrosamine **32** showed lack of cell retention ability. Bicyclic ring fused benzene ring substituted AM ester nitrosamine **39** and **46** show some similarity with nitrosamine **27** at some extent but as the time was increased, both of these compounds showed lack of cell retention. Because of the substituent was different between nitrosamine **27**, **39**, and **46**, it is relatively difficult to compared their NO release profile in Piccell and not conclusive result was obtained from these compounds. Therefore, direct analog of **27** was proposed to minimize the substituent effect in NO release efficiency and cell retention ability of this benzene ring substituted AM ester can be compared objectively.

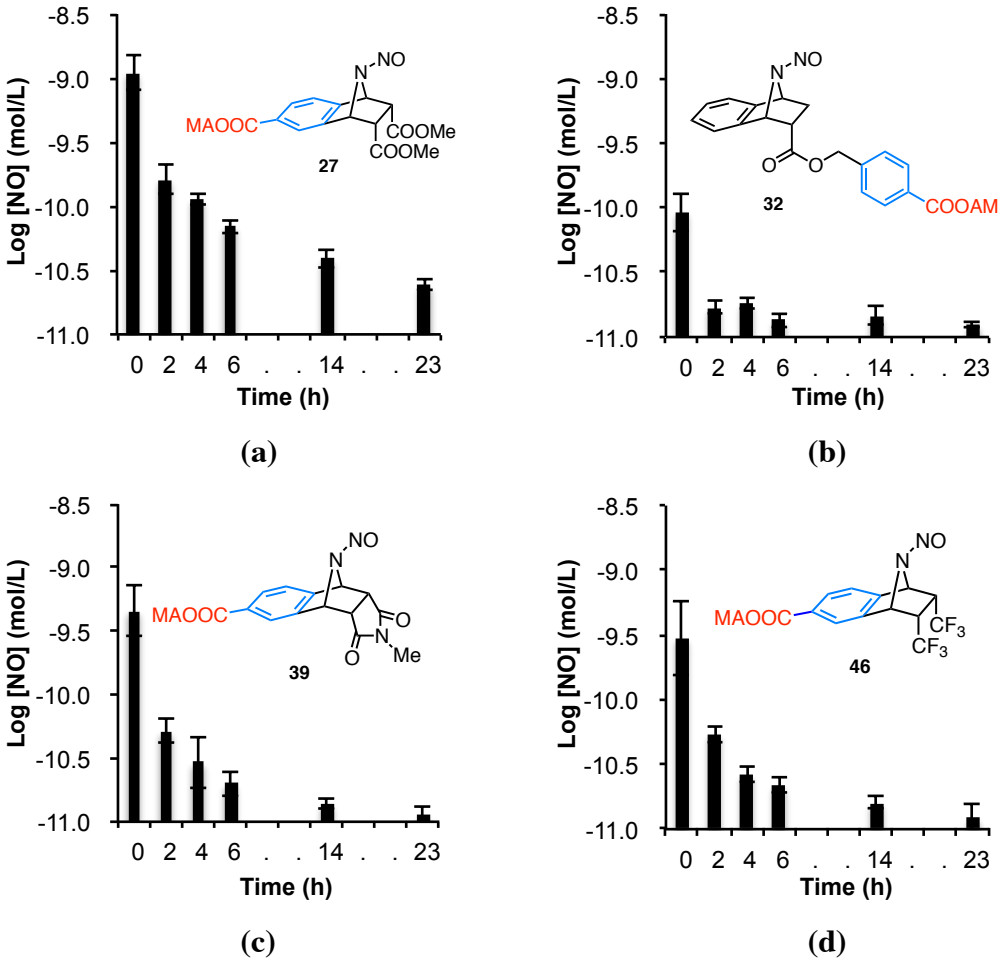
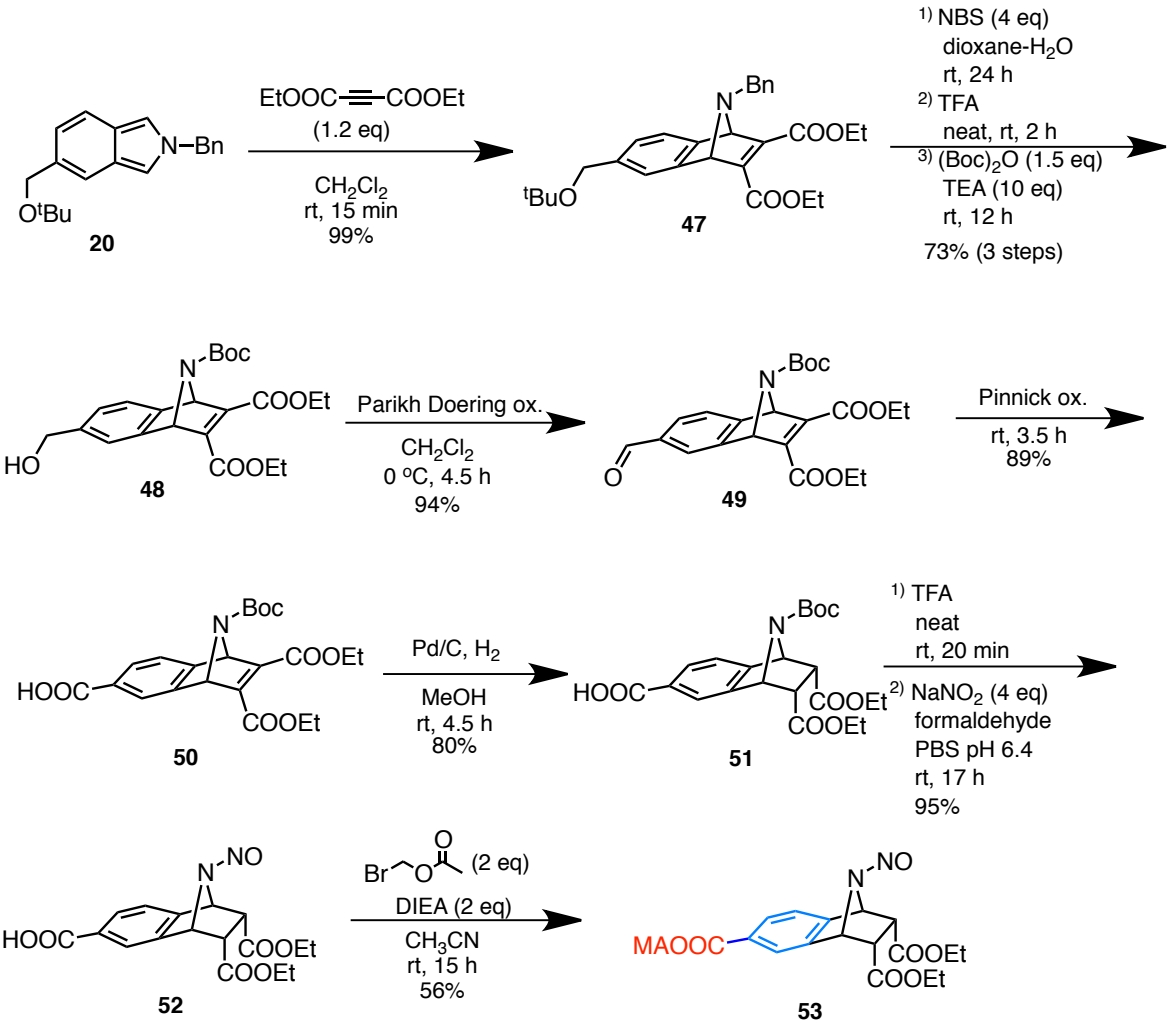


Fig. 2.11. Comparison of NO release from benzene ring substituted AM ester nitrosamine **27 (a)**, **32 (b)**, **39 (c)**, **46 (d)** (100 μM, 1 h incubation, 5 sec irradiation at 440 nm, wash 7 times before each measurement).

2.4.5. Synthesis of Direct Analog Nitrosamine 27

Direct analog of nitrosamine **27** was designed by changing the methyl ester group to ethyl ester group. Preparation of this compound was performed as shown **Scheme 2.10**. Following the same synthetic route of nitrosamine **27** in **Scheme 2.5.** and changing the dienophile from dimethyl acetylenedicarboxylate to diethyl acetylenedicarboxylate, direct analog of nitrosamine **27**, compound **53** can be obtained.



Scheme 2.10 Synthesis of direct analog of **27**, compound **53**.

2.4.6. Comparison of NO Release Efficiency between Nitrosamine 27 and 53 in Cuvette and Piccell

Efficiency of NO release from both nitrosamines **27** and **53** was compared in cuvette with DAF-2 as the NO probe. Both compounds, either as AM ester or carboxylic acid, show relatively similar NO release efficiency in cuvette (**Fig. 2.12. (a)**). However when their NO release in Piccell was studied, they showed very different profile.

As shown in **Fig. 2.12 (b)** and **(c)**, nitrosamine **27** show relatively stable NO release due to the retained compounds in cells. However, NO release from nitrosamine **53** declined rapidly one hour after incubation, which indicated weak retention ability. From this result, it is concluded that benzene ring substituted AM ester's cell retention ability was limited to nitrosamine **27** only and other derivatives did not show the same profile. It is thought that **27** may bind to some protein in Piccell and even after washing, it may retain in cells because of the interaction with the protein. Different from **27**, nitrosamine **53** did not bind to the same protein, after 1 hour and washing applied, most of the nitrosamine was leaked out from Piccell. Therefore it showed lack of cell retention compared to nitrosamine **27**.

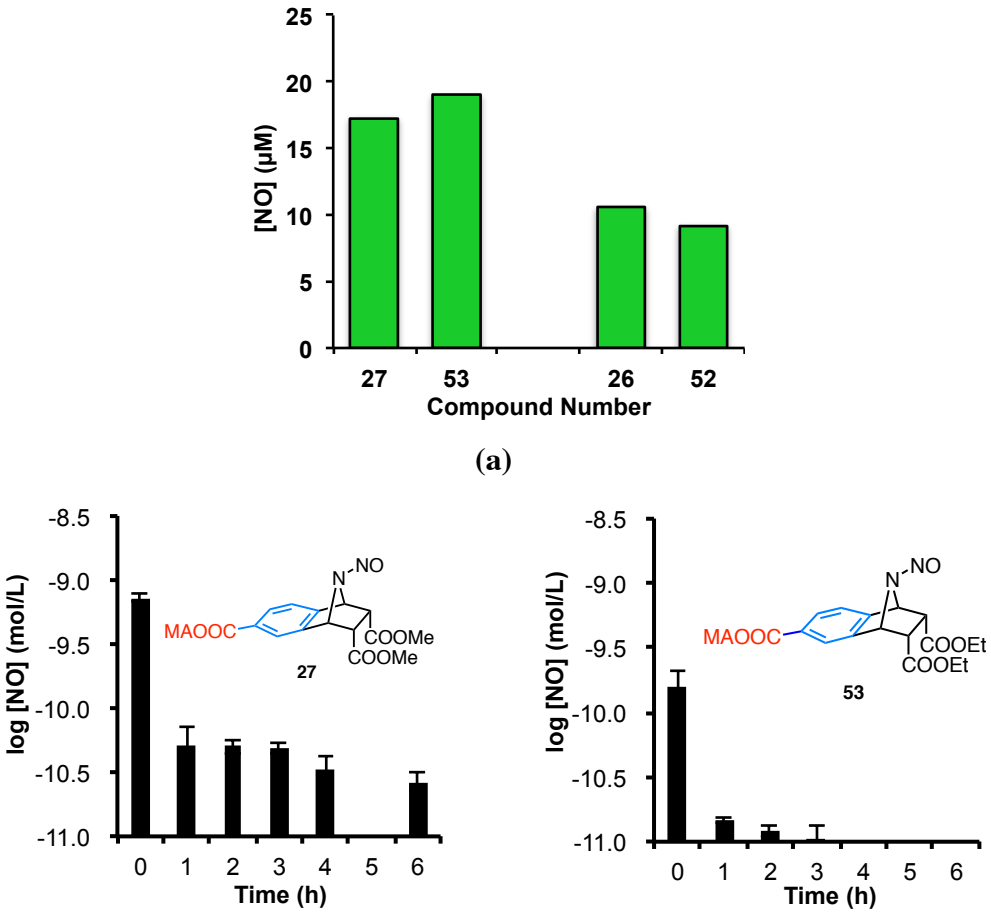


Fig. 2.12 (a). NO release efficiency of **27**, **53** and their related carboxylic acid nitrosamines (0.1 mM) in cuvette with DAF-2 (0.01 mM) as NO probe after irradiation with visible light (420 nm) for 10 mins. Comparison of cell retention profile of nitrosamine **27 (b)** with its analog nitrosamine **53 (c)** in Piccell (10 μM, 1 h incubation) with 5 sec irradiation (440 nm).

Because of the limited application of benzene substituted AM ester to improve cell retention ability, different approach to utilize AM ester as cell retention functional group was adapted and will be discussed in next chapter.

Chapter 3

Achieving High Retainable Nitrosamines

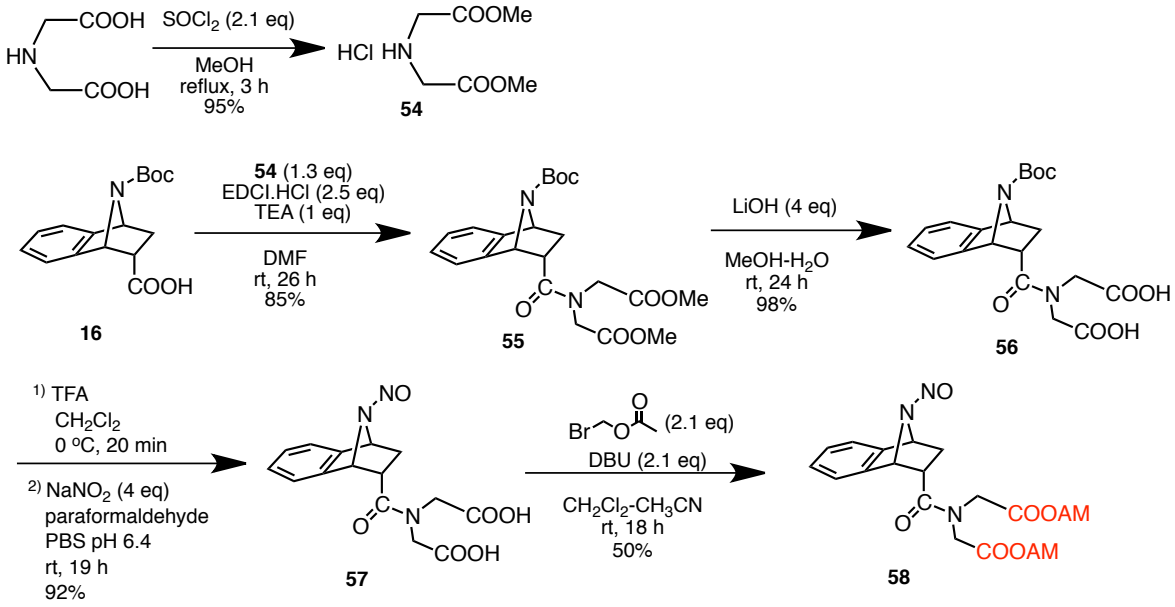
In the previous chapter, study of benzene ring substituted AM ester nitrosamines' cell retention property has been reported. The study was initiated by the founding of nitrosamine **27** as the lead compound with improved cell retention ability after the position of AM ester was changed. However analog of nitrosamine **27** did not show similar cell retention property, therefore it was suspected that application of this finding is very narrow and limited. In this chapter, another approach of AM ester application for increasing cell retention ability will be discussed.

3.1. Increasing AM-ester Numbers as Different Approach

3.1.1. Synthesis of Di-AM ester Nitrosamines

Mono-AM ester nitrosamine **11**, which show slightly improved cell retention ability in **Fig. 2.9.**, was chosen as lead compound. If one AM ester was installed and slightly improved its cell retention ability, it was hypothesized that increasing the number of AM ester will improve its retention in cells. To prove this, nitrosamine with two AM esters was synthesized and it was predicted would give better cell retention property. To facilitate two AM ester installation, previously used direct ester linkage with AM ester was change to amide linkage.

The synthetic route of di-AM ester nitrosamine was shown in **Scheme 3.1**. The synthesis started from esterification of commercially available iminodiacetic acid as methyl ester. With this amine in hand, amide bond was constructed with carboxylic acid **17**, followed by alkaline hydrolysis, TFA deprotection of *N*-Boc, Keefer's nitrosation, and AM ester synthesis to give desired di-AM ester nitrosamine **58**.



Scheme 3.1. Synthesis of di-AM ester nitrosamine **58**.

3.1.2. Comparison Study of Mono- and Di-AM ester Nitrosamines in Piccell

Study of di-AM ester **58** in Piccell showed higher NO release efficiency than mono-AM ester **11**. As the result, Piccell's signal was saturated for the first two hours. From the result in **Fig. 3.1.** about comparison of mono-AM ester and di-AM ester nitrosamine's NO release, di-AM ester **58** still showed decline of NO release from time to time but not as fast as mono-AM ester **11**. Nitrosamine **58** can be retained in Piccell for several hours even though the NO release keeps decreasing.

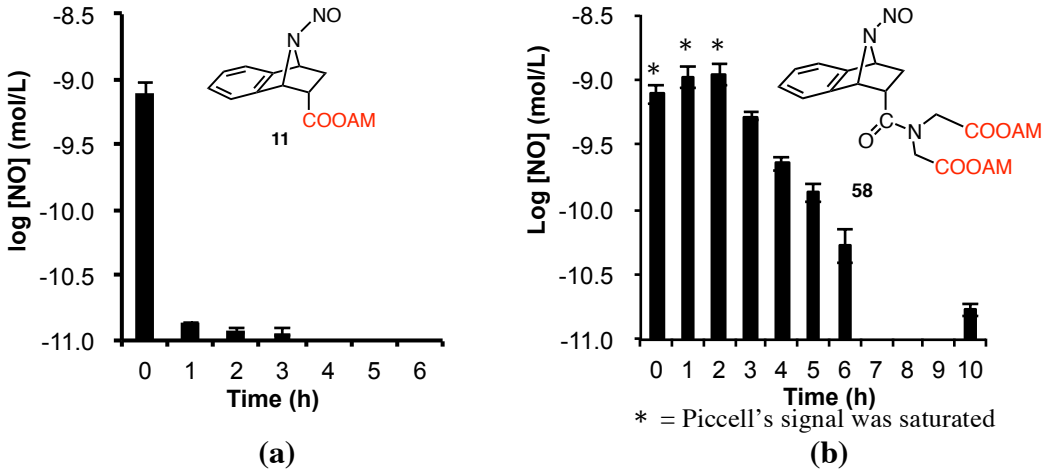


Fig. 3.1 Comparison of nitrosamine leakage between **(a)** nitrosamine **11** and **(b)** nitrosamine **58** in Piccell (10 μM, 1 h incubation) based on NO release upon irradiation (440 nm, 5 sec).

For better comparison between these mono- and di-AM ester nitrosamines, the irradiation's time interval was narrowed from every hour to every eight minutes. With narrower interval, decrease of NO release signal from mono-AM ester **11** can be observed. **Fig. 3.2.** showed that mono-AM ester **11** has higher slope with steep gradient compared to di-AM ester **58**. This result suggest that di-AM ester leakage was slower that the mono-AM ester.

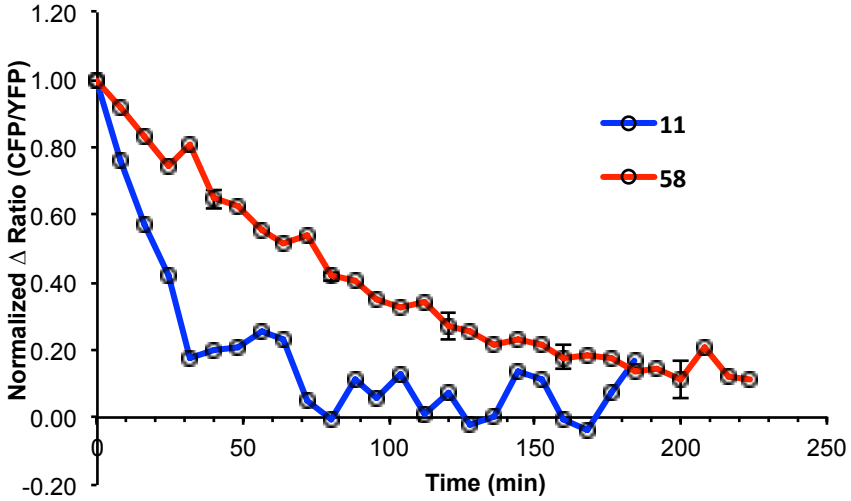


Fig. 3.2 Slope comparison between mono-AM ester **11** and di-AM ester **58** that related to leakage of these nitrosamine from Piccell.

3.1.3. Time Based Study of NO Release from Di-AM ester Nitrosamine in Piccell

To eliminate the possibility of declined-signal was not caused by repeated irradiation, ten minutes (**Fig. 3.3 (a)**) and sixty minutes (**Fig. 3.3 (b)**) interval irradiation was performed. For this experiment, lower concentration was used (1 μM) to prevent Piccell's signal saturation. As the result (**Fig. 3.3 (c)**), overlapped signal of ten minutes and sixty minutes interval shared the same profiles. Therefore, the possibility of declined-signal of NO in Piccell from time to time caused by repeated irradiation can be eliminated and the decline was most likely caused by leakage of nitrosamine from Piccell.

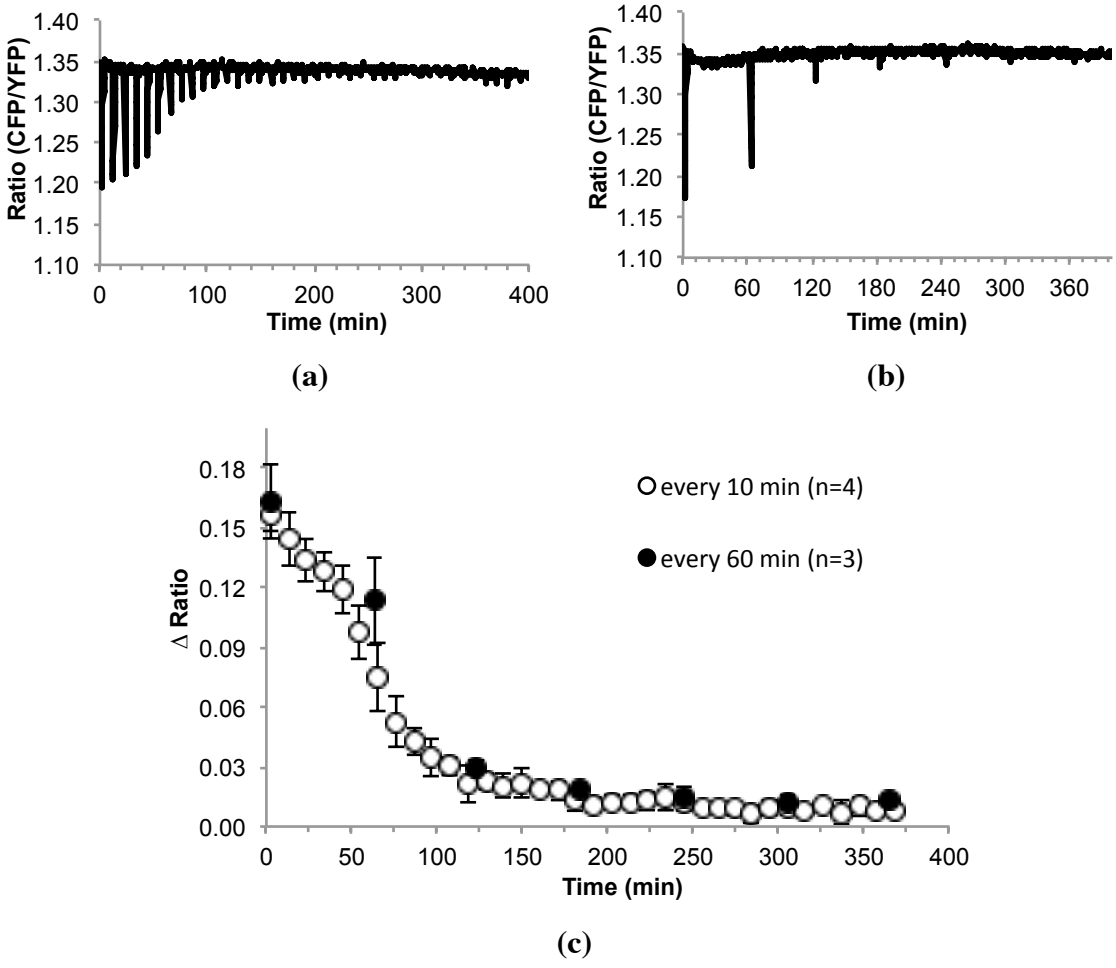


Fig. 3.3. NO release detection from di-AM ester nitrosamine **58** (1 μM, 1 h incubation, 5 sec irradiation at 440 nm) for ten minutes interval **(a)** and sixty minutes interval **(b)** of irradiation. **(c).** Overlapped signal of NO release from di-AM ester nitrosamine **58** with ten minutes and sixty minutes interval of irradiation.

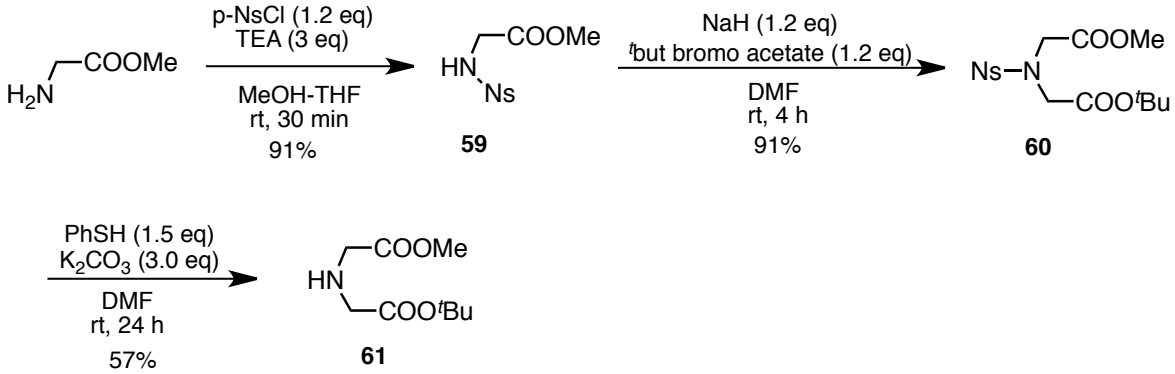
Since increasing the number of AM ester into bicyclic nitrosamine gave promising result to improve its cell retention ability, further study of correlation between number of installed-AM ester and cell retention ability was conducted.

3.2. Development of Right Wing AM ester Nitrosamines

To give better comparison of cell retention ability by different number of AM ester, nitrosamines with various number of AM ester, ranging from one to three, were synthesized. Besides, to minimize effect of functional groups to NO release efficiency, AM ester was linked to the bicyclic system through amide bond formation.

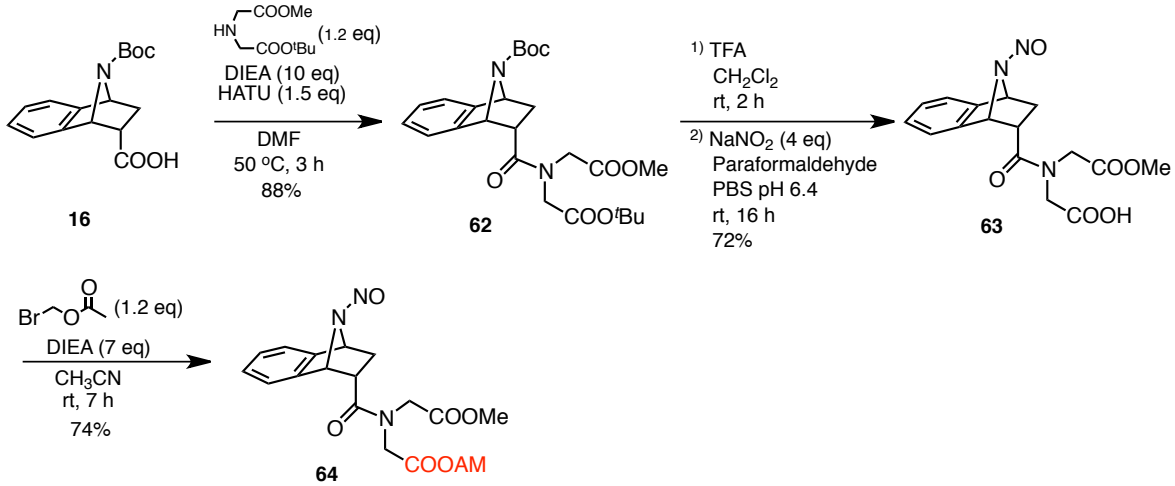
3.2.1. Synthesis of Right Wing Mono-AM ester Nitrosamine

Synthesis of mono-AM ester **64** was started with preparation of the amine **61** to facilitate the AM-ester. Amine **61** can be prepared in three step reactions from commercially available glysine methyl ester. Protection of the amine with nosyl group, followed by *N*-alkylation, and deprotection of nosyl with thiophenol will give desired amine **61** (**Scheme 3.2.**).



Scheme 3.2. Synthesis of amine **61**.

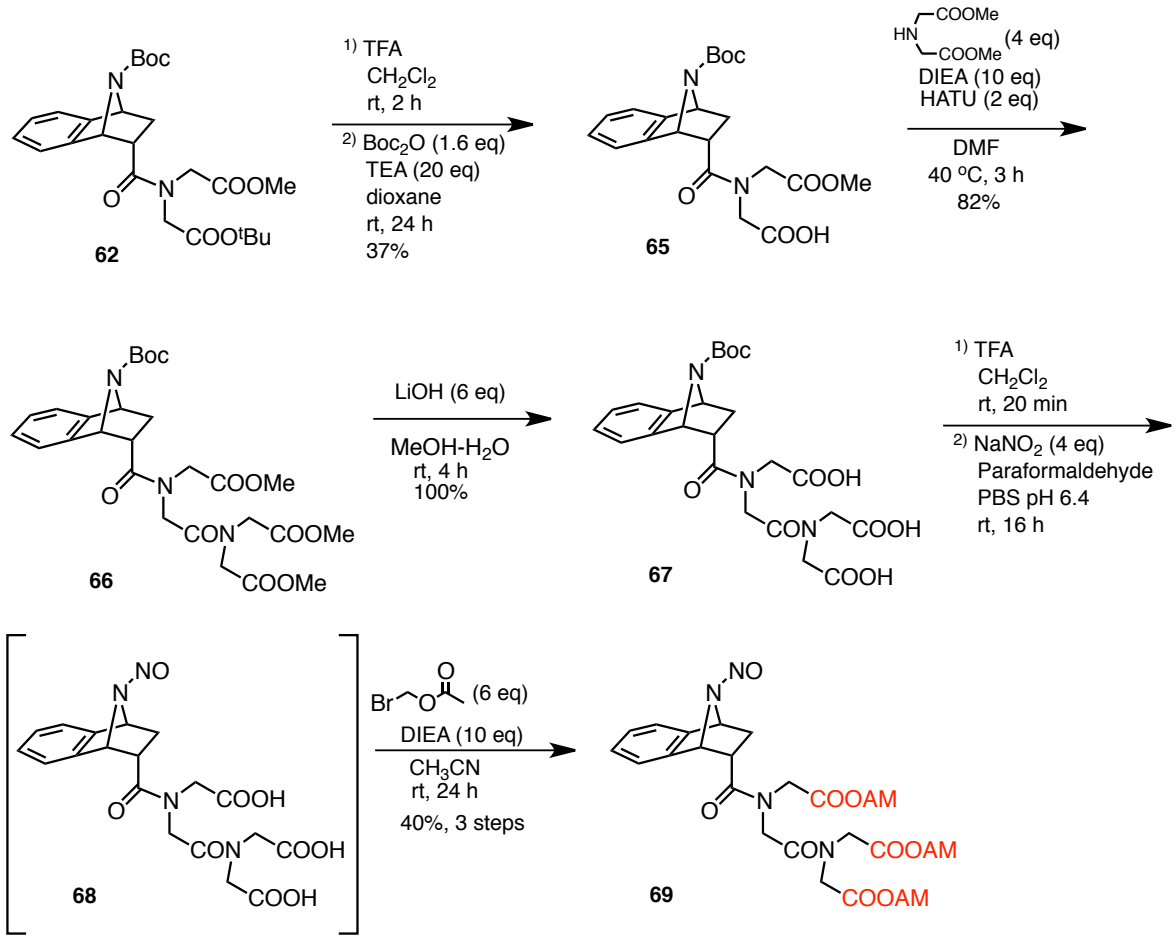
With this amine **61** in hand, amide bond formation with carboxylic acid **17** was performed with HATU as coupling reagent. Selective cleavage of *tert*-butyl ester and *N*-Boc deprotection by TFA, followed by Keefer's nitrosation and AM ester synthesis will give new mono-AM ester nitrosamine **64 (Scheme 3.3.)**.



Scheme 3.3. Synthesis of right wing mono-AM ester nitrosamine **64**.

3.2.2. Synthesis of Right Wing Tri-AM ester Nitrosamine

Utilizing the intermediate compound **62** in **Scheme 3.3.**, tri-AM ester can be synthesized as shown in **Scheme 3.4**. Selective hydrolysis of *tert*-butyl ester by TFA and re-protection of the amine with Boc will give compound **65**. Coupling with amine **54** to form another amide bond, which resulted in generation of three esters functionality and may facilitate three AM ester. Alkaline hydrolysis of **66**, followed by TFA deprotection, Keefer's nitrosation, and AM ester synthesis will give tri-AM ester nitrosamine **69** (**Scheme 3.4**).



Scheme 3.4. Synthesis of right wing tri-AM ester nitrosamine **69**.

3.2.3. Comparison of NO Release from Right Wing AM ester Nitrosamines in Cuvette and Piccell

With this, right wing mono-, di-, tri-AM ester nitrosamines were obtained. Study of their NO release in cuvette showed that functional group's effect in NO release was minimized and these compounds have relatively almost same NO release ability (**Fig. 3.4.**).

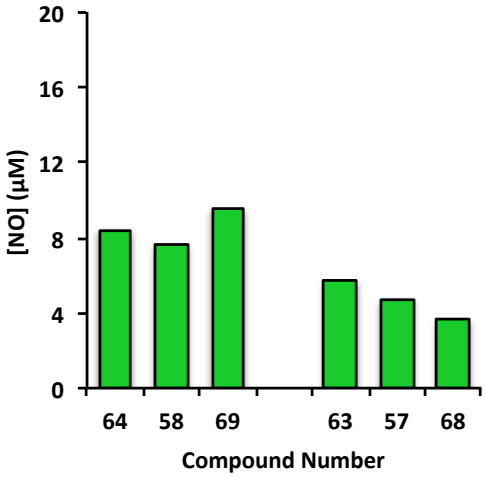


Fig. 3.4. NO release efficiency of right wing mono-, di-, tri- AM ester, **64**, **58** and **69** (0.1 mM) as AM ester and related carboxylic acid, respectively, in cuvette with DAF-2 (0.01 mM) as NO probe after irradiation with visible light (420 nm) for 10 mins.

Study of right wing mono-, di-, tri-AM ester nitrosamine's NO release in Piccell was shown in **Fig. 3.5**. Comparing the amount of NO release after irradiation, the result suggested that right wing tri-AM ester nitrosamine **69** showed relatively stable NO release for several hours, compared to its di-AM ester **58**, and mono-AM ester **64**. In tri-AM ester nitrosamine **69**, it was highly retained in cells that may have constant NO release for several hours (90±19.8 pM up to 5 hours after incubation and washed).

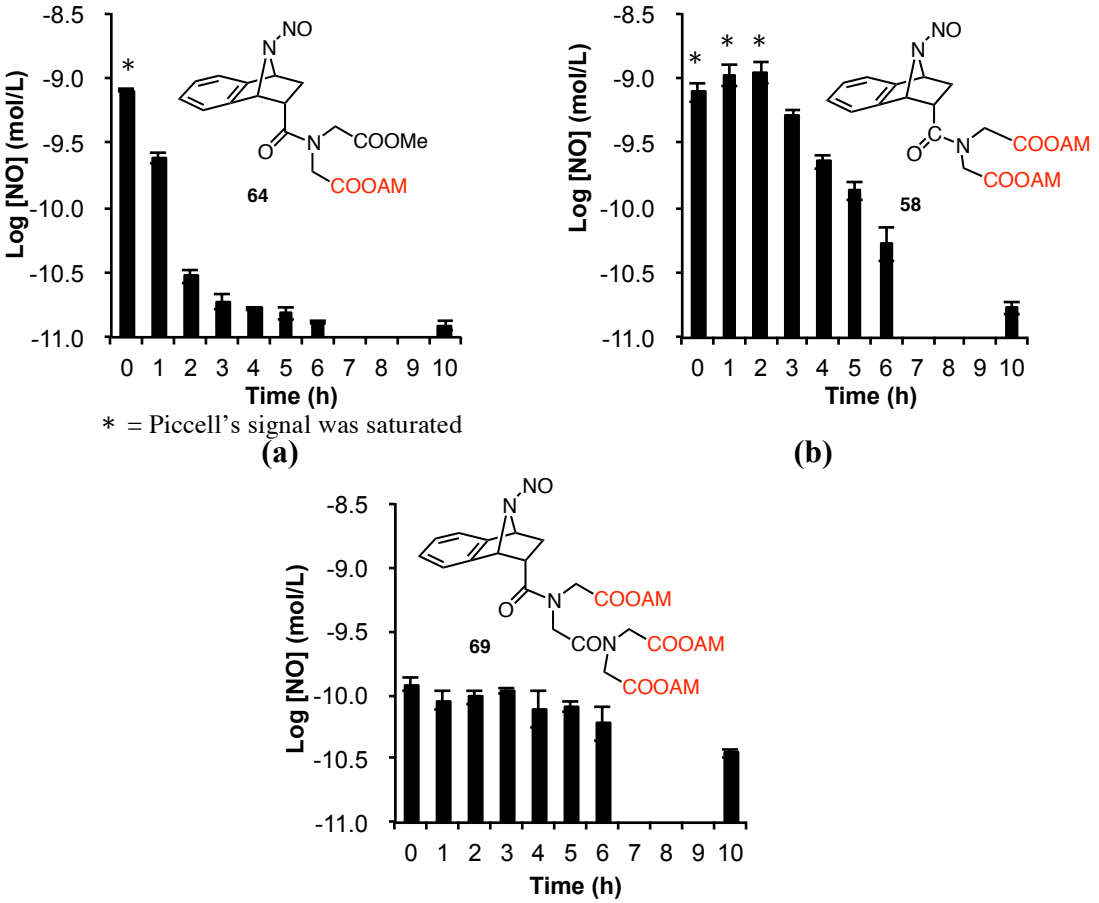


Fig. 3.5. NO release efficiency of right wing mono-, di-, tri- AM ester nitrosamines **64**, **58**, and **69** (10 μM, 1 h incubation with 5 sec irradiation (440 nm) and washed 7 times before each measurement), respectively in Piccell.

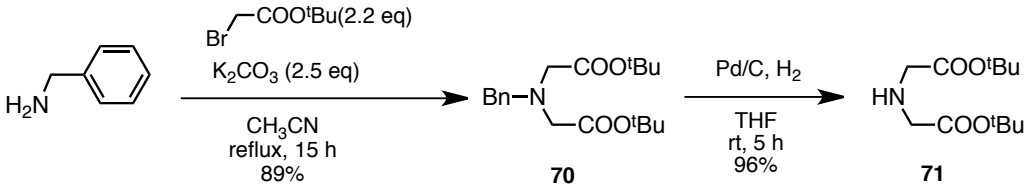
However, even though tri-AM ester nitrosamine **69** showed stable NO release, its NO release concentration was lower than the mono-, and di-AM ester nitrosamines. According to their NO release ability in cuvette, which showed relatively same efficiency, their NO release in Piccell supposed to be same as well. It is suspected that their cell penetration ability was different and their concentrations in Piccell were not the same. Differ in concentrations may lead to different amount of NO release when irradiation was applied, as for higher concentration will release more NO. Therefore tri-AM ester **69** due to lack of cell penetration, its NO release in Piccell also lower than mono- and di-AM ester nitrosamine **64** and **58**.

3.3. Verification of Increased AM Ester Numbers Strategy

Based on previous result in **Fig. 2.10.**, if the AM ester functional group was moved from right wing to left wing of the bicyclic ring system, different profile of cell retention was observed. Because of this reason, to verify the cell retention ability of tri-AM ester compounds, these AM esters were moved from right wing to left wing of bicyclic ring system. Besides, it also has been reported that right wing position is important position that may control the NO release efficiency, as different functional groups may showed different NO release efficiency.[33] Because of these reasons, left wing AM ester nitrosamines, ranging from one to three AM esters, were also synthesized to verify the applicability of tri-AM ester and to improve the NO release efficiency.

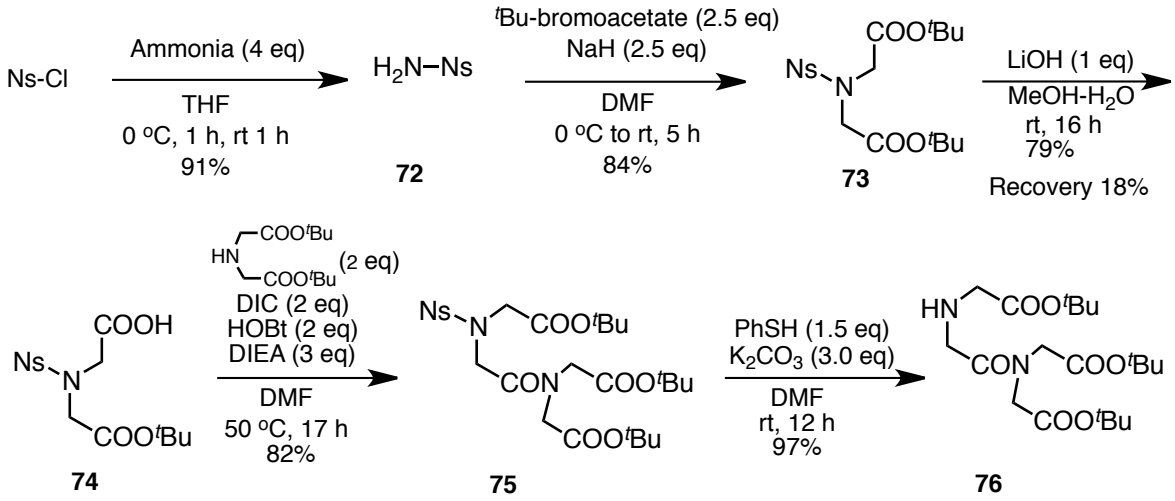
3.3.1. Synthesis of Left Wing Mono-AM ester Nitrosamine

The same strategy was applied to synthesize left wing AM ester nitrosamines, which amide bond was used to facilitate AM ester installation. The amine for mono-AM ester, amine **61**, was prepared as shown in **Scheme 3.2**. Amine for di- and tri-AM ester was prepared through **Scheme 3.5.** and **Scheme 3.6**. Benzyl amine was used as starting material and after *N*-alkylation followed by Palladium-Carbon hydrogenation for *N*-benzyl deprotection, amine **71** was obtained.



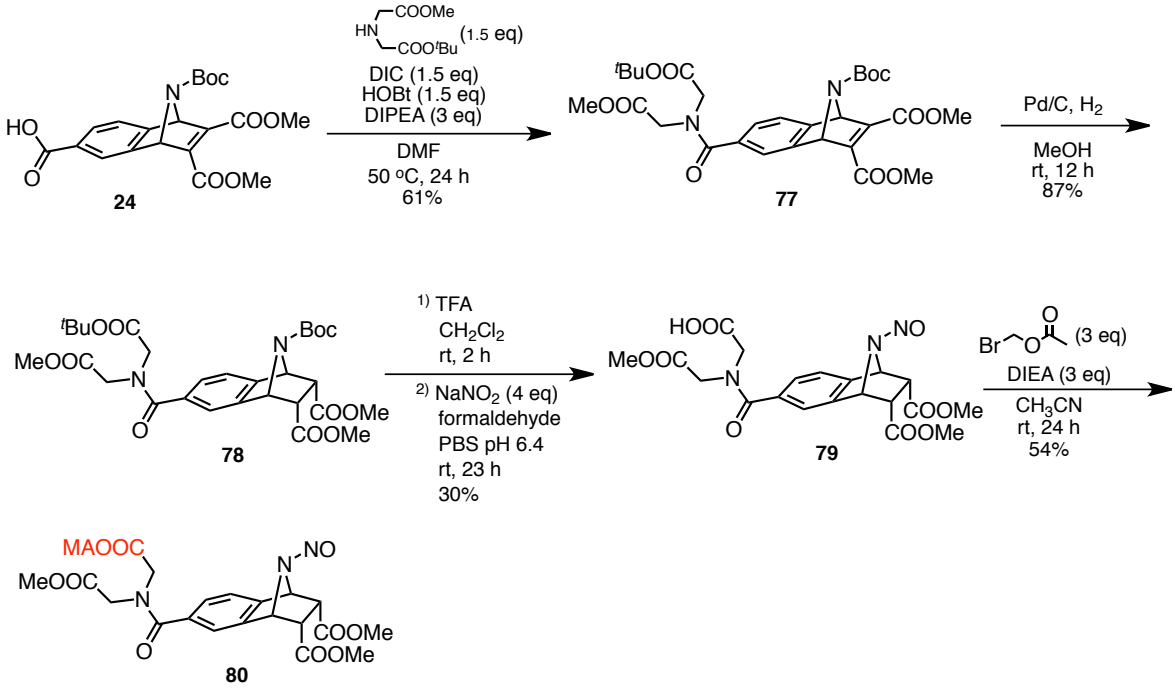
Scheme 3.5. Synthesis of amine **71** for di-AM ester nitrosamine.

For tri-AM ester nitrosamine, amine with three *tert*-butyl esters was synthesized. The reaction started from protecting ammonia as *N*-Nosyl followed by *N*-alkylation to give **73**. Alkaline hydrolysis of one ester with only 1 eq. of base, mono carboxylic acid **74** can be obtained. Coupling of this carboxylic acid with amine **71** followed by deprotection of *N*-Nosyl with thiophenol will give desired amine **76**.



Scheme 3.6. Synthesis of amine **76** for tri-AM ester nitrosamine.

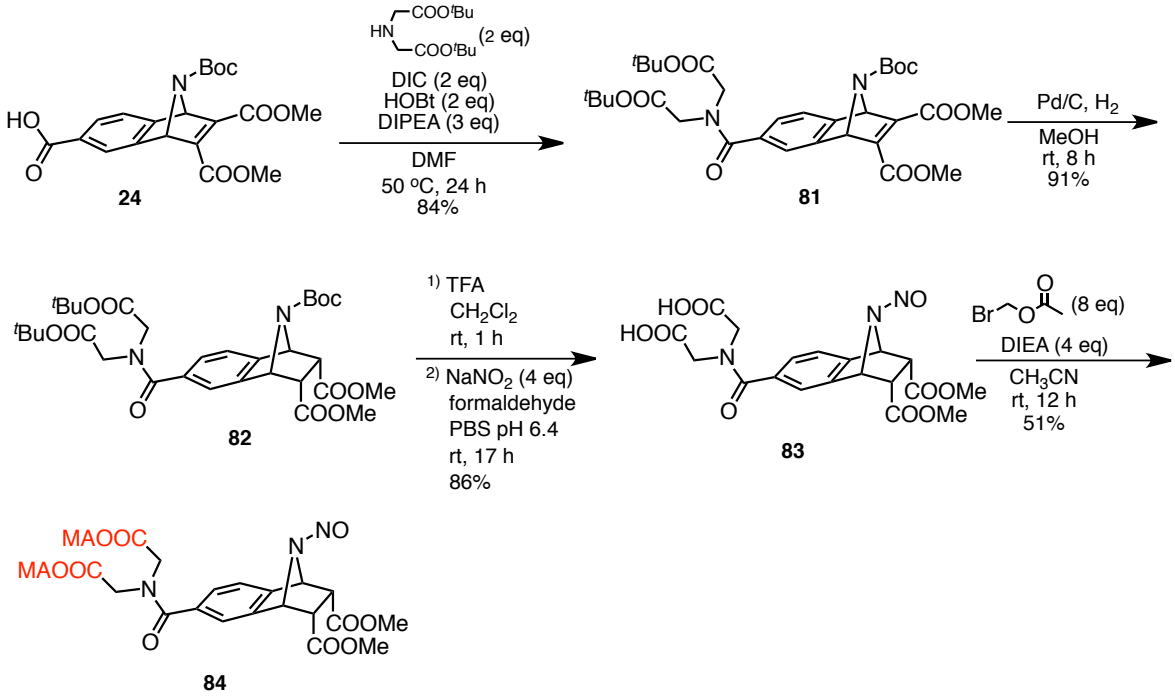
With these three types of amines for mono-, di-, and tri-AM ester nitrosamines can be synthesized. Synthesis of left wing mono-AM ester was completed based on the **Scheme 3.7**. Coupling of previously synthesized carboxylic acid **24** in **Scheme 2.5** with amine **61**, followed by Palladium/carbon hydrogenation, TFA deprotection of *N*-Boc and *tert*-butyl ester, Keefer's nitrosation and AM ester synthesis, right wing mono-AM ester nitrosamine **80** can be obtained.



Scheme 3.7. Synthesis of left wing mono-AM ester nitrosamine **80**.

3.3.2. Synthesis of Left Wing Di-AM ester Nitrosamine

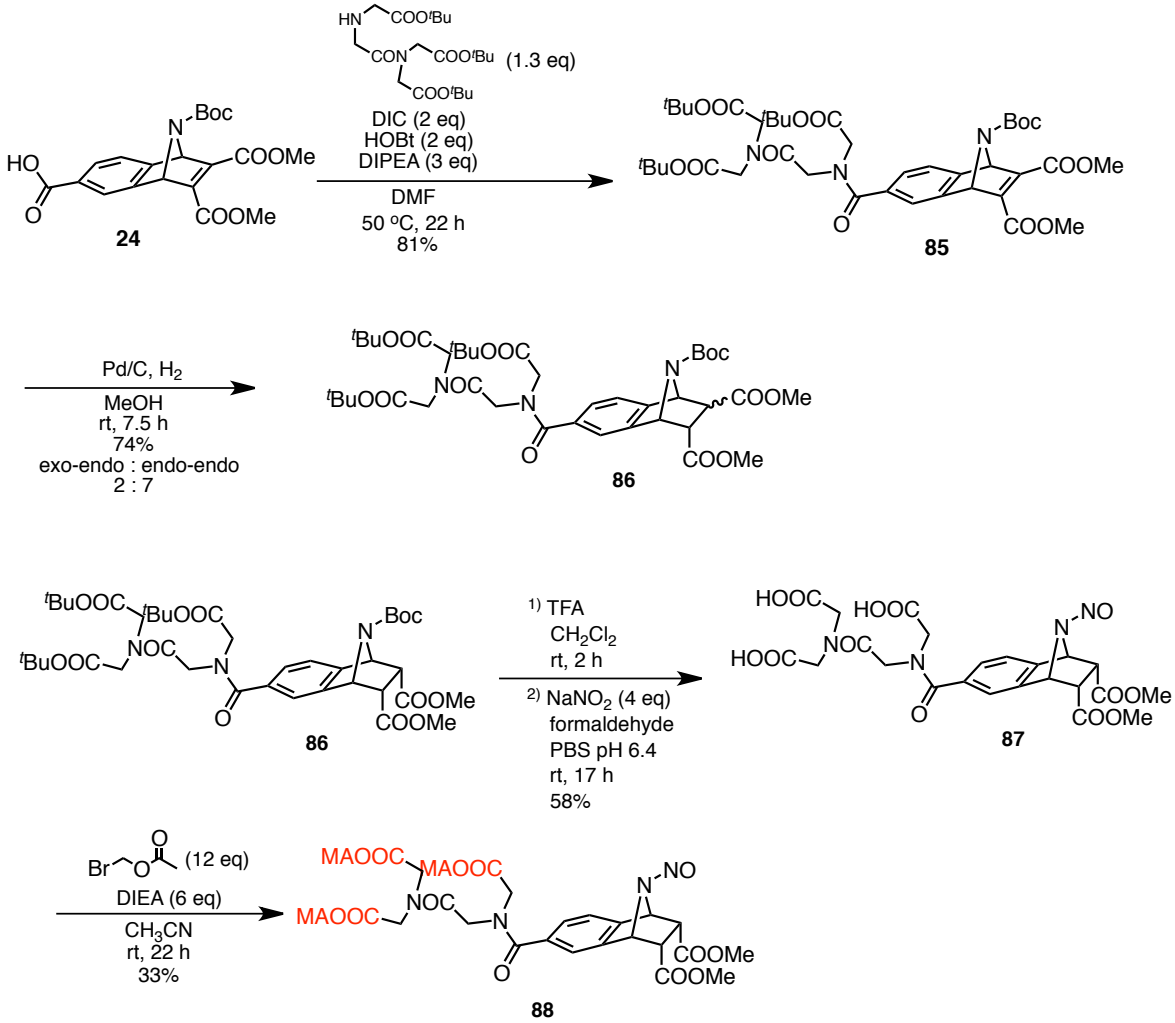
Synthesis of left wing di-AM ester nitrosamine was also performed with the same strategy. Amidation of carboxylic acid **24** with amine **71** will give amide **81**. Hydrogenation, TFA deprotection, nitrosation and AM ester synthesis will give left wing di-AM ester nitrosamine **84** (**Scheme 3.8.**).



Scheme 3.8. Synthesis of left wing di-AM ester nitrsoamine **84**.

3.3.3. Synthesis of Left Wing Tri-AM ester Nitrosamine

At last, applying the same strategy tri-AM ester nitrosamine **88** can be synthesized. In this process, Palladium/carbon hydrogenation gave *exo-endo* by-product with ratio 2:7, compared to the main *endo-endo* desired product. After separation, only the *endo-endo* **86** was used for the next step synthesis to synthesize tri-AM ester nitrosamines **88** as shown in **Scheme 3.9**.



Scheme 3.9. Synthesis of left wing tri-AM ester nitrosamine **88**.

3.3.4. Comparison of NO Release from Left Wing AM ester Nitrosamines in Cuvette and Piccell

In left wing AM ester nitrosamines, functional group effect in NO release was also minimized as their NO release efficiency did not change significantly regardless of the number of AM esters functional group. In cuvette, their NO release efficiency was almost the same among each other (**Fig. 3.6.**) and demonstrated higher efficiency than right wing AM ester nitrosamines.

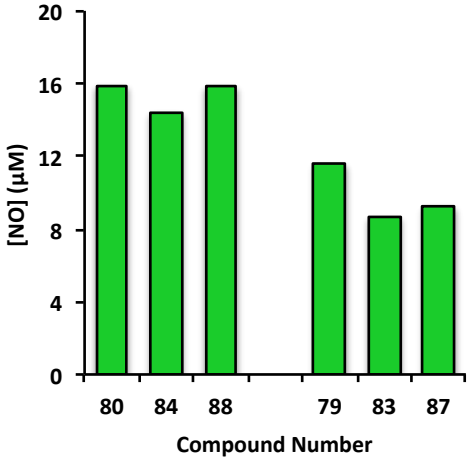


Fig. 3.6. NO release efficiency of left wing mono-, di-, tri- AM ester, **80**, **84** and **88** (0.1 mM) as AM ester and related carboxylic acid, respectively, in cuvette with DAF-2 (0.01 mM) as NO probe after irradiation with visible light (420 nm) for 10 mins.

NO release study of these left wing AM ester nitrosamines in Piccell was shown in **Fig. 3.7**. Overall, left wing and right wing AM ester nitrosamines shared the same trend, which as the number of AM esters was increased, its NO release became more stable. Rapid decline of NO release from mono-AM ester was still observed. Di-AM ester nitrosamine also showed continuous decline of NO release even though not as fast as mono-AM ester nitrosamine. Finally, tri-AM ester showed stable NO release and may release around 18±1.4 pM up to 3 hour after incubation and washed.

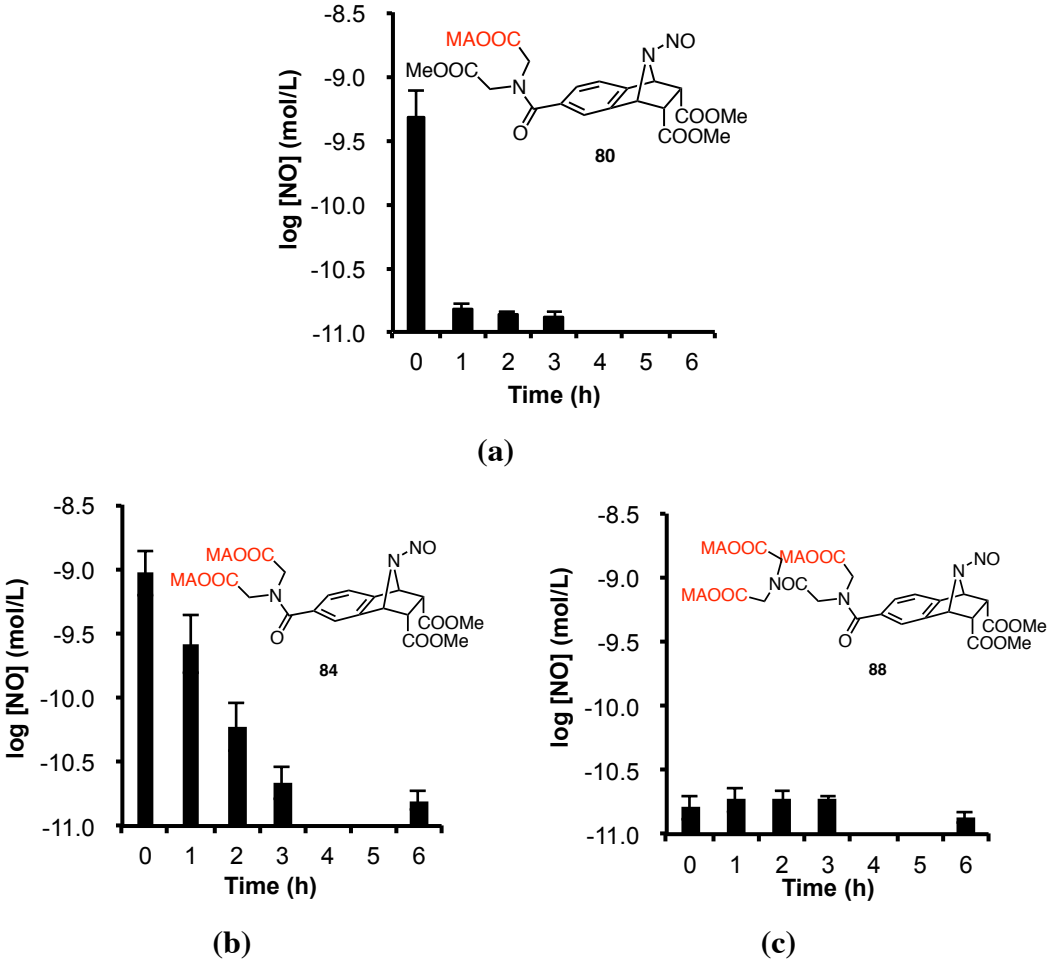


Fig. 3.7. NO release efficiency of left wing mono-, di-, tri- AM ester nitrosamines **80**, **84**, and **88** (10 μM, 1 h incubation with 5 sec irradiation (440 nm) and washed 7 times before each measurement), respectively in Piccell.

3.4. Rationalization of Right-Wing and Left-Wing AM ester Nitrosamines Results

Both left wing and right wing AM ester nitrosamines showed similarities and differences in their cell retention profiles. Various factors that may affect their cell retention profiles will be discussed.

3.4.1. Cell Penetration Difference Possibility

Comparison of Mono-, Di-, and Tri-AM ester Nitrosamines

One critical factor that may affect Piccell's signal is the amount of nitrosamines in cells. Concentration of nitrosamines in cells is depend on their ability to permeate through cell membrane, as highly permeable compound will lead to high concentration of nitrosamines in cells, thus upon irradiation, higher NO release concentration will be detected by Piccell.

Both right wing and left wing AM ester nitrosamines showed that mono- and di-AM ester nitrosamines' NO release at time zero was relatively high, but for tri-AM ester nitrosamine, the initial NO release was dropped significantly. It is suspected that nitrosamines with tri-AM ester have relatively low cell permeability compared to mono- and di-AM ester nitrosamines. If their cLogP was compared, there is a significant decrease of cLogP value in tri-AM ester nitrosamines. LogP is related to hydrophobicity-hydrophilicity of a compound, as low LogP value indicates highly hydrophilic property.

Calculated LogP (cLogP) values for right wing mono-, di-, and tri-AM ester nitrosamines are -0.05, -0.06, -1.45, respectively. In the mean while cLogP values for left wing mono-, di- and tri-AM ester nitrosamines are -0.66, -0.67, -2.07, respectively. cLogP value for mono-and di-AM ester nitrosamines were about the same values, which indicated that they have the same hydrophobicity. It is observed that mono- and di-AM ester nitrosamines' NO release in Piccell at time zero was about the same level to support this argument.

It is very obvious that cLogP values for right wing tri-AM ester nitrosamine was dropped from -0.06 to -1.45 and for left wing tri-AM ester nitrosamines was -0.67 to -2.07. These indicated that the polarity was significantly increased as the number of AM ester was increased to three. Because of higher polarity of tri-AM ester nitrosamines, it was more difficult to penetrate into cell and caused low concentration of nitrosamines in Piccell, which may affect their NO release efficiency upon irradiation. Therefore, due to

the change of cLogP of tri-AM ester nitrosamines, the amount of nitrosamines in cell was not as high as mono- and di-AM ester and leads to lower signal of NO release in Piccell.

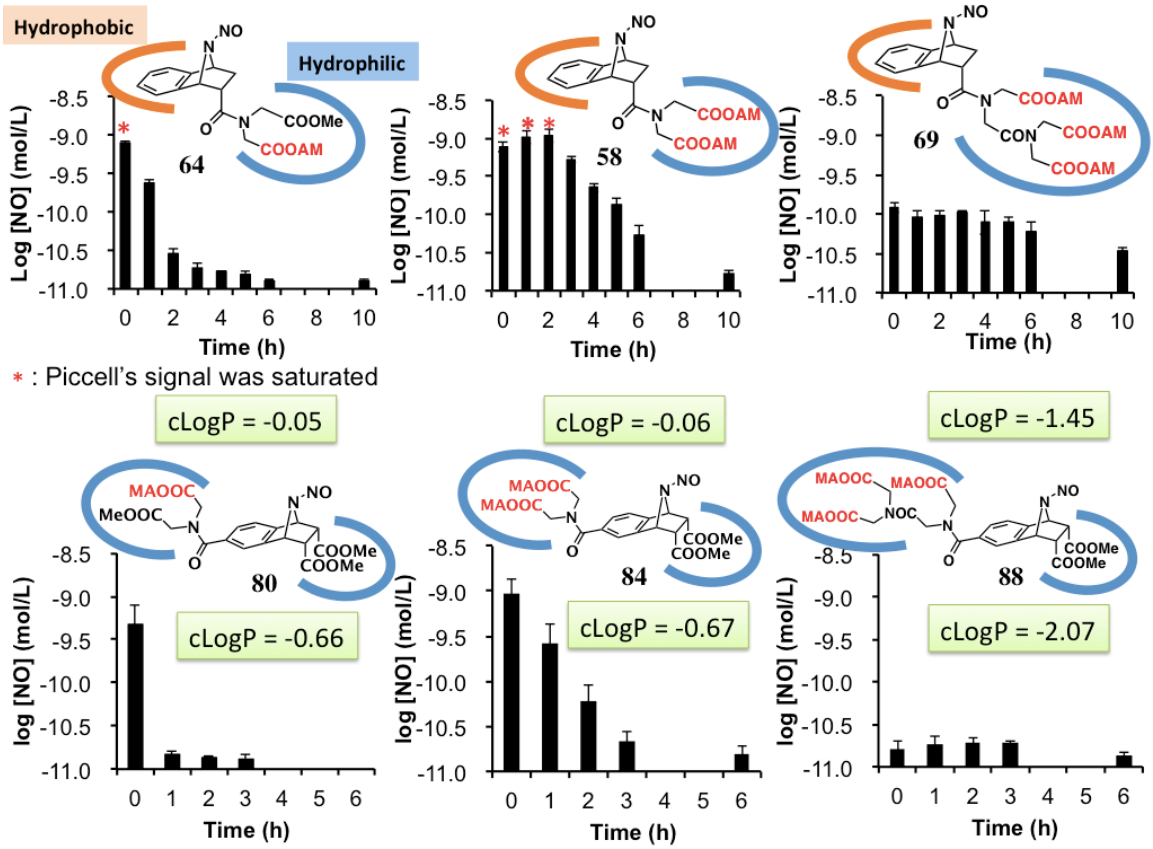


Fig. 3.8. Comparison of cLogP among right wing and left wing AM ester nitrosamines.

Comparison of Right Wing and Left Wing AM ester Nitrosamines

NO release efficiency in cuvette showed that relatively no significant difference in left wing and right wing AM ester nitrosamines series, which left wing AM ester nitrosamines series have higher NO release efficiency due to presence of two electron withdrawing methyl ester substituents (**Fig. 3.8.**). However, NO release efficiency in Piccell showed opposite result, which left wing AM ester nitrosamines have lower NO release efficiency.

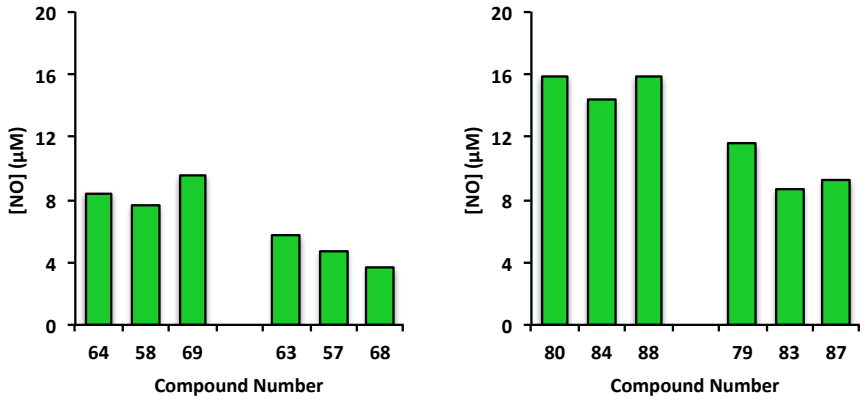


Fig. 3.9. Comparison of right wing **(a)** and left wing **(b)** AM ester nitrosamines' NO release in cuvette and their related carboxylic acid nitrosamines.

When cLogP values of nitrosamines with same number of AM ester were compared (-0.05 vs -0.66, -0.06 vs -0.67, and -1.45 vs -2.07 for right wing vs left wing of mono-, di-, and tri-AM ester, respectively), there is a common pattern, which left wing AM ester nitrosamines have smaller cLogP values than right wing AM ester nitrosamines. This indicated that left wing AM ester nitrosamines are more hydrophilic than right wing AM ester nitrosamines. It was probably caused by the hydrophobicity of benzene ring was cloaked by the hydrophilic AM ester functional group in left wing AM ester nitrosamines, especially tri-AM ester nitrosamines.

On the other hand, right wing AM ester nitrosamines' AM esters were in the right wing side of the bicyclic system and uncloaked benzene ring on the left wing side as the hydrophobic side, which may play some role in cell penetration to pass through the hydrophobic lipid bilayer cell membrane. Because of this, right wing AM ester nitrosamines may have better cell penetration than left wing AM ester nitrosamines and showed higher NO release performance. At the end, it is suggested to quantify the amount of each nitrosamine in cells right after incubation to provide a proof for the cell penetration difference of each nitrosamine.

3.4.2. Leakage Rate Difference Possibility

Rationalization of the leakage rate among mono-, di- and tri-AM ester nitrosamines can be explain through the cLogP values as well. It is generally stated that as AM ester will be hydrolyzed in cells by esterase to carboxylic anion that cannot permeate out from cell membrane. As shown in **Table 3.1.**, as number of carboxylic anion was increased, the hydrophobicity was decreased. Therefore, for mono-AM ester nitrosamines with higher cLogP values, they will leak out faster than di- and tri-AM ester nitrosamines, as results shown in Piccell.

Table 3.1. cLogP values of mono-, di-, and tri-carboxylic anion of left wing and right wing AM ester nitrosamines.

	Mono-AM ester	Di-AM ester	Tri-AM ester
Left wing AM ester	-2.7595	-3.5155	-3.7085
Right wing AM ester	-2.0610	-2.7259	-2.9304

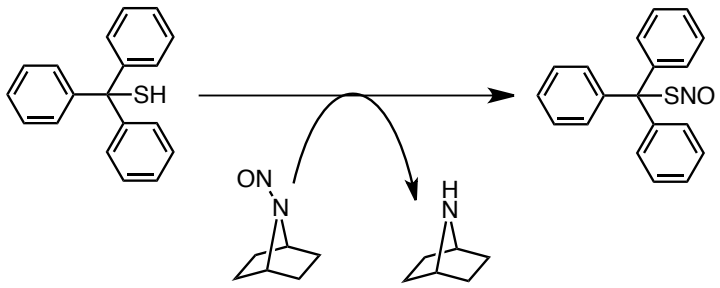
Besides, these cLogP values are also smaller than cLogP values in the AM ester state, which mean that these carboxylic anion nitrosamines cannot permeate through cell membrane as freely as the AM ester nitrosamines. As a result, these nitrosamines were trapped in cells.

3.4.3. Hydrolysis Rate Difference Possibility

It has been mentioned that AM ester and its carboxylic anion has different cLogP values. If nitrosamines enter cells as AM ester and was not hydrolyzed completely, there is a possibility it may permeate out from the cell as partially hydrolyzed nitrosamines. As the result, the final concentration of nitrosamine in cells is lower than expected; especially for nitrosamines with more AM ester functional group. Although Tsien has reported that his Ca^{2+} probe with AM ester functional group can be hydrolyzed in cells ~90% yield within 2 hours incubation, if the hydrolysis rate is different among mono-, di-, and tri-AM ester nitrosamines and they were not completely hydrolized, partially hydrolyzed tri-AM ester nitrosamines have a possibility to permeate out from cells and lead to low signal of NO detection by Piccell.

3.5. Possibility of *S*-transnitrosylation of Bicyclic Nitrosamines to Thiol

Nitric oxide may have direct protein functionalization or indirect activation through *S*-nitrosylation in which transfer of NO from *S*-nitrosylated species, such as GSNO, to target protein. Some of these bicyclic nitrosamines have been reported to have the ability to transfer the NO, from *N*-NO to the thiol species in cells, such as cysteine residue in protein, and showed biological activity. The ability of nitrosamines to transfer its NO to other species was called as trans-nitrosylation. This process can occurred naturally without any triggers to provoke the NO release. Bicyclic nitrosamines that was reported showed this trans-nitrosylation capability in cells, also demonstrated their trans-nitrosylation in cell free condition (in cuvette) with triphenylmethanethiol as the thiol source.[34, 35] As a model study, trans-nitrosylation in cuvette was performed with triphenylmethanethiol as thiol source because generated RSNOs are usually not stable especially for primary and secondary thiol.



Scheme 3.10. Trans-nitrosylation model study of bicyclic nitrosamines with triphenylmethanethiol in cuvette.

Nitrosylated-thiol (RSNO) has three characteristic bands that can be observed in UV-vis spectra: two intense bands in UV region and one weak band in the visible region. The first characteristic is at 225-261 nm region ($\varepsilon \sim 10^4$ $M^{-1}cm^{-1}$) which is attributed to the $\pi \rightarrow \pi^*$ transition. The second characteristic is 330-350 nm region ($\varepsilon \sim 10^3$ $M^{-1}cm^{-1}$) which is attributed to the $n_O \rightarrow \pi^*$ transition. The third band is in the 550-600 nm region ($\varepsilon \sim 20$ $M^{-1}cm^{-1}$) which is attributed to the $n_N \rightarrow \pi^*$ transition.[36] Based on these specific characterizations of RSNO, whether trans-nitrosylation of bicyclic nitrosamines occurred in cuvette can be distinguished.

Since AM ester nitrosamines will be hydrolyzed to carboxylic anion in cells, study of trans-nitrosylation in cuvette was conducted with their carboxylic acid instead of the AM ester itself. Mixture of right wing tri-carboxylic acid **68** with Ph_3CSH at 37 °C for 6 hours, no significant increase of absorbance was observed around 350 nm or 600 nm. This indicated that trans-nitrosylation did not occur (**Fig. 3.10.**).

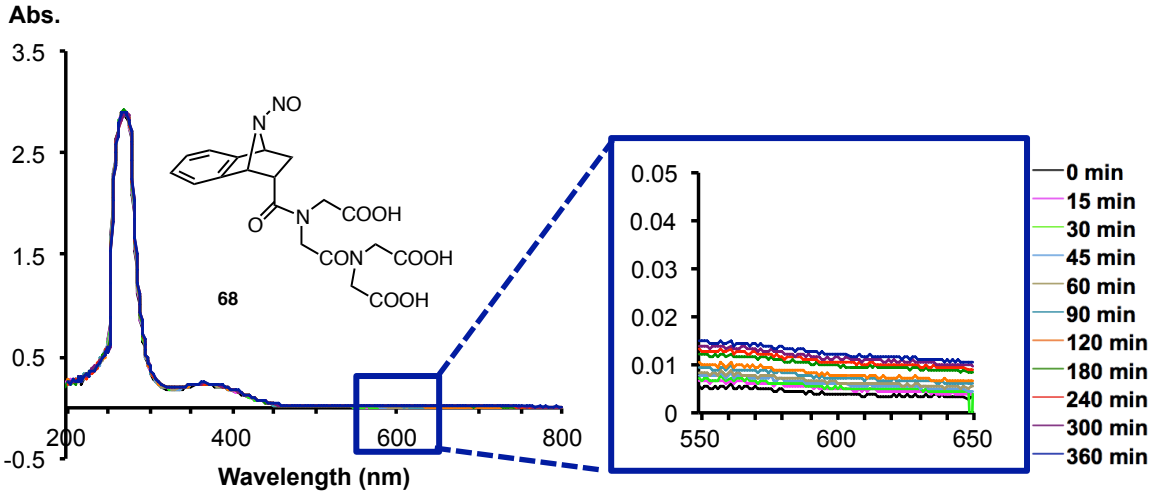


Fig. 3.10. UV-vis spectra of right wing tri-carboxylic acid nitrosamine **68** (3 mM) and triphenylmethanethiol (3 mM) in mixture of DMSO-$CHCl_3$ (1:4) without irradiation for 6 hours at 37 °C.

However, when irradiation was applied to trigger NO release from the nitrosamine, RSNO's characteristic band was observed. Significant increase of absorbance was detected around 350 nm and 600 nm (**Fig. 3.11.**). This result suggested that in the presence of NO, *S*-nitrosylation can occur naturally, but without irradiation to trigger the NO release, trans-nitrosylation will not occur.

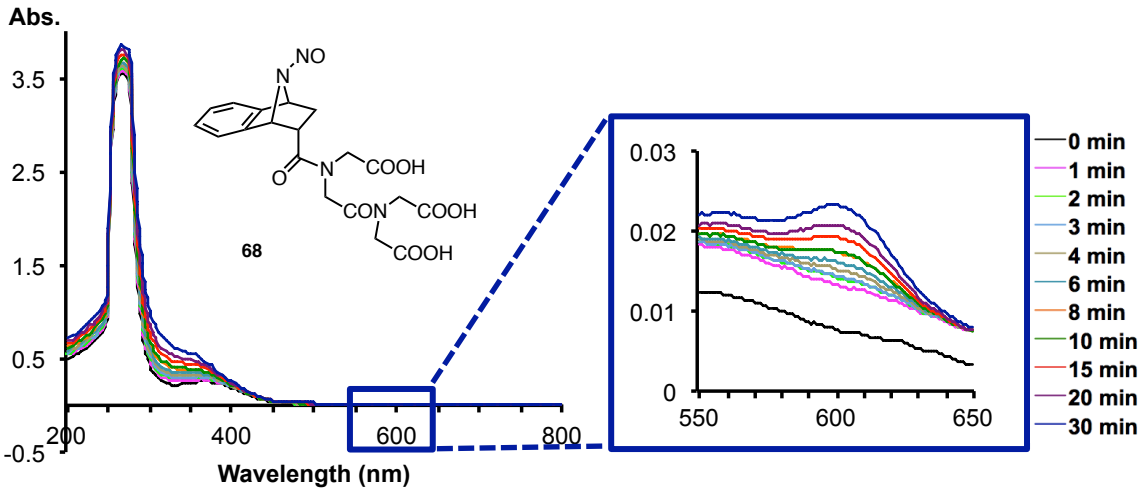


Fig. 3.11. UV-vis spectra of right wing tri-carboxylic acid nitrosamine **68** (3 mM) and triphenylmethanethiol (3 mM) in mixture of DMSO-$CHCl_3$ (1:4) with irradiation (420 nm) up to 30 minutes at room temperature.

Left wing tri-carboxylic acid nitrosamine **87** also showed the same result when mixed with Ph_3CSH. Trans-nitrosylation was not observed after 6 hours monitoring at 37 ºC (**Fig. 3.12.**), but once irradiation was applied, *S*-nitrosylation occurred and the characteristic bands can be observed (**Fig. 3.13.**).

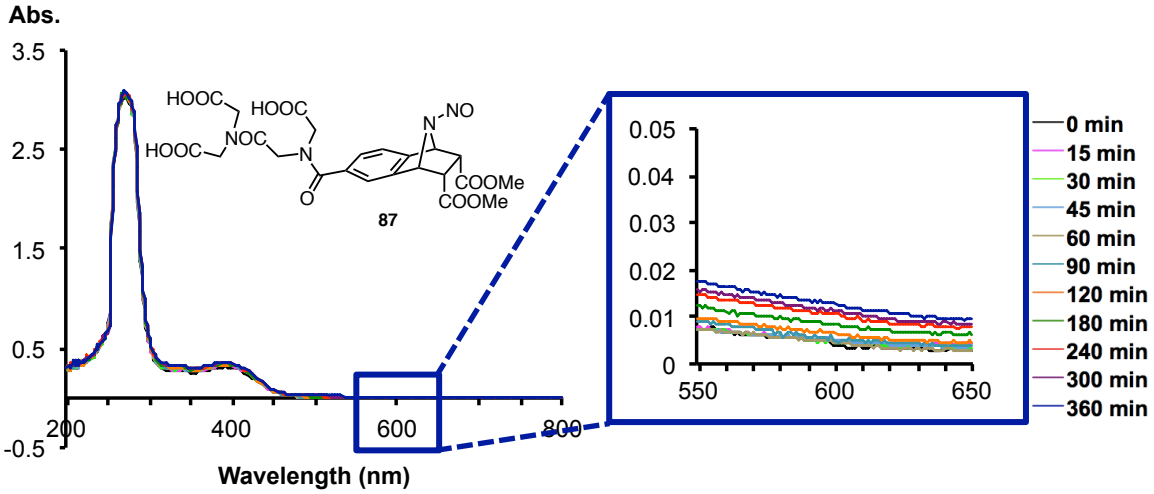


Fig. 3.12. UV-vis spectra of left wing tri-carboxylic acid nitrosamine **87** (3 mM) and triphenylmethanethiol (3 mM) in mixture of DMSO-$CHCl_3$ (1:4) without irradiation for 6 hours at 37 ºC.

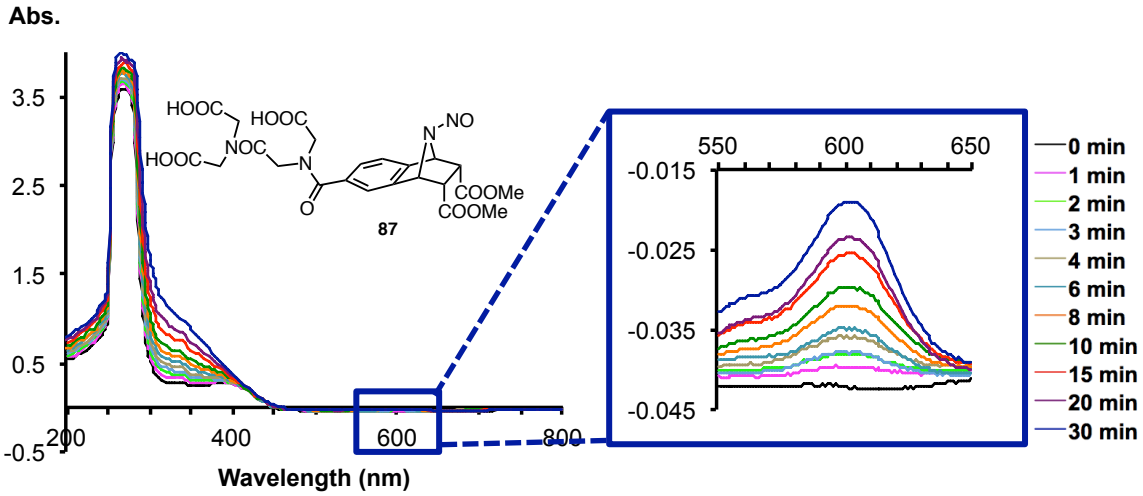


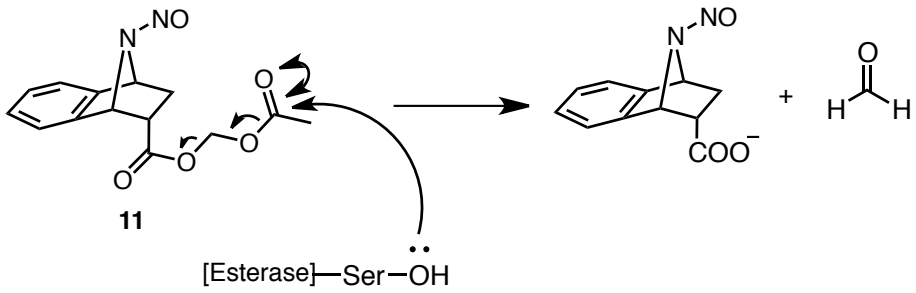
Fig. 3.13. UV-vis spectra of right wing tri-carboxylic acid nitrosamine **87** (3 mM) and triphenylmethanethiol (3 mM) in mixture of DMSO-$CHCl_3$ (1:4) with irradiation (420 nm) up to 30 minutes at room temperature.

From both left wing and right wing tri-carboxylic acid nitrosamines' result, it is suggested that there is a high possibility their tri-AM ester nitrosamines will not transfer the NO to thiol species in cells. These two nitrosamines have demonstrated that without irradiation the NO will keep intact to the nitrosamine. As the result, these compounds perfectly can be used as NOS mimics which their NO release can be fully controlled by

irradiation only. Without irradiation, they will not release NO or transfer NO naturally to another compounds, especially thiol species. However the reason why these nitrosamines won't transfer their NO to triphenylmethanethiol is still unknown and now is being studied.

3.6. Bicyclic Nitrosamines Toxicity in Piccell

Because development of these tri-AM ester nitrosamines are as artificial NOS mimics that will be applied to living cells, toxicity of these compounds will be an important issue. During the study of bicyclic nitrosamines in Piccell, no significant cell death was observed in the range of 1-10 μM of nitrosamines. However when the concentration of nitrosamines was increased from 10 μM to 100 μM for incubation in Piccell, right wing AM ester nitrosamines **64**, **58**, **69** showed toxic effect to Piccell. Three hours after incubation with high concentration of these nitrosamines, a small amount of Piccell was found dead. This finding may lead to two possibility which is whether the high concentration of nitrosamines itself is toxic or formaldehyde, that is released from hydrolysis of AM ester, is the toxic agent.



Scheme 3.11. Hydrolysis of AM ester by esterase

As shown in **Scheme 3.11.**, after hydrolysis was completed, one AM ester will generate one molecule of formaldehyde as the byproduct. Based on the toxicity observed in Piccell after incubated with 100 μM of right wing AM ester nitrosamines, the order of the toxicity is di-AM ester >> tri-AM ester > mono AM ester.

It is hypothesized that at high concentration, the hydrolysis byproduct (formaldehyde) caused toxicity to Piccell. Based on the right wing AM ester nitrosamines results in Piccell, mono- and di-AM ester has better permeability into cells than tri-AM ester. Since di-AM ester has two molecules of AM ester, it will produce two molecules of formaldehyde as well. Because of this, right wing di-AM ester nitrosamines showed higher toxicity than tri- and mono-AM ester nitrosamines.

On the other hand, toxicity in Piccell was not observed for left wing AM ester nitrosamines at 100 μM concentration. This can be explained by the amount of left wing AM ester nitrosamines that successfully penetrate into cells were not as much as right wing AM ester nitrosamines did. Therefore, formaldehyde generated from the left wing AM ester nitrosamines still can be tolerate by Piccell.

Chapter 4

Conclusion

4.1. Confirmation of NO Release in Cells

After incubation of bicyclic nitrosamines in Piccell and irradiation with visible light was applied, NO was released in living cells. In Piccell, not only generation of NO can be detected but also depletion of NO. Besides, controlling duration of the irradiation may control amount of NO release since cleavage of N-NO bond was triggered by irradiation. As the duration of visible light irradiation increased, amount of NO release was increase as well.

4.2. Highly Retainable Bicyclic Nitrosamine in Cells

Acetoxymethyl ester (AM ester) has been reported to have the ability to improve cell retention ability of calcium probe. Installing AM ester group to bicyclic nitrosamines also improved their cell retention ability. As the number of AM ester increased, nitrosamines' cell retention ability was improved. It is found that at least three AM ester groups were necessary for the bicyclic system to have relatively long retention time in cells with stable NO release upon irradiation with visible light. It has been demonstrated that moving the AM ester group to different location in the bicyclic system will not affect the cell retention ability of the compounds.

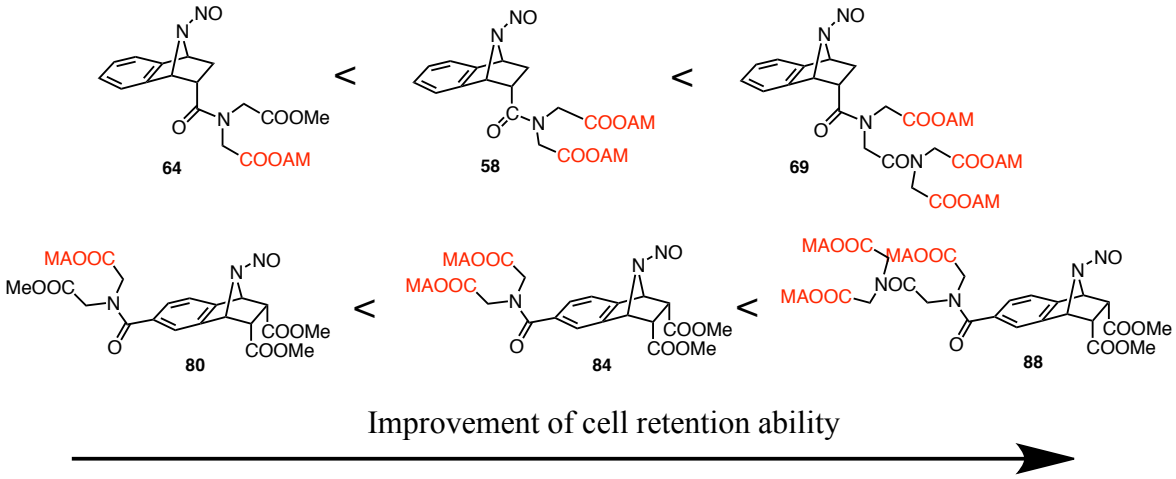
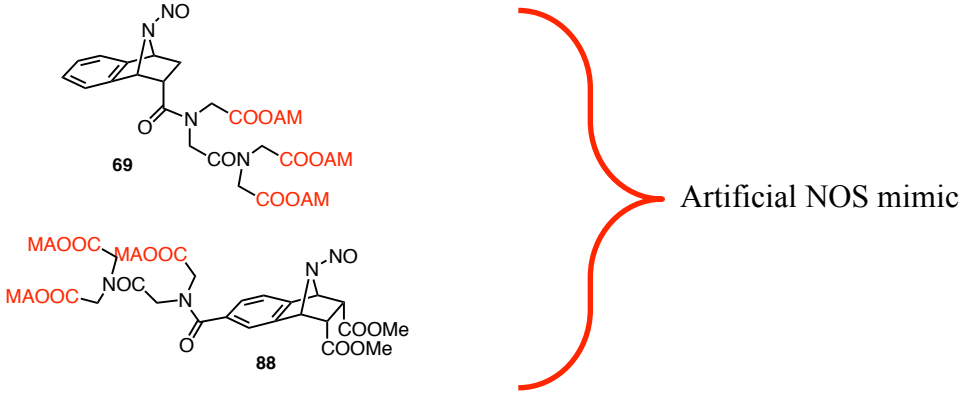


Fig. 4.1. Increase of AM ester numbers will increase nitrosamines' cell retention ability.

4.3. Bicyclic Nitrosamines as Artificial NOS Mimic

The ability of these bicyclic nitrosamines to release NO upon irradiation is completely controllable by irradiation. A transnitrosylation between nitrosamine and thiol was also performed in cuvette. After 36 hours of incubation, no transfer of NO was detected. Therefore it is a high possibility that these nitrosamines may not undergo transnitrosylation in cells to transfer NO to thiol, in which as long as irradiation was not applied, the NO will still intact to the nitrosamine. Integrating all the properties of tri-AM ester nitrosamines, which is high retention property, controllable NO release by irradiation, no transnitrosylation, low concentration of NO release, made these tri-AM ester nitrosamines can be used as artificial NOS mimics, especially eNOS and nNOS.



- Visible light triggered NO release
- Low concentration of NO release
- Controllable NO release concentration
- Highly retainable in cells
- No transnitrosylation to thiol, at least in cuvette

Fig. 4.2. Possibility of bicyclic nitrosamines to be used as artificial NOS mimics.

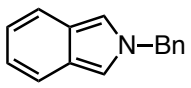
Chapter 5

Experimental section

5.1. Material

General Methods for Synthesis

All reagents are commercially available and used as supplied unless otherwise noted. All the NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR). *d*-$CDCl_3$ was used as a solvent, unless otherwise noted. Chemical shifts (δ) were reported in ppm with respect to undeuterated residual solvent (i.e. $CHCl_3$ (δ=7.26 ppm)). Coupling constants are given in hertz. Coupling patterns were indicated as s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; m, multiplet; brs, broad singlet peak. High-resolution mass spectrometry (HRMS) was obtained by electron spray ionization (ESI)-time-of-flight (TOF) detection mode and the mass spectra were recorded on a Bruker micrOTOF-05. Column chromatography was carried out on silica gel (silica gel 60N (100-210 mm), Kanto Chemicals, Japan). All the melting points were measured with a Yanaco Micro Melting Point Apparatus and were uncorrected. Combustion analyses were carried out in the microanalysis laboratory of this faculty.



12

To a suspension of $LiAlH_4$ (5.0100 g, 132.0158 mmol) in dry THF (100 mL) under Ar, a solution of MeOH (11 mL) in THF (100 mL) was added over 30 min at 0 ^{o}C, followed by cooling of the whole suspension to -78 ^{o}C. To this suspension, *N*-benzylphthalimide (10.0043 g, 42.1678 mmol) was added at -78 ^{o}C and the mixture was stirred at this temperature for 30 min, continued at 0 ^{o}C for 30 min. Then the reaction was quenched with saturated Na_2SO_4 solution. The inorganic salt formed was filtered by suction and washed with THF. The combine organic layer was dried over anhydrous Na_2SO_4 and evaporated. To the obtained residue, EtOH (20 mL) was added and the mixture was cooled to -28 ^{o}C for overnight. The precipitate was filtered and washed with cold EtOH. The solid was dried under vacuum to give **12** (5.1639 g, 59%) as off-white solid.

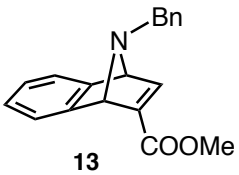
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.54-7.51 (2H, dd, *J*=3.2 Hz, 6.4 Hz), 7.36-7.30 (3H, m), 7.15 (4H, m), 6.94-6.92 (2H, dd, *J*=3.20 Hz, 6.4 Hz), 5.37 (2H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 137.23, 128.70, 127.89, 127.15, 124.47, 120.71, 119.52, 111.13, 54.70.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{15}H_{14}N^+$: 208.1121. Found 208.1122.

Anal. Calcd. for $C_{15}H_{13}N$; C, 86.92; H, 6.32; N, 6.76. Found C, 87.04; H, 6.73; N, 6.59.

Mp: 120-121 ^oC.



13

To a solution of **12** (5.1610 g, 24.8999 mmol) in CH_2Cl_2 (33 mL), a solution of methyl propiolate (3.1400 g, 37.3498 mmol) in CH_2Cl_2 (25 mL) was added dropwise for 10 min at 0 ^oC. The mixture was stirred at 0 ^oC to rt for 24 h. Then the solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 3:1) to afford **13** as yellow oil (5.7911 g, 80%).

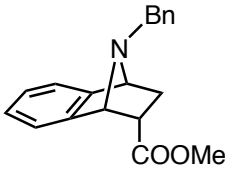
^{1}H-NMR (400 MHz, $CDCl_3$): δ =7.63-7.19 (8H, m), 7.03 (2H, brs), 4.85 (1H, brs), 4.69 (1H, brs), 3.71 (3H, s), 3.42 (2H, brs).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 163.78, 152.67, 149.24, 147.32, 145.69, 137.63, 128.86, 128.19, 127.02, 125.29, 125.11, 123.78, 121.13, 70.88, 69.52, 53.44, 51.34.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{19}H_{18}NO_2^+$: 292.1332. Found 292.1325.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{19}H_{17}NNaO_2^+$: 314.1151. Found 314.1151.

Anal. Calcd. for $C_{19}H_{17}NO_2$. $0.1H_2O$; C, 77.85; H, 5.91; N, 4.78. Found C, 77.73; H, 5.99; N, 4.60.

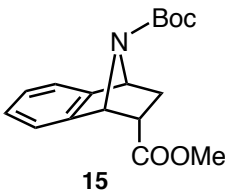


14

To a solution of **13** (5.7834 g, 19.8510 mmol) in MeOH (450 mL), 10% Pd-C (1.3607 g) in MeOH (130 mL) was added and stirred vigorously under H_2 for 2.5 h at room temperature. Pd-C was removed by filtration through Celite® and washed with MeOH. The solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 4:1 to 2:1) to give **14** (4.7663 g, 82%) as orange oil.

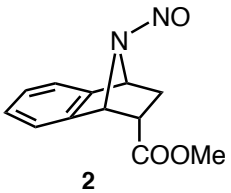
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.35-7.16 (9H, m), 4.44 (1H, d, *J*=4.4 Hz), 4.23 (1H, d, *J*=4.8 Hz), 3.51 (3H, s), 3.46-3.43 (1H, m), 3.38-3.30 (2H, m), 2.44-2.37 (1H, m), 1.70 (1H, dd, *J*=4.4 Hz, 12.0 Hz).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.51, 144.81, 140.54, 138.46, 128.83, 128.35, 127.36, 127.13, 126.53, 123.22, 121.57, 67.33, 65.67, 52.28, 51.52, 44.09, 30.17.
HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{19}H_{20}NO_2^+$: 294.1489. Found 294.1489.
Anal. Calcd. for $C_{19}H_{19}NO_2$; C, 77.79; H, 6.53; N, 4.77. Found C, 77.53; H, 6.61; N, 4.74.



15

To a solution of **14** (4.7663 g, 16.2473 mmol) in 93 mL dioxane and 36 mL of H_2O, NBS (4.3378 g, 24.3710 mmol) was added at room temperature. The mixture was stirred at room temperature for 24 h. The solvent was evaporated and to the residue obtained, TEA (22 mL) and $(Boc)_2O$ (5.3179 g, 24.3661 mmol) in dioxane (73 mL) was added and stirred at room temperature for 22 h. Then the solvent was evaporated and the residue was purified with flash column chromatography (*n*-hexane:EtOAc = 4:1) to give **15** (4.2801 g, 87%) as colourless needle crystal after recrystallization (CH_2Cl_2/*n*-hexane).
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) =7.24-7.10 (4H, m), 5.32 (1H, d, *J*=4.4 Hz), 5.14 (1H, d, *J*=4.0 Hz), 3.53 (3H, s), 3.42-3.37 (1H, m), 2.35 (1H, td, *J*=4.8 Hz, 11.2 Hz), 1.75 (1H, dd, *J*=4.0 Hz, 12.0 Hz), 1.40 (9H, s).
^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.65, 154.81, 144.87, 141.00, 127.26, 126.41, 121.13, 119.53, 80.50, 62.89, 61.54, 51.69, 44.37, 30.55, 28.14.
HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{17}H_{21}NNaO_4^+$: 326.1368. Found 326.1357.
Anal. Calcd. for $C_{17}H_{21}NO_4$; C, 67.31; H, 6.98; N, 4.62. Found C, 67.15; H, 7.01; N, 4.52.
Mp: 108.3-109.0 ^{o}C.



2

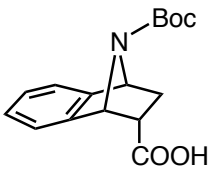
Compound **15** (151.5 mg, 0.4994 mmol) was dissolved in TFA (2.5 mL) at 0 ^{o}C and the mixture was stirred for 20 min at 0 ^{o}C. Then TFA was removed by evaporation. Sodium nitrite (142.3 mg, 2.0623 mmol), paraformaldehyde (20.9 mg) and PBS pH 6.4 (10 mL) was added and stirrred for 17 h at room temperature. The mixture was extracted with CH_2Cl_2, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give **2** (110.3 mg, 95%) as orange brick crystal after recrystallization (CH_2Cl_2/*n*-hexane).
^{1}H-NMR (400 MHz, $CDCl_3$): The two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 7.40-7.23 (4H, m), 6.19-6.06 (1H, m), 6.03-5.89 (1H, m), 3.60-3.56 (3H, m), 3.46-3.25 (1H, m), 2.50-2.25 (1H, m), 2.04-1.81 (1H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 170.37, 142.82, 141.66, 139.15, 137.90, 128.29, 127.48, 122.24, 121.61, 120.58, 119.92, 63.84, 63.02, 58.20, 57.62, 52.15, 45.13, 41.65, 32.16, 28.68.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{12}H_{12}N_2NaO_3^+$ $[M+Na]^+$ 255.0746; found 255.0751.

Anal. Calcd. for $C_{12}H_{12}N_2O_3$: calcd C, 62.06; H, 5.21; N, 12.06; O, 20.67; found C, 62.17; H, 5.33; N, 12.00.

Mp: 60.3-61.8 °C



16

To a solution of **15** (2.4962 g, 8.2287 mmol) in 350 mL of MeOH-H_2O (1:1), $LiOH.H_2O$ (699.8 mg, 16.6778 mmol) was added and the mixture was stirred at room temperature for 16 h. Then MeOH was evaporated and the aqueous solution's pH was adjusted to 3 with 0.5 N HCl to give cloudy solution, which was extracted with CH_2Cl_2 (3x50 mL). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to give **16** (2.2346 g, 94%) as white amorphous solid after recrystallization (CH_2Cl_2/*n*-hexane).

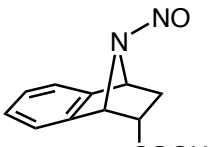
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 9.83 (1H, br), 7.26-7.08 (4H, m), 5.30-5.29 (1H, d, *J*=4.4 Hz), 5.13-5.12 (1H, d, *J*=4.0 Hz), 3.41-3.37 (1H, m), 2.36-2.29 (1H, td, *J*=4.4 Hz, 12.0 Hz), 1.68-1.64 (1H, dd, *J*=4.0 Hz, 12.0 Hz), 1.39 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 176.54, 154.77, 144.90, 140.56, 127.36, 126.46, 121.60, 119.45, 80.71, 62.78, 61.65, 44.26, 30.49, 28.15.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{16}H_{18}NO_4^-$: 288.1241. Found 288.1224.

Anal. Calcd. for $C_{16}H_{19}NO_4$; C, 66.42; H, 6.62; N, 4.84. Found C, 66.15; H, 6.62; N, 4.80.

Mp: 141.6-144.2 °C, with decomposition.



5

Compound **16** (101.1 mg, 0.3497 mmol) was dissolved in TFA (1.7 mL) at 0 °C and the mixture was stirred for 20 min at 0 °C. Then TFA was removed by evaporation. Sodium nitrite (98.2 mg, 1.4232 mmol), paraformaldehyde (11.5 mg) and PBS pH 6.4 (7.0 mL) was added and stirred for 17 h at room temperature. The mixture was acidified with HCl 1M until pH 3, extracted with CH_2Cl_2, and the organic phase was washed with brine, dried over anhydrous

Na_2SO_4, and evaporated to give **5** (63.7 mg, 84%) as yellow plate crystal after recrystallization (Et_2O/*n*-hexane).

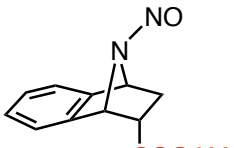
^{1}H-NMR (400 MHz, $CDCl_3$): The two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 7.41-7.21 (4H, m), 6.19 (0.5H, m), 6.05 (1H, m), 5.90 (0.5H, m), 3,46 (0.5H, m), 3.28 (0.5H, m), 2.48 (0.5H, m), 2.29 (0.5H, m), 1.95 (0.5H, m), 1.75 (0.5H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 175.09, 142.79, 141.63, 138.67, 137.40, 128.35, 127.46, 122.66, 122.02, 120.49, 119.83, 63.63, 63.03, 57.99, 57.67, 45.04, 41.58, 32.03, 28.54.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{11}H_9N_2O_3^-$: 217.0619. Found 217.0630.

Anal. Calcd. for $C_{11}H_{10}N_2O_3$; C, 60.55; H, 4.62; N, 12.84. Found C, 60.62; H, 4.62; N, 12.76.

Mp: 129-131 °C.



11

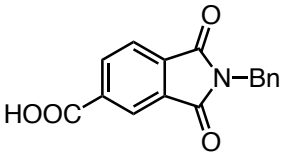
To a solution of compound **5** (32.6 mg, 0.1495 mmol) in 0.75 mL of MeOH-CH_3CN (2:1) was added DIEA (28 µL, 0.1637 mmol) and bromomethyl acetate (16 µL, 0.1631 mmol). The mixture was stirred at room temperature for 22 h and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 4:1) to give **11** (28.0 mg, 65%) as orange oil.

^{1}H-NMR (400 MHz, $CDCl_3$): The two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 7.41-7.21 (4H, m), 6.22 (0.5H, m), 6.06 (1H, m), 5.89 (0.5H, m), 5.65-5.58 (2H, m), 3.49 (0.5H, m), 3.29 (0.5H, m), 2.48 (0.5H, m), 2.30 (0.5H, m), 2.10 (3H, s), 2.03 (0.5H, m), 1.83 (0.5H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.30, 168.77, 142.78, 141.60, 138.70, 137.42, 128.38, 127.35, 122.44, 121.77, 120.62, 119.95, 79.45, 63.53, 62.93, 57.89, 57.56, 44.96, 41.47, 31.99, 28.46, 20.62.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{14}H_{14}N_2NaO_5^+$: 313.0795. Found 313.0779.

Anal. Calcd. for $C_{14}H_{14}N_2O_5$; C, 57.93; H, 4.86; N, 9.65. Found C, 58.02; H, 5.00; N, 9.50.



17

Benzylamine (16.5 mL, 151.0639 mmol) was added into mixture of 1,3,5-trimelltitic anhydride (14.4535 g, 75.2277 mmol) in acetic acid (200 mL) and refluxed for 2.5 h. The reaction was stopped

and the acetic acid was evaporated. Mixture of water-acetic acid (1:4, 150 mL) was added into the residue and heated until the entire solid was completely dissolved. Solution of HCl 3 M (150 mL) was added into the mixture and allowed to cool to room temperature. After cooled to room temperature, the mixture was cooled in ice bath. After filtration, white solid was obtained. Further purification by recrystallization (acetone/*n*-hexane) will give **17** (20.6220 g, 97%) as white solid amorphous colorless crystal.

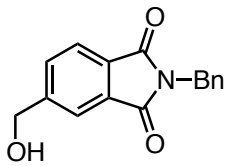
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.56 (1H, s), 8.47 (1H, d, *J*=8.0 Hz), 7.97 (1H, d, *J*=7.6 Hz), 7.46-7.43 (2H, m), 7.35-7.26 (3H, m), 4.88 (2H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.33, 166.93, 166.88, 136.22, 136.01, 135.91, 134.60, 132.47, 128.76, 128.71, 128.03, 125.05, 123.56, 41.98.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{16}H_{10}NO_4^-$: 280.0615. Found 280.0634.

Anal. Calcd. for $C_{16}H_{11}NO_4$; C, 68.32; H, 3.94; N, 4.98. Found C, 68.37; H, 4.06; N, 4.94.

Mp: 196-197 ^{o}C .



18

Borane-THF complex (59 mL, 56.05 mmol) was added into solution of **17** (10.0357 g, 35.6809 mmol) in THF (200 mL) in several portions and stirred at room temperature under Ar. After 58 h, the reaction was quenched with methanol and continued stirring at room temperature for another 30 min. The solvent was then evaporated and the residue obtained was purified by flash column chromatography (*n*-hexane:acetone = 3:1 to 0:1) to give **18** (8.8478 g, 93%) as colorless needle.

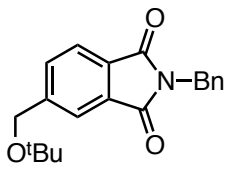
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.84 (1H, s), 7.79 (1H, d, *J*=3.6 Hz), 7.67 (1H, d, *J*=3.6 Hz), 7.45-7.43 (2H, m), 7.35-7.28 (3H, m), 4.84 (2H, s), 4.83 (2H, d, *J*=5.6 Hz), 2.44 (1H, t, *J*=5.6 Hz).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 168.00, 167.88, 148.07, 136.29, 132.46, 131.82, 131.04, 128.65, 128.53, 127.81, 123.41, 121.30, 64.21, 41.60.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{16}H_{13}NNaO_3^+$: 290.0788. Found 290.0789.

Anal. Calcd. for $C_{16}H_{13}NO_3$; C, 71.90; H, 4.90; N, 5.24. Found C, 71.91; H, 5.06; N, 5.29.

Mp: 134-135 ^{o}C.



19

To a mixture of **18** (10.3211 g, 38.6154 mmol) ang $Mg(ClO_4)_2$ (862.0 mg, 3.8618 mmol) in $CHCl_3$ (50 mL) was added $(Boc)_2O$ (43.4085 g, 198.8935 mmol), which was divided in several portions. After stirring at 50 ^oC for 44 h, the mixture was poured into water and extracted with CH_2Cl_2. The combine organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 5:1 to 2:1) to give **19** (9.9819 g, 80%) as colorless plate after recrystallization (CH_2Cl_2/*n*-hexane), and starting material (1.1912 g, 12%).

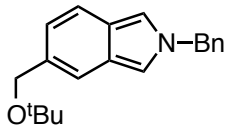
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.84 (1H, m), 7.78 (1H, d, *J*=8.0 Hz), 7.68-7.66 (1H, m), 7.43-7.41 (2H, m), 7.33-7.25 (3H, m), 4.84 (2H, s), 4.56 (2H, s), 1.30 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 168.13, 168.02, 147.48, 136.46, 132.40, 132.30, 130.86, 128.63, 128.49, 127.74, 123.21, 121.96, 74.08, 63.38, 41.57, 27.59.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{20}H_{21}NNaO_3^+$: 346.1414. Found 346.1410.

Anal. Calcd. for $C_{20}H_{21}NO_3$; C, 74.28; H, 6.55; N, 4.33. Found C, 74.19; H, 6.53; N, 4.33.

Mp: 103.0-103.5 ^oC.



20

To a suspension of $LiAlH_4$ (5.7488 g, 17.7769 mmol) in dry THF (47 mL) under Ar, a solution of MeOH (5 mL) in THF (47 mL) was added over 30 min at 0 ^oC, followed by cooling of the whole suspension to -78 ^oC. To this suspension, **19** (5.7488 g, 17.7769 mmol) in THF (20 mL) was added at -78 ^oC and the mixture was stirred at this temperature for 30 min, continued at 0 ^oC for 30 min. Then the reaction was quenched with hydrated Na_2SO_4 crystal ($Na_2SO_4.xH_2O$). The inorganic salt formed was filtered and washed with THF. The combine organic layer was dried over anhydrous Na_2SO_4 and evaporated to give **20** (3.0348 g, 58%) as colorless amorphous crystal after recrystallization (THF/*n*-hexane).

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.48-7.46 (2H, m), 7.32-7.27 (2H, m), 7.11-7.07 (3H, m), 6.90 (1H, dd, *J*=8.8 Hz, 1.6 Hz), 5.34 (2H, s), 4.45 (2H, s), 1.31 (9H, s).

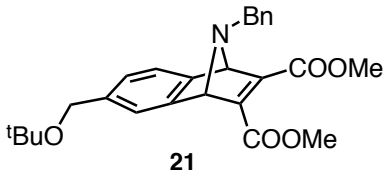
^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 137.46, 131.98, 128.70, 127.84, 127.02, 124.59, 124.16, 121.62, 119.64, 117.76, 111.30, 111.19, 73.13, 65.09, 54.69, 27.79.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{20}H_{24}NO^+$: 294.1852. Found 294.1839.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{20}H_{23}NNaO^+$: 316.1672. Found 316.1670.

Anal. Calcd. for $C_{20}H_{23}NO$; C, 81.87; H, 7.90; N, 4.77. Found C, 81.81; H, 7.83; N, 4.76.

Mp: 111.5-112.0 ^oC.



21

To a solution of **20** (997.8 mg, 3.4008 mmol) in 20 mL CH_2Cl_2 was added dimethyl acetylene dicarboxylate (0.5 mL, 4.0673 mmol) and stirred at rt for 30 min. After the solvent was evaporated, the crude product was purified with flash column chromatography (*n*-hexane:EtOAc = 9:2) to give **21** (1.4522 g, 98%) as pale yellow oil.

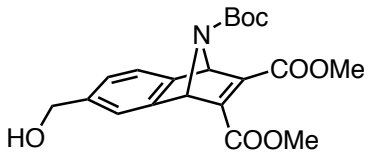
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.39 (1H, brs), 7.35-7.27 (6H, m), 7.06 (1H, d, *J*=6.8 Hz), 4.93 (1H, s), 4.89 (1H, s), 4.42 (2H, s), 3.80 (3H, s), 3.79 (3H, s), 3.60 (2H, brs), 1.31 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 164.00, 150.15, 146.57, 145.17, 137.45, 129.05, 128.36, 127.24, 124.74, 122.47, 73.34, 72.81, 63.96, 53.26, 52.08, 27.59.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{26}H_{30}NO_5^+$: 436.2118. Found 436.2117.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{26}H_{29}NNaO_5^+$: 458.1938. Found 458.1936.

Anal. Calcd. for $C_{26}H_{29}NO_5$; C, 71.70; H, 6.71; N, 3.22. Found C, 71.87; H, 6.84; N, 3.19.



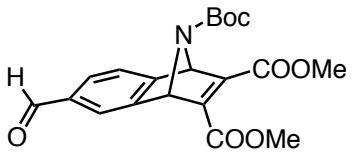
22

To a solution of **21** (3.0908 g, 7.0970 mmol) in 60 mL dioxane and 20 mL of H_2O, was added NBS (4.9627 g, 27.8835 mmol), which has been divided into two portions, and stirred at room temperature for 24 h. The solvent was evaporated and to the residue obtained, TFA (5 mL) was added and continue to stir at rt. After 2 h, TFA was evaporated. $(Boc)_2O$ (2.3422 g, 10.7317 mmol) and TEA (10 mL, 71.7462 mmol) in dioxane (60 mL) was added into the mixture and stirred at room temperature for 12 h. Then the solvent was evaporated and the residue was purified with flash column chromatography (*n*-hexane:EtOAc = 2:1 to 1:1) to give **22** (819.2 mg, 30%) as yellow foam solid and **23** (959.0 mg, 35%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.43 (1H, s), 7.37 (1H, d, *J*=7.2 Hz), 7.04 (1H, d, *J*=7.2 Hz), 5.75 (2H, s), 4.62 (2H, s), 3.78 (6H, s), 1.93 (1H, brs), 1.37 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 162.97, 162.90, 154.28, 151.04, 150.79, 146.21, 144.92, 139.15, 124.53, 121.86, 121.00, 81.70, 68.85, 64.97, 52.40, 52.39, 28.04.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{20}H_{23}NNaO_7^+$: 412.1367. Found 412.1371.



23

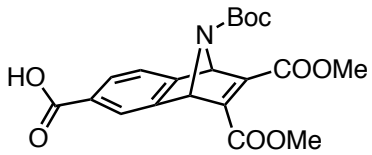
A solution of **22** (819.2 mg, 2.1038 mmol) in CH_2Cl_2 (20 mL) was added into solution of oxalyl chloride (270 μL, 3.1503 mmol) and DMSO (300 μL, 4.2276 mmol) that has been stirred for 20 min at -78°C under Ar and continued to stir at -78 °C for another 40 min. Then TEA (1.2 mL, 8.6095 mmol) was added into the mixture and warmed to rt. After 1 h, the reaction was quenched with water, extracted with CH_2Cl_2, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 2:1) to give **23** (728.7 mg, 89%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 9.96 (1H, s), 7.91 (1H, s), 7.63 (1H, dd, *J*=1.2Hz, 7.6Hz), 7.57 (1H, d, *J*=7.6 Hz), 5.82 (2H, s), 3.81 (3H, s), 3.80 (3H, s), 1.38 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 191.32, 162.64, 162.50, 154.08, 152.40, 150.84, 149.86, 146.97, 134.93, 131.05, 122.30, 120.82, 82.19, 68.72, 52.59, 52.56, 28.03.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{20}H_{21}NNaO_7^+$: 410.1210. Found 410.1211.

Anal. Calcd. for $C_{20}H_{21}NO_7$; C, 62.01; H, 5.46; N, 3.62. Found C, 62.03; H, 5.65; N, 3.56.



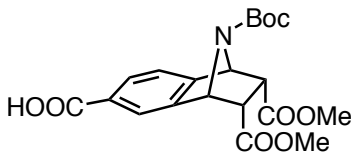
24

To a solution of **23** (326.5 mg, 0.8428 mmol) in *tert*-butanol (29 mL) and water (19 mL) was added 2-methyl-2-butene (1.3383 g, 19.0831 mmol), $NaClO_2$ (779.9 mg, 8.6234 mmol), and $NaH_2PO_4.2H_20$ (947.1 mg, 6.0708 mmol). The reaction was stirred at room temperature and after 4 h *tert*-butanol was evaporated. The aqueous solution was then acidified with HCl, extracted with EtOAc, dried over anhydrous Na_2SO_4, and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 2:1 to 1:1) to give **24** (321.9 mg, 95%) as white foam solid.

^{1}H-NMR (400 MHz, MeOD): δ (ppm) = 8.03 (1H, s), 7.85 (1H, dd, *J*=1.2 Hz, 7.6 Hz), 7.55 (1H, d, *J*=7.6 Hz), 5.82 (2H, s), 3.80 (3H, s), 3.79 (3H, s), 1.39 (9H, s).

^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 169.09, 164.29, 164.25, 156.14, 152.36, 152.12, 151.47, 147.62, 130.19, 129.99, 123.61, 122.90, 83.27, 70.18, 53.05, 53.02, 28.27.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{20}H_{20}NO_8^-$: 402.1194. Found 402.1222.



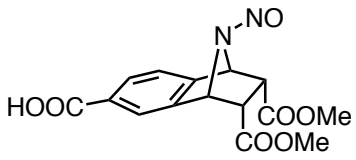
25

To a solution of **24** (688.5 mg, 1.7068 mmol) in MeOH (36 mL), 10% Pd-C (139.4 mg) was added and stirred vigorously under H_2 for 5.5 h at rt. Pd-C was removed by filtration through Celite® and washed with MeOH. The solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 2:1 to 1:1) to give **25** (510.0 mg, 74%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 10.50 (1H, brs), 8.05 (1H, s), 8.02 (1H, d, *J*=7.6 Hz), 7.48 (1H, d, *J*=8.0 Hz), 5.34 (2H, brs), 3.74-3.68 (2H, m), 3.51 (6H, s), 1.40 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.56, 169.71, 169.55, 154.23, 147.60, 142.32, 129.66, 128.16, 123.59, 122.69, 81.55, 63.30, 51.91, 51.77, 47.33, 47.07, 28.07

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{20}H_{22}NO_8^-$: 404.1351. Found 404.1352.



26

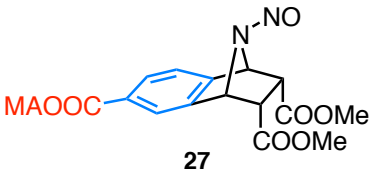
Compound **25** (184.4 mg, 0.4549 mmol) was dissolved in TFA (1.0 mL) and the mixture was stirred for 20 minutes at rt. Then TFA was removed by evaporation. Sodium nitrite (127.9 mg, 2.0116 mmol), formaldehyde solution 37% (57.7 mg) and PBS pH 6.4 (9.1 mL) was added into the mixture and stirred for 24 h at rt. The mixture was acidified with HCl 1M until pH 3, extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography (*n*-hexane:EtOAc = 1:1) to give **26** (138.4 mg, 91%) as orange solid after recrystalisation (*n*-hexane/CH_2Cl_2).

^{1}H-NMR (400 MHz, MeOD): The two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 8.15 (0.5H, brs), 8.03 (0.5H, brs), 7.98 (1H, brs), 7.60 (0.5H, d, *J*=6.0 Hz), 7.49 (0.5H, d, *J*=6.0 Hz), 6.39 (1H, brs), 6.08 (1H, brs), 3.87 (1H, brs), 3.62 (1H, brs), 3.50 (6H, brs).

^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 170.50, 170.43, 169.20, 146.46, 145.19, 142.21, 140.89, 131.39, 130.48, 125.49, 124.99, 124.48, 124.00, 65.77, 60.41, 52.46, 52.44, 49.33, 45.63

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{15}H_{13}N_2O_7^-$: 333.0728. Found 333.0737.

Mp: 168.5-169.5 ^oC.



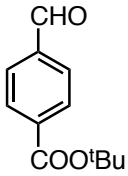
27

To a solution of compound **26** (98.4 mg, 0.2944 mmol) in 5.7 mL of acetonitrile was added DIEA (101 μL, 0.5908 mmol) and bromomethyl acetate (57.7 μL, 0.5884 mmol). The mixture was stirred at room temperature for 24 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:EtOAc = 2:1 to 1:1) to give **27** (58.9 mg, 49%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 8.15 (0.5H, brs), 8.04 (1.5H, brs), 7.58 (0.5H, brs), 7.49 (0.5H, brs), 6.27 (1H, brs), 6.00 (1H, brs), 5.98-5.93 (2H, m), 3.71 (1H, brs), 3.53 (7H, brs), 2.12 (3H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.60, 168.51, 168.46, 164.50, 145.19, 143.95, 140.31, 139.02, 130.10, 128.63, 124.46, 123.93, 123.55, 123.09, 79.45, 64.32, 59.05, 52.21, 52.09, 48.37, 48.18, 44.41, 44.21, 20.69.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{18}H_{18}N_2NaO_9^+$: 429.0905. Found 429.0904.



28

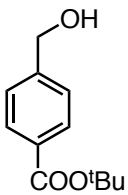
p-formyl benzoic acid (513.8 mg, 3.4224 mmol), DMAP (418.3 mg, 3.4091 mmol) and $(Boc)_2O$ (1.5635 g, 7.1638 mmol) was stirred for 5 min at room temperature then dry *t*-butanol (9 mL) was added and continued stirring for 23 h at room temperature. After the reaction was finished, *t*-butanol was evaporated and water was added into the mixture followed by EtOAc extraction. The organic phase was combined, washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was purified by flash column chromatography (*n*-hexane:Et_2O = 8:1) to give **28** (459.7 mg, 65%) as white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 10.09 (1H, s), 8.13 (2H, d, *J*=8.4 Hz), 7.92 (2H, d, *J*=8.4 Hz), 1. 62 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 191.61, 164.54, 138.77, 136.97, 129.91, 129.28, 81.89, 28.02.

Anal. Calcd. for $C_{12}H_{14}O_3$; C, 69.88; H, 6.84; N,-. Found C, 69.65; H, 6.82; N,-.

Mp: 50.5-51.8 ^{o}C.



29

In solution of **28** (1.2080 g, 5.8573 mmol) in MeOH (25 mL), $NaBH_4$ (235.6 mg, 6.2279 mmol) was added and stirred at 0 ^{o}C for 30 min. After the reaction was completed, MeOH was evaporated and the residue remained was purified by flash column chromatography (*n*-hexane:EtOAc = 4:1 to 3:1) to give **29**

(608.1 mg, 50%) as white solid.

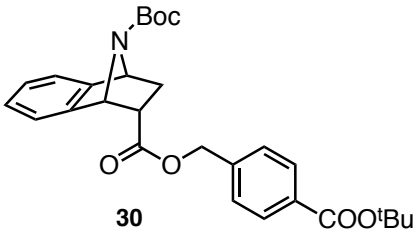
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.43 (2H, d, *J*=8.4 Hz), 7.37 (2H, d, *J*=8.8 Hz), 4.72(2H, s), 2.35 (1H, brs), 1.58 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 165.78, 145.67, 130.76, 129.42, 126.16, 80.98, 64.23, 28.03.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{12}H_{16}NaO_3^+$: 231.0992. Found 231.0991.

Anal. Calcd. for $C_{12}H_{16}O_3$; C, 69.21; H, 7.74; N, -. Found C, 68.97; H, 7.75; N, -.

Mp: 48.0-49.3 °C



30

To a solution of compound **17** (284.7 mg, 0.9840 mmol) in dichloromethane (5 mL), **29** (230.3 mg, 1.1059 mmol), DMAP (35.8 mg, 0.2917 mmol) and EDCI (288.7 mg, 1.5060 mmol) was added and stirred at rt under Ar for 18 h. After the reaction was complete, mixture was poured into water, extracted with CH_2Cl_2, washed with brine, and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 5:1) to give **30** (417.8 g, 89%) as white solid after recrystallized from Et_2O/*n*-hexane.

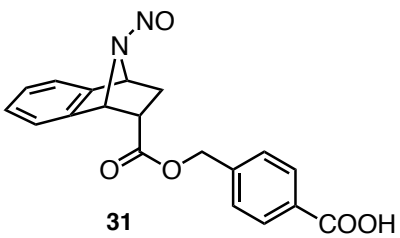
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) =7.96 (2H, d, *J*=8.1 Hz), 7.27-7.23 (3H, m), 7.16 (1H, td, *J*=7.2 Hz, 1.2 Hz), 7.04-6.96 (2H, m), 5.32 (1H, brs), 5.13 (1H, brs), 4.96 (2H, s), 3.48-3.44 (1H, m), 2.36 (1H, td, *J*=11.2 Hz, 4.4 Hz), 1.77 (1H, dd, *J*=12.0 Hz, 4.0 Hz), 1.61 (9H, s), 1.40 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 170.82, 165.28, 154.74, 144.88, 140.75, 139.92, 131.89, 129.59, 127.81, 127.26, 126.39, 121.23, 119.49, 81.11, 80.52, 65.73, 62.91, 61.57, 44.46, 30.61, 28.14, 28.13.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{28}H_{33}NNaO_6^+$: 502.2200. Found 502.2201.

Anal. Calcd. for $C_{28}H_{33}NO_6$; C, 70.13; H, 6.94; N, 2.92. Found C, 69.98; H, 7.05; N, 3.00.

Mp: 111.0-113.2 °C.



31

Compound **30** (383.3 mg, 0.7993 mmol) was dissolved in TFA (3.0 mL) and the mixture was stirred for 2 h at room temperature. Then TFA was removed by evaporation. Sodium nitrite (229.3 mg, 3.3232 mmol), formaldehyde

solution 37% (65.0 mg) and PBS pH 6.4 (16 mL) was added into the mixture and stirred for 17 h at room temperature. The mixture was acidified with HCl 1M until pH 3, extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 3:1 to 2:1) to give **31** (230.2 mg, 82%) as brown-orange solid after recrystallized from CH_2Cl_2/*n*-hexane.

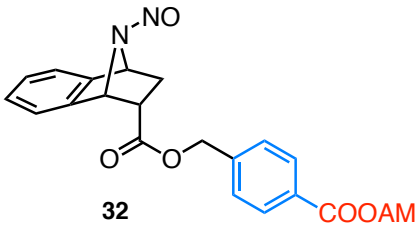
^{1}H-NMR (400 MHz, MeOD-d_4) δ = 8.02 (1H, s), 8.00 (1H, s), 7.45-7.24 (4H, m), 7.11-7.02 (2H, m), 6.27 (0.5H, brs), 6.10 (0.5H, brs), 6.06 (0.5H, brs), 5.92 (0.5H, brs), 5.07 (1H, s), 5.04 (1H, s), 3.65 (0.5H, brs), 3.41 (0.5H, brs), 2.51 (0.5H, brs), 2.29 (0.5H, brs), 1.99 (0.5H, d, *J*=10.4 Hz), 1.81 (0.5H, d, *J*=12.0 Hz).

^{13}C-NMR (100 MHz, MeOD-d_4): δ (ppm) = 171.22, 169.43, 144.66, 143.44, 142.10, 140.61, 139.30, 131.89, 130.97, 129.33, 129.26, 128.40, 123.42, 122.89, 121.53, 120.98, 67.08, 65.25, 64.49, 59.55, 59.03, 46.10, 42.79, 32.89, 29.54.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{19}H_{15}N_2O_5^-$: 351.0986. Found 351.0988.

Anal. Calcd. for $C_{19}H_{16}N_2O_5$; C, 64.77; H, 4.58; N, 7.95. Found C, 64.53; H, 4.74; N, 7.85.

Mp: 162-164 °C with decomposition.



32

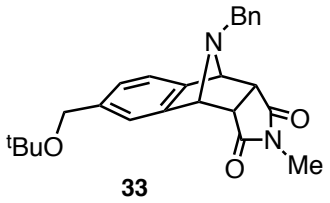
To a solution of compound **31** (153.0 mg, 0.4342 mmol) in 10 mL of acetonitrile was added DIEA (148.6 µL, 0.8692 mmol) and bromomethyl acetate (85 µL, 0.8890 mmol). The mixture was stirred at room temperature for 14 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 3:1 to 2:1) to give **32** (159.0 mg, 86%) as orange oil.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 8.07 (1H, s), 8.05 (1H, s), 7.38-7.25 (4H, m), 7.09 (1.5H, brs), 6.97 (0.5H, brs), 6.19 (0.5H, brs), 6.04 (1H, brs), 6.00 (2H, s), 5.89 (0.5H, s), 5.04 (1H, s), 5.01 (1H, s), 3.50 (0.5H, brs), 3.31 (0.5H, brs), 2.49 (0.5H, brs), 2.30 (0.5H, brs), 2.14 (3H, s), 2.5 (0.5H, brs), 1.84 (0.5H, brs).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.62, 169.56, 164.74, 142.83, 141.66, 140.95, 138.86, 137.58, 130.33, 129.05, 128.28, 128.19, 127.39, 122.26, 121.64, 120.53, 119.88, 79.66, 66.02, 63.81, 62.98, 58.15, 57.61, 45.21, 41.68, 32.17, 28.65, 20.72.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{22}H_{20}N_2NaO_7^+$: 447.1163. Found 447.1156.

Anal. Calcd. for $C_{22}H_{20}N_2O_7$; C, 62.26; H, 4.75; N, 6.60. Found C, 62.10; H, 4.90; N, 6.46.



33

To a solution of **20** (1.1752 g, 4.0054 mmol) in CH_2Cl_2 (20 mL), *N*-methyl maleimide (655.0 mg, 5.8956 mmol) was added and stirred at room temperature for 9 h. After the reaction was completed, the solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 3:1 to 2:1) to give **33** (1.2219 g, 75%) as colorless plate solid after recrystallized from CH_2Cl_2/*n*-hexane.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.32-7.26 (3H, m), 7.24-7.18 (5H, m), 4.56-4.53 (2H, m), 4.42 (2H, s), 3.66 (2H, t, *J*=2.4 Hz), 3.36 (2H, d, *J*=4.4 Hz), 2.27 (3H, s), 1.27 (9H, s).

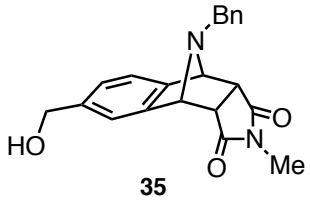
^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 175.79, 140.26, 140.22, 138.91, 138.18, 128.63, 128.44, 127.29, 126.77, 123.27, 122.47, 73.61, 67.05, 66.75, 63.90, 52.39, 47.55, 47.51, 27.72, 23.74.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{25}H_{29}N_2O_3^+$: 405.2173. Found 405.2173.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{25}H_{28}N_2NaO_3^+$: 427.1992. Found 427.1997.

Anal. Calcd. for $C_{25}H_{28}N_2O_3$; C, 74.23; H, 6.98; N, 6.93. Found C, 74.07; H, 7.22; N, 6.75.

Mp: 107.0-108.0 ^{o}C .



35

To solution of **33** (871.5 mg, 2.1492 mmol) in CH_2Cl_2 (20 mL) was added TfOH (0.4 mL, 4.5309 mmol) and stirred at 0 ^{o}C for 5 h. After reaction was completed, the mixture was poured into ice-cold saturated Na_2CO_3 solution, extracted with CH_2Cl_2, washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:Acetone = 3:1) to give **35** (709.8 mg, 95%) as white solid after recrystallized from CH_2Cl_2/*n*-hexane.

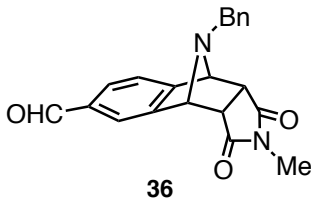
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.33-7.18 (8H, m), 4.68 (2H, s), 4.57-4.56 (2H, m), 3.69-3.68 (2H, m), 3.67 (2H, s), 2.27 (3H, s), 1.81 (1H, brs).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 175.87, 175.68, 141.03, 140.58, 139.34, 137.89, 128.62, 128.44, 127.35, 126.45, 123.46, 122.01, 66.87, 66.69, 64.75, 52.39, 47.37, 23.73.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{21}H_{21}N_2O_3^+$: 349.1547. Found 349.1550.

Anal. Calcd. for $C_{21}H_{20}N_2O_3$; C, 72.40; H, 5.79; N, 8.04. Found C, 72.16; H, 5.94; N, 7.92.

Mp: 127.0-128.5 ^{o}C.



36

A solution of **35** (358.4 mg, 1.0287 mmol) in CH_2Cl_2 (8 mL) was added into solution of oxalyl chloride (176 μL, 2.0570 mmol) and DMSO (150 μL, 2.1138 mmol) that has been stirred for 20 min at -78°C under Ar and continued to stir at -78°C for another 40 min. Then TEA (573 μL, 4.1111 mmol) was added into the mixture and warmed to rt. After 1 h, the reaction was quenched with water, extracted with CH_2Cl_2, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 4:1) to give **36** (326.9 mg, 92%) as colorless amorphous crystal after recrystallized from acetone/*n*-hexane.

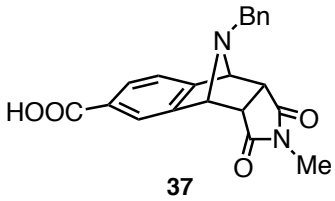
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 9.99 (1H, s), 7.80-7.78 (2H, m), 7.44 (1H, d, *J*=7.6 Hz), 7.35-7.27 (3H, m), 7.17 (2H, d, *J*=6.8 Hz), 4.66-4.64 (2H, m), 3.75 (2H, t, *J*=2.8 Hz), 3.36 (2H, s), 2.28 (3H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 191.34, 175.15, 175.10, 147.11, 141.59, 137.45, 136.30, 130.76, 128.57, 127.58, 124.04, 123.84, 66.81, 66.50, 52.51, 47.14, 47.12, 23.84.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{21}H_{19}N_2O_3^+$: 347.1390. Found 347.1400.

Anal. Calcd. for $C_{21}H_{18}N_2O_3$; C, 72.82; H, 5.24; N, 8.09. Found C, 72.56; H, 5.41; N, 8.00.

Mp: 143.0-144.5 °C.



37

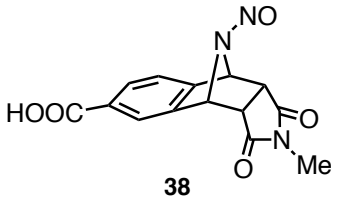
To a solution of **36** (172.4 mg, 0.4977 mmol) in *tert*-butanol (15 mL) and water (10 mL) was added 2-methyl-2-butene (803.1 mg, 11.4516 mmol), $NaClO_2$ (465.9 mg, 5.1514 mmol), and $NaH_2PO_4.2H_20$ (559.0 mg, 3.5831 mmol). The reaction was stired at room temperature and after 5 h *tert*-butanol was evaporated. The aqueous solution was then acidified with HCl, extracted with EtOAc, dried over anhydrous Na_2SO_4, and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 2:1 to 1:1) to give **37** (116.0 mg, 58%) as white solid after recrystallized from acetone/*n*-hexane.

^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) = 7.82 (1H, dd, *J*=7.6 Hz, 1.6 Hz), 7.72 (1H, s), 7.34-7.23 (6H, m), 4.61 (2H, dd, *J*=8.8 Hz, 4.0 Hz), 3.84 (2H, s), 3.38 (2H, s), 2.11 (3H, s).

^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) = 175.41, 175.29, 166.99, 145.71, 141.36, 138.17, 129.74, 128.99, 128.40, 128.32, 127.05, 123.14, 122.68, 66.09, 65.92, 51.58, 46.63, 46.57.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{21}H_{17}N_2O_4^-$: 361.1194. Found 361.1186.

Mp: 163.0-165.5°C



38

To a solution of compound **37** (252.9 mg, 0.6979 mmol) in mixture of dioxane (8 mL) and water (4 mL) was added NBS (501.7 mg, 2.8187 mmol) that was divided into two portion and the second portion was added into the mixture after 12 h. After stirring at rt for 24 h, the mixture was evaporated to dry. Sodium nitrite (194.4 mg, 2.8174 mmol), formaldehyde 37% in water (70.3 mg, 0.8670 mmol) and PBS pH 6.4 (15 mL) was added and stirred for 24 h at room temperature. The mixture was acidified with HCl solution, extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified with flash column chromatography ($CHCl_3$:MeOH = 20:1) to give **38** (153.3 mg, 73%) as amorphous yellow solid after recrystallized from acetone/*n*-hexane.

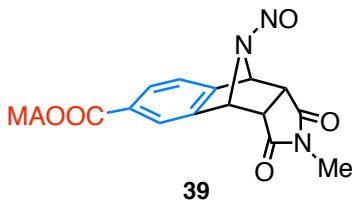
^{1}H-NMR (400 MHz, Acetone-d_6): isomer due to the N-NO bond was present in 1:1 ratio. δ = 8.06-8.01 (2H, m), 7.56 (0.5 H, brs), 7.50 (0.5 H, brs), 6.71 (1H, brs), 6.27 (1H, brs), 4.08 (1H, brs), 3.81 (1H, brs), 2.27 (3H, s).

^{13}C-NMR (100 MHz, Acetone-d_6): δ (ppm) = 173.94, 173.88, 166.72, 144.67, 143.58, 140.62, 139.47, 131.48, 130.97, 124.25, 123.63, 123.01, 64.40, 59.05, 48.76, 45.74, 24.25

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{14}H_{10}N_3O_5^-$: 300.0626. Found 300.0646.

Anal. Calcd. for $C_{14}H_{11}N_3O_5.0.35H_2O$; C, 54.67; H, 3.83; N, 13.66. Found C, 54.64; H, 3.82; N, 13.36.

Mp: >300°C, with decomposition.



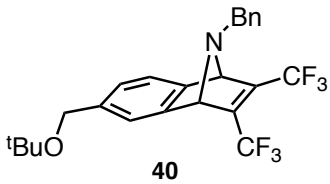
39

To a solution of compound **38** (49.3 mg, 0.1636 mmol) in 1 mL of acetonitrile was added DIEA (56 µL, 0.3276 mmol) and bromomethyl acetate (32 µL, 0.3263 mmol). The mixture was stirred at room temperature for 20 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 3:1) to give **39** (26.3 mg, 43%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 8.05-8.03 (2H, m), 7.46 (1H, brs), 6.45 (1H, br)s, 6.18 (1H, brs), 5.96 (2H, q, *J*=6.4 Hz), 3.83 (1H, brs), 3.69 (1H, brs), 2.53 (3H, s), 2.14 (3H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.63, 172.58, 169.50, 164.04, 142.50, 138.68, 131.00, 129.57, 123.53, 122.61, 79.89, 63.49, 58.19, 48.04, 44.87, 24.41, 20.69.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{17}H_{15}N_3NaO_7^+$: 396.0802. Found 396.0800.



40

Hexafluoro-2-butyne gas was flowed into CH_2Cl_2 (20 mL) at -78°C and **20** (660.7 mg, 2.2519 mmol) in CH_2Cl_2 (5 mL) was added into the solution. The acetone-dry ice cooling bath was removed and the mixture was stirred for 3 h at rt. After the reaction was completed, the solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 7:1) to give **40** (978.1 mg, 95%) as yellow oil.

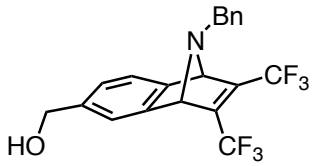
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) =7.37-7.28 (5H, m), 7.24 (2H, m), 7.09 (1H, d, *J*=7.2 Hz), 4.85 (2H, br), 4.43 (2H, s), 3.55 (2H, brs), 1.30 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 145.59, 144.14, 138.45, 136.73, 129.00, 128.57, 127.61, 125.11, 122.85, 122.01, 120.15, 73.47, 72.10, 63.81, 53.28, 27.55.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{24}H_{24}F_6NO^+$: 456.1757. Found 456.1745.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{24}H_{23}F_6NNaO^+$: 478.1576. Found 478.1567.

Anal. Calcd. for $C_{24}H_{23}F_6NO$; C, 63.29; H, 5.09; N, 3.08. Found C, 63.11; H, 5.22; N, 3.06.



41

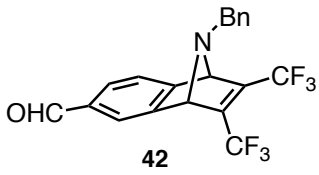
To solution of **40** (258.0 mg, 0.5665 mmol) in CH_2Cl_2 (5 mL) was added TfOH (0.1 mL, 1.1327 mmol) and stirred at 0 °C for 9 h. After reaction was completed, the mixture was poured into ice-cold saturated Na_2CO_3 solution, extracted with EtOAc, washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 5:1) to give **41** (204.6 mg, 90%) as colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) =7.40 (1H, s), 7.36-7.30 (4H, m), 7.26-7.24 (2H, m), 7.10 (1H, d, *J*=7.2 Hz), 4.87 (2H, brs), 4.68 (2H, s), 3.59 (2H, brs), 1.67 (1H, brs).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 145.89, 144.60, 139.55, 136.41, 129.07, 128.64, 127.74, 124.72, 122.77, 121.94, 120.07, 71.99, 64.65, 53.26.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{20}H_{16}F_6NO^+$: 400.1131. Found 400.1115.

Anal. Calcd. for $C_{20}H_{15}F_6NO$; C, 60.15; H, 3.79; N, 3.51. Found C, 59.88; H, 4.07; N, 3.57.



42

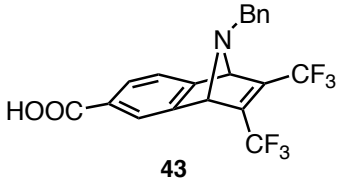
A solution of **41** (76.8 mg, 0.1923 mmol) in CH_2Cl_2 (2 mL) was added into solution of oxalyl chloride (33 μL, 0.3850 mmol) and DMSO (28 μL, 0.3946 mmol) in CH_2Cl_2 (1 mL) that has been stirred for 20 min at -78 ^oC under Ar and continued to stir at -78 ^oC for another 40 min. Then TEA (107 μL, 0.7677 mmol) was added into the mixture and warmed to rt. After 1 h, the reaction was quenched with water, extracted with CH_2Cl_2, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 7:1) to give **42** (69.4 mg, 91%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 9.99 (1H, s), 7.88 (1H, s), 7.68 (1H, d, *J*=7.2 Hz), 7.53 (1H, d, *J*=7.2 Hz), 7.38-7.32 (3H, m), 7.25-7.24 (2H, m), 4.96 (2H, brs), 3.62 (2H, brs).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 191.12, 151.97, 146.66, 136.08, 135.25, 131.36, 128.96, 128.72, 127.90, 125.23, 122.93, 122.54, 121.50, 119.83, 117.11, 71.84, 53.13.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{20}H_{14}F_6NO^+$: 398.0974. Found 398.0975.

Anal. Calcd. for $C_{20}H_{13}F_6NO$; C, 60.46; H, 3.30; N, 3.53. Found C, 60.29; H, 3.55; N, 3.61.



43

To a solution of **42** (69.4 mg, 0.1747 mmol) in *tert*-butanol (3 mL) and water (1.5 mL) was added 2-methyl-2-butene (255.8 mg, 3.6475 mmol), $NaClO_2$ (158.4 mg, 1.7514 mmol), and $NaH_2PO_4.2H_20$ (200.8 mg, 1.2871 mmol). The reaction was stirred at room temperature and after 5 h *tert*-butanol was evaporated. The aqueous solution was then acidified with HCl, extracted with EtOAc, dried over anhydrous Na_2SO_4, and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:Acetone = 3:1) to give **43** (71.8 mg, 99%) as amorphous white solid after recrystallized from CH_2Cl_2/*n*-hexane.

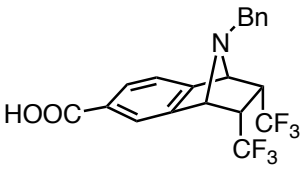
^{1}H-NMR (400 MHz, Acetone-d_6): δ = 8.14 (1H, s), 7.93 (1H, d, *J*=7.6 Hz), 7.68 (1H, d, *J*=7.2 Hz), 7.37-7.28 (5H, m), 5.27 (2H, d, *J*=12.8 Hz), 3.67 (2H, brs).

^{13}C-NMR (100 MHz, Acetone-d_6): δ (ppm) = 167.38, 151.69, 147.29, 137.81, 129.99, 129.90, 129.81, 129.45, 128.49, 126.54, 124.38, 123.86, 121.17, 118.48, 72.83, 53.67.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{20}H_{12}F_6NO_2^-$: 412.0778. Found 412.0780.

Anal. Calcd. for $C_{20}H_{13}F_6NO_2$; C, 58.12; H, 3.17; N, 3.39. Found C, 57.86; H, 3.45; N, 3.66.

Mp: 166.0-167.0 ^oC



44

To a solution of **43** (314.2 mg, 0.7602 mmol) in MeOH (15 mL), 10% Pd-C (57.0 mg) was added and stirred vigorously under H_2 for 12 h at rt. Pd-C was removed by filtration through Celite® and washed with MeOH. The solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 3:1) to give **44** (232.0 mg, 73%) as colorless oil.

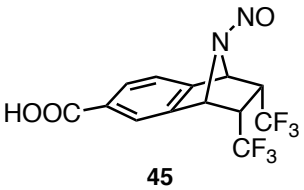
^{1}H-NMR (400 MHz, $CDCl_3$): δ = 9.18 (1H, brs), 8.17-8.14 (2H, m), 7.53 (1H, d, *J*=7.2 Hz), 7.35-7.30 (3H, m), 7.17-7.15 (2H, m), 4.49 (1H, s), 4.47 (1H, s), 3.62 (2H, s), 3.28 (2H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.40, 147.20, 141.91, 137.26, 130.47, 129.20, 128.74, 128.64, 127.69, 125.97, 125.83, 124.55, 123.05, 66.47, 66.31, 51.00, 46.85, 46.55, 46.30, 46.02.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{20}H_{16}F_6NO_2^+$: 416.1080. Found 416.1080.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{20}H_{15}F_6NNaO_2^+$: 438.0899. Found 438.0897.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{20}H_{14}F_6NO_2^-$: 414.0934. Found 414.0947.



45

To a solution of compound **44** (232.0 mg, 0.5586 mmol) in mixture of dioxane (7.5 mL) and water (2.5 mL) was added NBS (415.4 mg, 2.3339 mmol) that was divided into two portions and the second portion was added into the mixture after 12 h. After stirring at rt for 24 h, the mixture was evaporated to dry. Sodium nitrite (163.0 mg, 2.3623 mmol), formaldehyde 37% in water (53.2 mg, 0.6561 mmol) and PBS pH 6.4 (11.2 mL) was added and stirred for 23 h at room temperature. The mixture was extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified with flash column chromatography (*n*-

hexane:acetone = 3:1) to give **45** (170.1 mg, 86%) as yellow solid after recrystallized from $CHCl_3$/*n*-hexane.

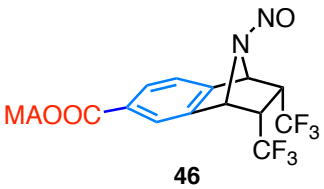
^{1}H-NMR (400 MHz, Acetone-d_6): δ = 8.25 (1H, brs), 8.13 (0.5H, s), 8.11 (0.5H, s), 7.77 (1H, brs), 6.80 (1H, brs), 6.29 (1H, brs), 4.20 (1H, brs), 3.84 (1H, brs).

^{13}C-NMR (100 MHz, Acetone-d_6): δ (ppm) = 166.94, 145.61, 144.58, 141.57, 140.43, 131.64, 130.99, 125.27, 124.57, 124.02, 64.32, 58.89, 47.87, 47.63, 44.31, 44.03.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{13}H_7F_6N_2O_3^-$: 353.0366. Found 353.0378.

Anal. Calcd. for $C_{13}H_8F_6N_2O_3$; C, 44.08; H, 2.28; N, 7.91. Found C, 44.10; H, 2.48; N, 8.15.

Mp: 157-160 °C.



46

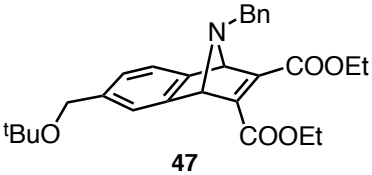
To a solution of compound **45** (150.7 mg, 0.4255 mmol) in 1 mL of acetonitrile was added DIEA (56 μL, 0.8482 mmol) and bromomethyl acetate (83.4 μL, 0.8505 mmol). The mixture was stirred at room temperature for 19 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:Acetone = 7:1) to give **46** (82.6 mg, 46%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.18 (1H, brs), 8.13 (1H, d, *J*=7.6 Hz), 7.60 (1H, brs), 6.37 (1H, brs), 6.00 (3H, q, *J*=6.0 Hz), 3.53 (1H, brs), 3.35 (1H, brs), 2.15 (3H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.60, 164.23, 143.91, 143.26, 139.30, 138.54, 130.89, 129.61, 127.51, 124.73, 124.30, 123.22, 121.96, 119.18, 79.72, 63.40, 58.12, 47.52, 43.50, 20.66.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{16}H_{12}F_6N_2NaO_5^+$: 449.0543. Found 449.0542.

Anal. Calcd. for $C_{16}H_{12}F_6N_2O_5$; C, 45.08; H, 2.84; N, 6.57. Found C, 44.92; H, 3.16; N, 6.33.



47

To a solution of **20** (2.5147 g, 8.5708 mmol) in 20 mL CH_2Cl_2 was added diethyl acetylene dicarboxylate (1.64 mL, 10.3126 mmol) and stirred at rt for 15 min. After the solvent was evaporated, the crude product was purified with flash column chromatography (*n*-hexane:EtOAc = 5:1 to 3:1) to give **47** (3.9314 g, 99%) as red orange oil.

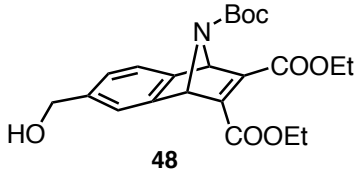
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.37 (1H, s), 7.33-7.26 (6H, m), 7.03 (1H, d, *J*=6.4 Hz), 4.90 (1H, s), 4.86 (1H, s), 4.40 (2H, s), 4.22 (4H, quint, *J*=7.2 Hz), 3.61 (2H, brs), 1.31-1.26 (15H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 163.38, 149.38, 146.51, 145.07, 137.29, 128.86, 128.11, 127.01, 124.46, 121.62, 73.02, 72.59, 63.75, 60.82, 53.03, 27.36, 13.76.

HRMS (ESI-TOF, $[M+H]^+$): Calcd. for $C_{28}H_{34}NO_5^+$: 464.2431. Found 464.2432.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{28}H_{33}NNaO_5^+$: 486.2251. Found 486.2252.

Anal. Calcd. for $C_{28}H_{33}NO_5$. $0.4H_2O$; C, 71.44; H, 7.24; N, 2.98. Found C, 71.43; H, 7.17; N, 2.95.



48

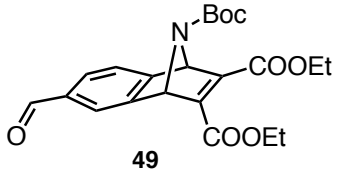
To a solution of **47** (2.2682 g, 4.8929 mmol) in 16 mL dioxane and 7 mL of H_2O, NBS (1.2836 g, 7.2116 mmol) was added and stirred at room temperature for 24 h. The solvent was evaporated and to the residue obtained, TFA (5 mL) was added and continue to stir at rt. After 2 h, TFA was evaporated. $(Boc)_2O$ (1.7268 g, 3.5597 mmol) and TEA (6.82 mL, 48.9309 mmol) in dioxane (40 mL) was added and stirred at room temperature for 12 h. Then the solvent was evaporated and the residue was purified with flash column chromatography (*n*-hexane:acetone = 3:1) to give **48** (1.4874 g, 73%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.43 (1H, s), 7.37 (1H, d, *J*=7.2 Hz), 7.04 (1H, dd, *J*=0.8 Hz,7.2 Hz), 5.75 (2H, s), 4.63 (2H, s), 4.23 (4H, quint, *J*=7.2 Hz), 1.38 (9H, s), 1.29 (6H, td, *J*=7.2 Hz, 2.0 Hz).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 162.14, 153.92, 150.39, 150.08, 145.45, 143.95, 139.12, 123.73, 121.04, 120.28, 80.99, 68.32, 63.62, 60.90, 60.88, 27.36, 13.36.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{22}H_{27}NNaO_7^+$: 440.1680. Found 440.1669.

Anal. Calcd. for $C_{22}H_{27}NO_7$; C, 63.30; H, 6.52; N, 3.36. Found C, 63.10; H, 6.59; N, 3.27.



49

To a solution of **48** (1.1236 g, 2.6915 mmol) in CH_2Cl_2 (15 mL) were added DMSO (0.6 mL) and DIEA (1.88 mL, 10.7936 mmol) and stirred at 0 ^oC. Pyridine-sulfur trioxide complex (861.4 mg, 5.4122 mmol) was added into the mixture and continued stirring for 4.5 h at 0 ^oC. The reaction was worked up by adding water, extracted with CH_2Cl_2, washed with brine and dried over anhydrous Na_2SO_4. The solvent

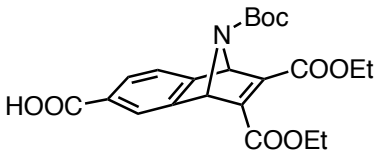
was evaporated and crude product was purified with flash column chromatography (*n*-hexane:acetone = 5:1 to 3:1) to give **49** (1.0472 g, 94%) as pale yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 9.45 (1H, s), 1.89 (1H, s), 7.61 (1H, dd, *J*=1.2 Hz, 7.6 Hz), 7.56 (1H, d, *J*=7.6 Hz), 5.81 (2H, s), 4.24 (4H, qd, *J*=7.2 Hz, 1.6 Hz), 1.36 (9H, s), 1.29 (6H, td, *J*=7.2 Hz, 2.8 Hz).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 190.95, 161.89, 153.80, 152.16, 150.26, 149.23, 146.76, 134.56, 130.67, 121.88, 120.27, 81.57, 68.36, 61.29, 27.60, 13.62.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{22}H_{25}NNaO_7^+$: 438.1523. Found 438.1524.

Anal. Calcd. for $C_{22}H_{25}NO_7$. $0.2H_2O$; C, 63.06; H, 6.11; N, 3.34. Found C, 63.02; H, 6.12; N, 3.19.



50

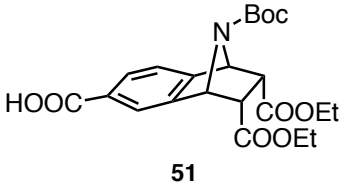
To a solution of **49** (663.4 mg, 1.5969 mmol) in *tert*-butanol (60 mL) and water (30 mL) was added 2-methyl-2-butene (3.0560 g, 43.5762 mmol), $NaClO_2$ (1.4502 g, 16.0349 mmol), and $NaH_2PO_4.2H_20$ (1.7543 g, 11.2448 mmol). The reaction was stirred at room temperature and after 3.5 h *tert*-butanol was evaporated. The aqueous solution was then acidified with HCl, extracted with EtOAc, dried over anhydrous Na_2SO_4, and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 3:1) to give **50** (616.4 mg, 89%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.09 (1H, s), 7.92 (1H, dd, *J*=7.6 Hz, 1.6 Hz), 7.51 (7.6 Hz), 5.82 (2H, s), 4.25 (4H, qd, *J*=7.2 Hz, 3.6 Hz), 1.38 (9H, s), 1.31 (6H, td, *J*=7.2 Hz, 4.8 Hz).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 170.39, 162.01, 153.94, 151.49, 150.43, 149.51, 146.16, 129.18, 127.21, 122.54, 121.46, 81.66, 68.39, 61.34, 61.32, 27.60, 13.62.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{22}H_{25}NNaO_8^+$: 454.1472. Found 454.1458.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{22}H_{24}NO_8^-$: 430.1507. Found 430.1514.



51

To a solution of **50** (284.9 mg, 0.6604 mmol) in MeOH (10 mL), 10% Pd-C (54.8 mg) was added and stirred vigorously under H_2 for 4 h at rt. Pd-C was removed by filtration through Celite® and washed with MeOH. The solvent was evaporated

and the residue obtained was purified with flash column chromatography (*n*-hexane:Acetone = 3:1) to give **51** (228.6 mg, 80%) as white foam solid.

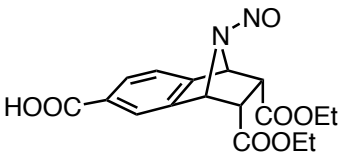
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.05 (1H, s), 8.01 (1H, dd, *J*=1.2 Hz, 7.6 Hz), 7.47 (1H, d, *J*=7.6 Hz), 5.33 (2H, s), 4.02-3.86 (4H, m), 3.72-3.67 (2H, m), 1.41 (9H, s), 1.15-1.10 (6H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.29, 169.18, 169.04, 154.16, 147.58, 142.30, 129.35, 128.00, 123.67, 122.59, 81.41, 63.25, 60.83, 60.70, 47.40, 47.18, 27.98, 13.81.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{22}H_{27}NNaO_8^+$: 456.1629. Found 456.1632.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{22}H_{26}NO_8^-$: 432.1664. Found 432.1689.

Anal. Calcd. for $C_{22}H_{27}NO_8$. $0.5H_2O$; C, 59.72; H, 6.38; N, 3.17. Found C, 59.72; H, 6.36; N, 3.07.



52

Compound **51** (387.9 mg, 0.8949 mmol) was dissolved in TFA (2.0 mL) and the mixture was stirred for 20 minutes at rt. Then TFA was removed by evaporation. Sodium nitrite (248.3 mg, 3.5985 mmol), formaldehyde solution 37% (96.3 mg, 1.1865) and PBS pH 6.4 (18 mL) was added into the mixture and stirred for 17 h at rt. The mixture was extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography (*n*-hexane:Acetone = 3:1) to give **52** (306.8 mg, 95%) as red orange solid after recrystallization (*n*-hexane/EtOAc).

^{1}H-NMR (400 MHz, $CDCl_3$, Two isomers with respect to the N-NO bond were present in a ratio of 1:1: δ (ppm) = 8.19 (0.5H, brs), 8.09 (1.5H, brs), 7.62 (0.5H, brs), 7.52 (0.5H, brs), 6.29 (1H, brs), 6.04 (1H, brs), 4.00-3.95 (4H, m), 3.71 (1H, brs), 3.50 (1H, brs), 1.15 (6H, quint, *J*=7.2 Hz).

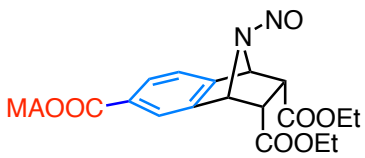
^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.07, 168.09, 167.96, 145.40, 144.14, 140.35, 139.04, 130.20, 128.84, 124.77, 124.23, 123.70, 123.21, 64.49, 61.40, 61.28, 59.25, 48.71, 48.52, 44.67, 44.45, 13.92.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{17}H_{18}N_2NaO_7^+$: 385.1006. Found 385.0981.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{17}H_{17}N_2O_7^-$: 361.1041. Found 361.1063.

Anal. Calcd. for $C_{17}H_{18}N_2O_7$; C, 56.35; H, 5.01; N, 7.73. Found C, 56.08; H, 5.09; N, 7.44.

Mp: 148.0-149.5 ^{o}C.



53

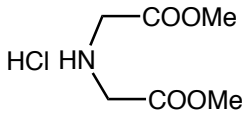
To a solution of compound **52** (211.7 mg, 0.5843 mmol) in 4 mL of acetonitrile was added DIEA (200 μL, 1.1699 mmol) and bromomethyl acetate (115 μL, 1.1727 mmol). The mixture was stirred at room temperature for 15 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:EtOAc = 3:1 to 2:1) to give **53** (142.4 mg, 56%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): Two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 8.17 (0.5H, brs), 8.05 (1.5H, brs), 7.59 (0.5H, brs), 7.52 (0.5H, brs), 6.26 (1H, brs), 6.02 (1H, brs), 5.99-5.95 (2H, m), 3.95 (4H, brs), 3.69 (1H, brs), 3.49 (1H, brs), 2.13 (3H, s), 1.15 (6H, quint, *J*=6.8 Hz).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.45, 167.96, 167.89, 164.43, 145.25, 144.00, 140.33, 139.03, 129.84, 128.37, 124.48, 123.95, 123.55, 123.05, 79.36, 64.31, 61.20, 61.10, 59.06, 48.47, 48.30, 44.49, 44.28, 20.57, 13.79, 13.77.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{20}H_{22}N_2NaO_9^+$: 457.1218. Found 457.1200.

Anal. Calcd. for $C_{20}H_{22}N_2O_9$; C, 55.30; H, 5.10; N, 6.45. Found C, 55.05; H, 5.25; N, 6.52.



54

To a solution of iminodiacetic acid (800.1 g, 6.0113 mmol) in MeOH (80 mL), thionyl chloride (0.88 mL) was added dropwise at rt. The mixture was refluxed at 80 °C for 3 h. After the mixture was cooled down to rt, MeOH was evaporated to give **54** (1.1340 g, 95%) as colorless needle crystal after recrystallization (MeOH/EtOAc).

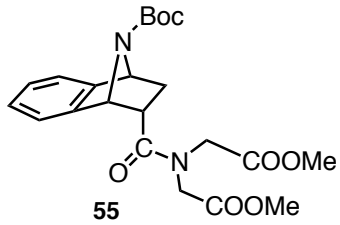
^{1}H-NMR (400 MHz, MeOD) : δ (ppm) = 4.08 (4H, s), 3.86 (6H, s).

^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 168.01, 53.64, 47.88.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_6H_{11}NNaO_4^+$: 184.0580. Found 184.0535.

Anal. Calcd. for $C_6H_{12}ClNO$; C, 36.47; H, 6.12; N, 7.09. Found C, 36.17; H, 5.84; N, 7.17.

Mp: 173.0-174.5 °C.



55

To a solution of **16** (398.5 g, 1.3783 mmol) in DMF (11 mL), EDCI.HCl (652.2 mg, 3.4022 mmol), compound **54** (348.6 g, 1.7640 mmol) and TEA (195 μL, 1.3991 mmol) was added at rt under Ar. The mixture was stirred at rt for 26 h. Water was added into the mixture, extracted with EtOAc, washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified

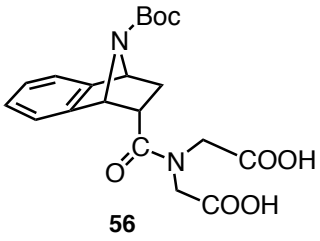
with flash column chromatography (*n*-hexane:acetone = 1:1) to give **55** (506.1 mg, 85%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.34-7.10 (4H, m), 5.15 (1H, s), 5.12 (1H, s), 4.49-4.35 (2H, m), 4.03 (2H, s), 3.85 (3H, s), 3.68 (3H, s), 3.46 (1H, s), 2.30 (1H, s), 1.90 (1H, s), 1.42 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 170.54, 169.63, 155.00, 144.63, 139.77, 127.37, 126.39, 122.16, 119.02, 80.62, 63.02, 61.80, 52.68, 52.09, 49.57, 47.66, 42.03, 31.84, 28.18.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{22}H_{28}N_2NaO_7^+$: 455.1789. Found 455.1771.

Anal. Calcd. for $C_{22}H_{28}N_2O_7$; C, 61.10; H, 6.53; N, 6.48. Found C, 60.92; H, 6.59; N, 6.34.



56

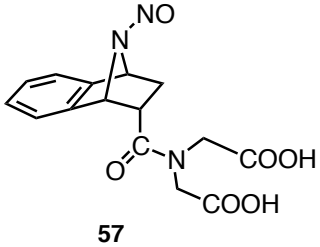
To a solution of **55** (149.9 mg, 0.3466 mmol) in 30 mL of MeOH-H_2O (1:1), $LiOH.H_2O$ (58.9 mg, 1.4037 mmol) was added and the mixture was stirred at room temperature for 24 h. Then MeOH was evaporated and the aqueous solution's pH was adjusted to 3 with 0.5 N HCl and was extracted with EtOAc. The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to give **56** (137.1 mg, 98%) as white powder after recrystallization (CH_2Cl_2/*n*-hexane).

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 10.33 (2H, brs), 7.30-7.07 (4H, m), 5.27 (1H, s), 5.13 (1H, d, *J*=4.0 Hz), 4.41 (2H, m), 4.02 (2H, s), 3.51 (1H, s), 2.30 (1H, m), 1.91 (1H, m), 1.41 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.38, 171.90, 171.76, 155.05, 144.52, 139.78, 127.39, 126.60, 122.20, 119.08, 81.65, 62.70, 61.80, 50.55, 49.49, 42.11, 31.99, 28.20.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{20}H_{23}N_2O_7^-$: 403.1511. Found 403.1523.

Mp: > 300 ^{o}C with decomposition



57

Compound **56** (87.3 mg, 0.2159 mmol) was dissolved in TFA (1.3 mL) at 0 ^{o}C and the mixture was stirred for 20 min at 0 ^{o}C. Then TFA was removed by evaporation. Sodium nitrite (60.3 mg, 0.8739 mmol), paraformaldehyde (11.3 mg) and PBS pH 6.4 (4.4 mL) was added and stirred for 19 h at room temperature. The mixture was acidified with HCl 1 M until pH 3, extracted with EtOAc,

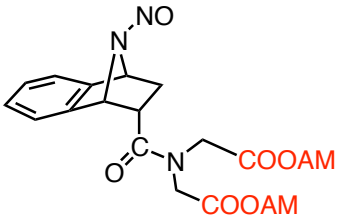
and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give **57** (66.5 mg, 92%) as pale yellow solid after recrystallization (EtOAc/*n*-hexane).

^{1}H-NMR (400 MHz, MeOD): The two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 7.51-7.12 (4H, m), 6.16 (0.5H, m), 6.08 (0.5H, m), 6.00 (0.5H, m), 5.91 (0.5H, m), 4.69 (0.5H, m), 4.58 (0.5H, m), 4.44 (0.5H, m), 4.34 (0.5H, m), 3.98 (2H, m), 3.77 (0.5H, m), 3.55 (0.5H, m), 2.47 (0.5H, m), 2.20 (0.5H, m), 2.07 (0.5H, m), 1.88 (0.5H, m).

^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 172.71, 172.63, 172.27, 171.89, 171.76, 144.49, 143.25, 140.16, 138.73, 129.09, 129.01, 128.19, 124.31, 123.77, 121.04, 120.46, 65.57, 64.57, 59.51, 59.04, 50.91, 49.43, 43.82, 40.42, 33.81, 30.40.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{15}H_{14}N_3O_6^-$: 332.0888. Found 332.0903.

Mp: 174-176 ^oC, with decomposition.



58

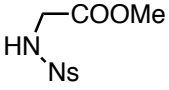
To a solution of compound **57** (37.7 mg, 0.1131 mmol) in 4 mL of CH_2Cl_2-CH_3CN (3:1) was added DBU (36 µL, 0.2412 mmol) and bromomethyl acetate (24 µL, 0.2447 mmol). The mixture was stirred at room temperature for 18 h and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 2:1) to give **58** (26.9 mg, 50%) as orange oil.

^{1}H-NMR (400 MHz, $CDCl_3$): The two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 7.49-7.20 (4H, m), 6.03 (1H, m), 5.88 (3H, m), 5.71 (2H, s), 4.43 (2H, m), 4.05 (2H, m), 3.45 (0.5H, m), 3.30 (0.5H, m), 2.41 (0.5H, m), 2.25 (1H, m), 2.16 (3H, m), 2.10 (3H, s), 1.91 (0.5H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.38, 167.93, 167.70, 142.46, 141.18, 138.09, 136.43, 128.28, 127.39, 123.18, 122.70, 120.05, 119.41, 79.87, 79.42, 64.13, 63.13, 58.03, 57.74, 49.53, 47.88, 42.92, 39.10, 33.51, 29.73, 20.60, 20.57.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{21}H_{23}N_3NaO_{10}^+$: 500.1270. Found 500.1286.

Anal. Calcd. for $C_{21}H_{23}N_3O_{10}$; C, 52.83; H, 4.86; N, 8.80. Found C, 52.97; H, 4.87; N, 8.73.



59

To a solution of Glycine methyl ester HCl (220.0 mg, 1.7522 mmol) and TEA (753 μL, 5.4025 mmol) in MeOH (2 mL) was added p-NsCl (482.7 mg, 2.1781 mmol) in THF (8 mL) dropwise. The mixture was stirred at room temperature and after 30 minutes, the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 4:1 to 3:1) to give **59** (435.6 mg, 91%) as pale yellow needle after recrystallized from *n*-hexane/acetone.

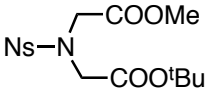
^{1}H-NMR (400 MHz, Acetone-d_6): δ (ppm) = 8.46-8.42 (2H, m), 8.17-8.14 (2H, m), 7.33 (1H, brs), 3.95 (2H, d, *J*=6.0 Hz), 3.57 (3H, s).

^{13}C-NMR (100 MHz, Acetone-d_6): δ (ppm) = 170.96, 152.06, 148.66, 130.37, 126.16, 53.45, 45.86.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_9H_{10}N_2NaO_6S^+$: 297.0152. Found 297.0152.

Anal. Calcd. for $C_9H_{10}N_2O_6S$; C, 39.42; H, 3.68; N, 10.21. Found C, 39.39; H, 3.67; N, 10.36.

Mp: 147-148 ^oC.



60

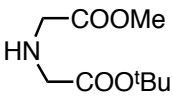
To a solution of **59** (124.9 mg, 0.4554 mmol) in DMF (3 mL) was added NaH 60% in oil (22.3 mg, 0.5575 mmol) and *tert*-butyl bromo acetate (81 μL, 0.5481 mmol). The mixture was stirred at room temperature for 4 h and then quenched by addition of water at 0 ^oC. Extraction of the mixture with EtOAc, washed with brine, dried over anhydrous Na_2SO_4, and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 3:1) to give **60** (160.5 mg, 91%) as yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.32 (2H, d, *J*=9.2 Hz), 8.01 (2H, d, *J*=8.8 Hz), 4.21 (2H, s), 4.10 (2H, s), 3.68 (3H, s), 1.38 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 168.77, 167.15, 150.03, 145.38, 128.54, 124.08, 82.67, 52.37, 49.03, 48.35, 27.84.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{15}H_{20}N_2NaO_8S^+$: 411.0833. Found 411.0829.

Anal. Calcd. for $C_{15}H_{20}N_2O_8S$; C, 46.39; H, 5.19; N, 7.21. Found C, 46.31; H, 5.15; N, 6.95.



61

Potassium carbonate (3.8470 g, 27.8345 mmol) and PhSH (1.4 mL, 13.7217 mmol) was added into the solution of **60** (2.6734 g, 9.2700 mmol) in DMF (13 mL) and was stirred at room temperature. After 24 h, saturated

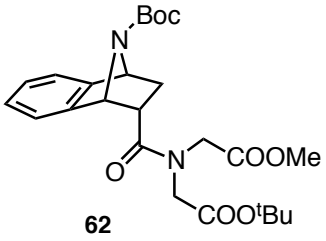
$NaHCO_3$ solution was added into the mixture and extracted with EtOAc, dried over anhydrous Na_2SO_4, and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 4:1) to give **61** (1.0650 g, 57%) as orange oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.73 (3H, s), 3.46 (2H, s), 3.35 (2H, s), 1.87 (1H, br), 1.46 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.26, 170.98, 81.38, 51.83, 50.91, 50.01, 28.08.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_9H_{17}NNaO_4^+$: 226.1050. Found 226.1049.

Anal. Calcd. for $C_9H_{17}NO_4$; C, 53.19; H, 8.43; N, 6.89. Found C, 53.40; H, 8.40; N, 6.85.



62

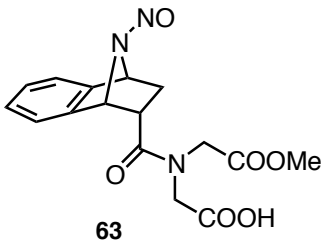
To a solution of **17** (572.3 mg, 1.9780 mmol), **61** (481.2 mg, 2.3677 mmol), and DIEA (1.8 mL, 19.6642 mmol) in DMF (5.0 mL), HATU (1.1246 g, 2.9577 mmol) was added and stirred at 50 ^{o}C for 3 h. After the reaction was completed, water was added into the mixture, extracted with EtOAc, washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 4:1 to 2:1) to give **62** (826.7 mg, 88%) as pale orange foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-alkyl bond was present in 3:2 ratio. δ (ppm) = 7.36 -7.32 (1H, m), 7.24-7.22 (2H, m), 7.18-7.07 (2H, m), 5.12 (2H, brs), 4.38-3.91 (4H, m), 3.83 (1.2H, s), 3.66 (1.8H, s), 3.45 (1H, brs), 2.29 (1H, brs), 1.90 (1H, m), 1.53 (5.5H, s), 1.41 (5.4H, s), 1.41 (3.4H, s), 1.40 (3.5H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 170.44, 170.32, 169.72, 169.69, 168.35, 168.23, 154.92, 144.55, 139.77, 127.26, 127.24, 126.31, 126.25, 122.21, 122.11, 118.93, 82.97, 81.84, 80.50, 80.47, 63.07, 61.75, 52.54, 51.97, 50.35, 49.51, 48.27, 47.71, 42.14, 31.70, 28.17, 28.10, 28.04, 27.96.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{25}H_{34}N_2NaO_7^+$: 497.2258. Found 497.2263.

Anal. Calcd. for $C_{25}H_{34}N_2O_7$; C, 63.27; H, 7.22; N, 5.90. Found C, 63.02; H, 7.19; N, 5.84.



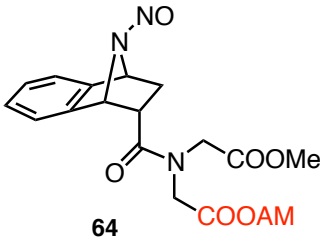
63

Compound **62** (560.9 mg, 1.1820 mmol) was dissolved in TFA (4.0 mL) and the mixture was stirred for 2 h at room temperature. Then TFA was removed by evaporation. Sodium nitrite (325.0 mg, 4.7101 mmol), paraformaldehyde (38.5 mg) and PBS pH 6.4 (23.6 mL) was added into the mixture and stirred for 16 h at room temperature. The mixture was acidified with HCl 1M until pH 3, extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 2:1) to give **63** (294.2 mg, 72%) as yellow foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): The two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 8.49 (1H, brs), 7.45 (0.5H, m), 7.36 (1H, m), 7.29-7.19 (2.5H, m), 6.05-5.90 (2H, m), 4.50 (2H, m), 4.03-4.00 (2H, m), 3.85 (1.5H, s), 3.67 (1.5H, s), 3.51 (0.5H, m), 3.34 (0.5H, m), 2.42 (0.5H, m), 2.23 (0.5H, m), 2.16-2.13 (0.5H, m), 1.95-1.92 (0.5H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.49, 171.67, 170.03, 169.87, 169.71, 169.64, 169.57, 169.42, 142.30, 142.23, 140.96, 137.98, 136.40, 136.33, 128.30, 127.44, 123.23, 122.74, 120.08, 119.46, 64.24, 63.32, 63.23, 58.32, 58.19, 57.91, 57.84, 52.98, 52.31, 49.79, 49.71, 48.24, 42.80, 42.65, 39.13, 33.37, 33.24, 29.71.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{16}H_{16}N_3O_6^-$: 346.1045. Found 346.1065.



64

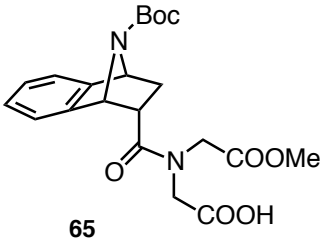
To a solution of compound **63** (204.0 mg, 0.5874 mmol) in 5 mL of acetonitrile was added DIEA (1.0 mL, 4.3330 mmol) and bromomethyl acetate (69 µL, 0.7036 mmol) in 0.8 mL acetonitrile. The mixture was stirred at room temperature for 7 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 3:2 to 1:1) to give **64** (182.5 mg, 74%) as yellow foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 7.48 (0.5H, m), 7.36 (1H, m), 7.29-7.20 (2.5H, m), 6.03-6.00 (1H, m), 5.91-5.85 (2H, m), 5.68 (1H, s), 4.56-4.32 (2H, m), 4.09-3.97 (2H, m), 3.84 (1.7H, s), 3.65 (1.3H, s), 3.49-3.46 (0.5H, m), 3.32-3.30 (0.5H, m), 2.42-2.36 (0.5H, m), 2.23-2.12 (2.3H, m), 2.07 (1.7H, s), 1.93-1.90 (0.5H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.51, 169.40, 169.32, 169.22, 169.12, 168.04, 167.70, 142.37, 141.09, 138.10, 136.46, 128.14, 127.26, 123.15, 123.11, 122.65, 119.94, 119.31, 79.79, 79.69, 79.28, 64.06, 63.04, 58.03, 57.95, 57.63, 52.78, 52.09, 49.49, 47.77, 42.79, 39.04, 38.99, 33.36, 29.60, 20.54, 20.50.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{19}H_{21}N_3NaO_8^+$: 442.1221. Found 442.1220.

Anal. Calcd. for $C_{19}H_{21}N_3O_8$; C, 54.41; H, 5.05; N, 10.02. Found C, 54.53; H, 4.99; N, 9.73.



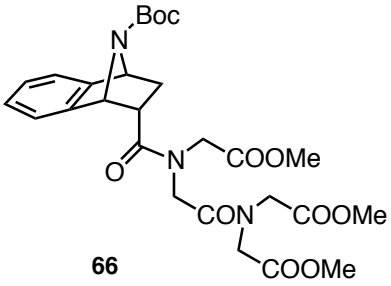
65

TFA (1 mL) was added into **62** (171.6 mg, 0.3616 mmol) and stirred at room temperature. After 2 h, TFA was evaporated and dioxane (5 mL) was added into the flask, followed by $(Boc)_2O$ (128.0 mg, 0.5865 mmol) and TEA (1 mL, 7.1764 mmol). The mixture was stirred at room temperature for 24 h. Then dioxane was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 3:1 to 1:1) to give **65** (55.4 mg, 37%) as pale orange foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 9.75 (1H, brs), 7.33-7.30 (1H, m), 7.25-7.22 (1H, m), 7.19-7.15 (1H, m), 7.13-7.09 (1H, m), 5.25 (1H, brs), 5.14 (1H, brs), 4.51-4.31 (2H, m), 4.04 (2H, m), 3.84 (1.5H, s), 3.67 (1.5H, s), 3.48 (1H, brs), 2.30 (1H, brs), 1.91 (1H, brs), 1.42 (4.5H, s), 1.41 (4.5H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.17, 171.22, 170.61, 169.86, 169.53, 154.99, 144.52, 144.28, 139.61, 127.33, 127.31, 126,42, 122.13, 122.08, 119.01, 118.93, 81.42, 80.88, 62.85, 61.77, 52.71, 52.10, 49.69, 49.64, 48.10, 47.93, 42.03, 31.84, 28.14, 28.10.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{21}H_{25}N_2O_7^-$: 417.1667. Found 417.1693.



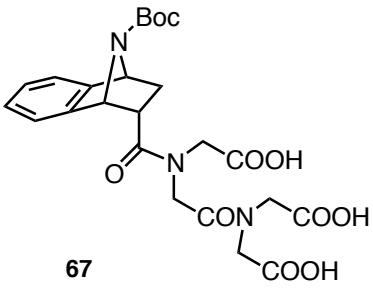
66

To a solution of **65** (58.1 mg, 0.1389 mmol), **54** (109.3 mg, 0.5531 mmol), and DIEA (127 μL, 1.3885 mmol) in DMF (3.0 mL), HATU (106.1 mg, 0.2791 mmol) was added and stirred at 40 ^oC for 3 h. After the reaction was completed, water was added into the mixture, extracted with EtOAc, washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 3:1) to give **66** (64.0 mg, 82%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the amide bond was present in 3:2 ratio. δ (ppm) = 7.37-7.30 (1H, m), 7.23-7.06 (3H, m), 5.15-5.08 (2H, m), 4.56-3.99 (8H, m), 3.82 (1.7H, s), 3.81 (1.3H, s), 3.71 (1.3H, s), 3.69 (1.7H, s), 3.68 (1.7H, s), 3.64 (1.2H, s), 3.45-3.42 (1H, m), 2.25 (1H, brs), 1.87 (1H, brs), 1.39 (5H, s), 1.38 (4H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 170.73, 170.35, 170.14, 169.98, 169.09, 169.03, 169.01, 168.94, 168.73, 168.69, 154.99, 154.83, 144.54, 139.98, 139.72, 127.15, 127.04, 126.27, 126.22, 122.41, 122.11, 118.87, 118.76, 80.48, 80.31, 62.82, 61.79, 52.85, 52.46, 52.32, 52.14, 51.85, 49.58, 49.49, 49.08, 48.79, 48.74, 48.16, 47.54, 46.42, 41.99, 31.75, 28.07, 28.03.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{27}H_{35}N_3NaO_{10}^+$: 584.2215. Found 584.2210.



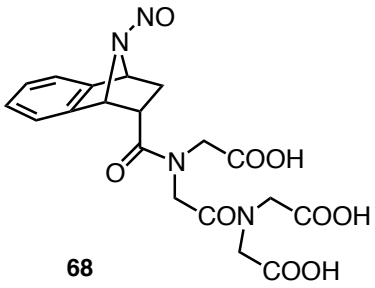
67

To a solution of **66** (796.2 mg, 1.4178 mmol) in 20 mL of MeOH-H_2O (1:1), $LiOH.H_2O$ (369.7 mg, 8.8108 mmol) was added and the mixture was stirred at rt for 3 h. Then MeOH was evaporated and the aqueous solution's pH was adjusted to 3 with HCl 1 N to give cloudy solution, which was extracted with EtOAc. The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to give **67** (416.7 mg, 100%) as white foam solid.

^{1}H-NMR (400 MHz, MeOD): The two isomers with respect to the amide bond were present in a ratio of 3:2. δ (ppm) = 7.40-7.34 (1H, m), 7.26-7.22 (1H, m), 7.17-7.06 (2H, m), 5.25 (0.6H, d, *J*=4.4 Hz), 5.18 (0.4H, d, *J*=4.0 Hz), 5.09-5.06 (1H, m), 4.76-4.60 (2H, m), 4.36-4.07 (5H, m), 3.90 (1H, brs), 3.61-3.56 (1H, m), 2.35-2.26 (1H, m), 1.82-1.74 (1H, m), 1.42 (3.7H, s), 1.39 (5.3H, s).

^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 173.35, 172.65, 172.59, 172.33, 172.22, 172.02, 171.57, 171.40, 156.79, 156.60, 146.46, 146.14, 142.08, 141.37, 128.16, 127.96, 127.36, 127.17, 123.75, 123.31, 119.95, 82.02, 81.89, 64.36, 62,89, 50.85, 50.53, 50.14, 49.85, 49.78, 48.47, 43.38, 32.67, 28.46, 28.42.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{24}H_{28}N_3O_{10}^-$: 518.1780. Found 518.1785.



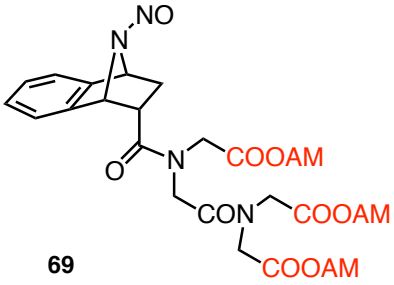
68

Compound **67** (416.7 mg, 0.8021 mmol) was dissolved in TFA (2.0 mL) and the mixture was stirred for 20 min at room temperature. Then TFA was removed by evaporation. Sodium nitrite (221.3 mg, 3.2072 mmol), paraformaldehyde (26.3 mg) and PBS pH 6.4 (16 mL) was added into the mixture and stirred for 16 h at room temperature. The mixture was acidified with HCl 1M until pH 3, extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography ($CHCl_3$:MeOH = 20:1 to 10:1) to give **68** (57.6 mg, 16%) as yellow foam solid.

^{1}H-NMR (400 MHz, MeOD): The two isomers with respect to the N-NO bond were present in a ratio of 1:1. δ (ppm) = 7.57-7.17 (4H, m), 5.22-5.90 (2H, m), 4.99-3.87 (8H, m), 3.75-3.73 (0.5H, m), 3.51 (0.5H, br), 2.50-2.45 (0.5H, m), 2.22-2.18 (0.5H, m), 2.10-2.01 (0.5H, m), 1.92-1.88 (0.5H, m).

^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 172.61, 172.51, 172.41, 172.26, 172.09, 171.73, 171.55, 171.27, 170.95, 144.62, 143.26, 140.36, 139.18, 130.51, 129.67, 129.09, 128.94, 128.29, 128.94, 128.29, 128.20, 125.19, 124.78, 124.48, 124.06, 123.94, 121.67, 120.98, 120.49, 120.34, 65.54, 64.60, 64.24, 63.56, 59.78, 59.54, 59.10, 58.95, 58.37, 51.06, 50.64, 50.57, 50.31, 49.95, 49.84, 43.86, 43.70, 40.79, 40.45, 34.02, 33.89, 30.75, 30.48.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{19}H_{19}N_4O_9^-$: 447.1158. Found 447.1160.



69

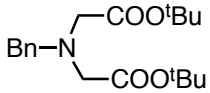
To a solution of compound **68** (315.3 mg, 0.7032 mmol) in 17 mL of CH_3CN was added DIEA (1.2 mL, 7.0195 mmol) and bromomethyl acetate (413 µL, 4.2187 mmol). The mixture was stirred at room temperature for 24 h and the solvent was evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 2 : 1) to give **69** (186.7 mg, 40%) as orange foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 7.51-7.16 (4H, m), 6.05-5.67 (8H, m), 4.74-3.68 (8H, m), 3.53-3.29 (1H, m), 2.40-2.35 (0.5H, m), 2.13-2.05 (10H, m), 1.94-1.90 (0.5H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 169.85, 169.60, 169.50, 169.43, 169.34, 168.79, 168.68, 168.45, 168.29, 167.42, 167.30, 167.26, 142.40, 141.14, 138.36, 138.17, 136.67,

136.60, 128.00, 127.16, 123.33, 123.14, 122.73, 122.63, 119.87, 119.78, 119.24, 119.14, 80.17, 79.63, 79.53, 79.37, 79.21, 63.97, 63.88, 63.06, 62.97, 58.03, 57.89, 57.56, 57.50, 49.44, 49.14, 49.00, 48.81, 48.19, 47.89, 46.64, 42.67, 42.53, 38.97, 33.28, 33.14, 29.60, 29.43, 20.49, 20.46.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{28}H_{32}N_4NaO_{15}^+$: 687.1756. Found 687.1759.



70

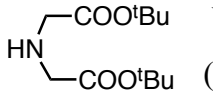
To a solution of *N*-benzylamine (6.3843 g, 59.5828 mmol) in acetonitrile was added potassium carbonate (20.5844 g, 148.9411 mmol) and *tert*-butyl bromoacetate (18.5 mL, 125.1922 mmol). After refluxed for 15 h, the reaction was stopped and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:Et_2O = 10:1) to give **70** (17.7196 g, 89%) as colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.41-7.39 (2H, m), 7.33-7.30 (2H, m), 7.27-7.23 (1H, m), 3.89 (2H, s), 3.42 (4H, s), 1.46 (18H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 170.53, 138.54, 129.02, 128.21, 127.13, 80.78, 57.44, 55.03, 28.10.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{19}H_{29}NNaO_4^+$: 358.1989. Found 358.1989.

Anal. Calcd. for $C_{19}H_{29}NO_4$; C, 68.03; H, 8.71; N, 4.18. Found C, 67.85; H, 8.61; N, 4.31.



71

Wet 20% $Pd(OH)_2$ on carbon (823.1 mg) was added into solution of **70** (4.0808 g, 12.1656 mmol) in dry THF (150 mL) and stirred at room temperature under H_2. After 5 h, the mixture was filtered through celite and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 2:1) to to give **71** (2.8574 g, 96%) as colorless brick.

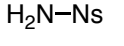
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.32 (4H, s), 1.93 (1H, brs), 1.45 (18H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.02, 81.20, 50.90, 28.06.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{12}H_{23}NNaO_4^+$: 268.1519. Found 268.1518.

Anal. Calcd. for $C_{12}H_{23}NO_4$; C, 58.75; H, 9.45; N, 5.71. Found C, 58.71; H, 9.32; N, 5.73.

Mp: 36.0-37.5 ^oC.



72

4-Nitrobenzensulfonyl chloride (2.2118 g, 9.9801 mmol) was added to a solution of concentrated ammonium hydroxide (3 mL, 39.87 mmol) in THF (5 mL) at 0°C. The reaction was stirred at 0°C for 1 h and then at room temperature for additional 1

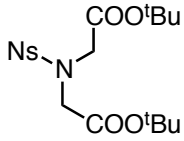
h. The THF was removed under vaccum, and the residue was purified by recrystallization to give **72** (1.8316 g, 91%) as white amorphous (acetone/*n*-hexane).

^{1}H-NMR (400 MHz, Acetone-*d*6): δ (ppm) = 8.43 (2H, d, *J*=8.8 Hz), 8.17 (2H, d, *J*=9.2 Hz), 6.97 (2H, brs).

^{13}C-NMR (100 MHz, Acetone-*d*6): δ (ppm) = 150.67, 150.39, 128.42, 125.10.

Anal. Calcd. for $C_6H_6N_2O_4S$; C, 35.64; H, 2.99; N, 13.86. Found C, 35.90; H, 3.11; N, 13.89.

Mp: 179.0-180.3 ^{o}C.



73

To a solution of **72** (196.6 mg, 0.9724 mmol) in DMF (10 mL) was added NaH (98.3 mg, 2.4575 mmol) at 0 ^{o}C, followed by *tert*-butyl bromo acetate (360 μL, 2.4381 mmol). The mixture was stirred for 5 h at 0 ^{o}C and gradually warm to rt. After the reaction was finished, water was added into the mixture, followed by EtOAc extraction. The organic phase was then washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 5:1) to give **73** (351.1 mg, 84%) as colorless needle after recrystallization from CH_2Cl_2/*n*-hexane.

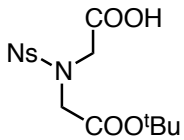
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.32 (2H, d, *J*=9.2 Hz), 8.02 (2H, d, *J*=9.2 Hz), 4.09 (4H, s), 1.41 (18H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 167.37, 150.13, 145.66, 128.56, 124.08, 82.79, 49.17, 27.93.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{18}H_{26}N_2NaO_8S^+$: 453.1302. Found 453.1309.

Anal. Calcd. for $C_{18}H_{26}N_2O_8S$; C, 50.22; H, 6.09; N, 6.51. Found C, 50.17; H, 6.01; N, 6.54.

Mp: 136.5-138 ^{o}C.



74

To a solution of **73** (3.9660 g, 9.2131 mmol) in MeOH (250 mL) was added LiOH. H_2O (389.8 mg, 9.2898 mmol) in water (10 mL) and stirred at room temperature for 16 h. Water was added into the mixture and MeOH was then evaporated. Extraction of the aqueous solution with EtOAc was performed to remove unreacted starting material. Remained aqueous solution was acidified with HCl and extracted with EtOAc. The organic phase was washed with brine, dried over anhydrous Na_2SO_4 and evaporated to give pale yellow foam solid. Further

purification by recrystallization (*n*-hexane/acetone) gave **74** (2.7163 g, 79%) as white amorphous solid.

^{1}H-NMR (400 MHz, MeOD): δ (ppm) = 8.39 (2H, d, *J*=9.2 Hz), 8.10 (2H, d, *J*=8.8 Hz), 4.21 (2H, s), 4.13 (2H, s), 1.40 (9H, s).

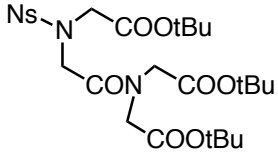
^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 171.66, 169.32, 151.65, 146.97, 129.92, 125.27, 83.57, 50.41, 28.17.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{14}H_{18}N_2NaO_8S^+$: 397.0676. Found 397.0678.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{14}H_{17}N_2O_8S^-$: 373.0711. Found 373.0733.

Anal. Calcd. for $C_{14}H_{18}N_2O_8S$; C, 44.92; H, 4.85; N, 7.48. Found C, 44.58; H, 4.71; N, 7.47.

Mp: 212.0-213.5 °C.



75

To a solution of **74** (2.6315 g, 7.0292 mmol), **71** (3.4440 g, 14.0391 mmol), and DIEA (1.93 mL, 21.0844 mmol) in DMF (20 mL), $HOBt.H_2O$ (1.8967 g, 14.0372 mmol) and DIC (2.16 mL, 14.0349 mmol) were added and stirred at 50 °C for 17 h. After the reaction was completed, water was added into the mixture, extracted with EtOAc, washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 5:1) to give **75** (3.4788 g, 82%) as colorless brick after recrystallization from CH_2Cl_2/*n*-hexane.

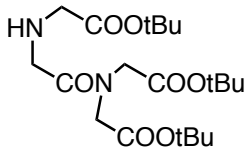
^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.32-8.28 (2H, m), 8.03-7.99 (2H, m), 4.40 (2H, s), 4.06 (2H, s), 3.97 (2H, s), 3.93 (2H, s), 1.45 (9H, s), 1.44 (9H, s), 1.43 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 167.70, 167.67, 167.63, 167.49, 149.95, 145.40, 128.71, 124.02, 83.42, 82.61, 82.31, 50.42, 49.21, 48.95, 47.98, 28.01.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{26}H_{39}N_3NaO_{11}S^+$: 624.2198. Found 624.2197.

Anal. Calcd. for $C_{26}H_{39}N_3O_{11}S$; C, 51.90; H, 6.53; N, 6.98. Found C, 51.90; H, 6.45; N, 6.99.

Mp: 115-116 °C.



76

To a solution of **75** (218.8 mg, 0.3637 mmol) in DMF (1 mL), was added K_2CO_3 (151.0 mg, 1.0925 mmol) and PhSH (56 μL, 0.5489 mmol). The mixture was stirred at rt for 12 h. Saturated $NaHCO_3$ solution was added to the mixture and extracted with EtOAc. The

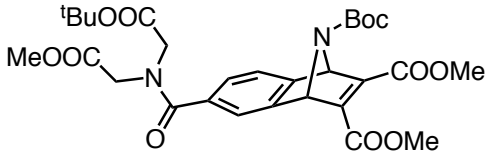
organic phase was washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 3:1) to give **76** (147.3 mg, 97%) as colorless oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 4.06 (2H, s), 3.98 (2H, s), 3.45 (2H, s), 3.31 (2H, s), 2.14 (1H, brs), 1.45 (9H, s), 1.44 (9H, s), 1.43 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.31, 170.99, 168.25, 167.90, 82.63, 81.92, 81.07, 51.02, 50.15, 49.83, 48.88, 28.06, 28.04, 27.98.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{20}H_{36}N_2NaO_7^+$: 439.2415. Found 439.2414.

Anal. Calcd. for $C_{20}H_{36}N_2O_7$; C, 57.67; H, 8.71; N, 6.73. Found C, 57.40; H, 8.48; N, 6.65.



77

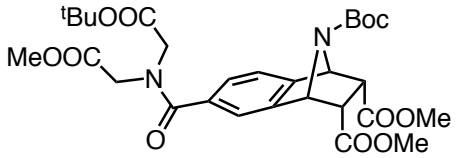
To a solution of **24** (169.3 mg, 0.4197 mmol), **61** (127.9 mg, 0.6293 mmol), and DIEA (115.3 μL, 1.2585 mmol) in DMF (4 mL), HOBt.H_2O (86.8 mg, 0.6424 mmol) and DIC (96.8 μL, 0.6289 mmol) were added and stirred at 50 ^{o}C for 24 h. After the reaction was completed, water was added into the mixture, extracted with EtOAc, washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 2:1) to give **77** (150.0 mg, 61%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the amide bond was present in 3:2 ratio. δ (ppm) = 7.49 (0.6H, s), 7.47 (0.4H, s), 7.42 (0.4H, s), 7.40 (0.6H, s), 7.17-7.13 (1H, m), 5.74 (2H, brs), 4.26 (1.2 H, s), 4.15 (0.8 H, s), 4.05 (0.8H, s), 3.94 (1.2H, s), 3.78 (3H, s), 3.77 (3H, s), 3.75 (1.8 H, s), 3.72 (1.2H, s), 1.46 (4H, s), 1.44 (5H, s), 1.36 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.66, 171.59, 169.54, 169.38, 168.14, 167.85, 162.75, 162.34, 154.04, 150.54, 147.88, 146.32, 132.76, 132.67, 125.10, 124.98, 121.81, 120.70, 82.67, 82.13, 81.90, 68.73, 52.44, 52.35, 52.16, 51.41, 48.04, 47.47, 28.02, 27.96, 27.87.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{29}H_{36}N_2NaO_{11}^+$: 611.2211. Found 611.2211.

Anal. Calcd. for $C_{29}H_{36}N_2O_{11}$; C, 59.18; H, 6.16; N, 4.76. Found C, 59.00; H, 6.09; N, 4.61.



78

To a solution of **77** (134.8 mg, 0.2290 mmol) in MeOH (5 mL), 10% Pd-C (29.9 mg) was added and stirred vigorously under H_2 for 12 h at rt. Pd-C was

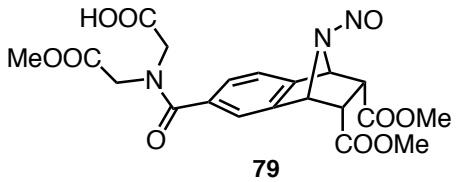
removed by filtration through Celite® and washed with MeOH. The solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 2:1 to 1:1) to give **78** (118.1 mg, 87%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the amide bond was present in 2:3 ratio. δ (ppm) = 7.45 (0.4H, s), 7.44 (0.6H, s), 7.38-7.29 (2H, m), 5.28 (1H, s), 5.24 (1H, s), 4.26 (1.5H, s), 4.11-3.91 (2.5H, m), 3.76 (1.6H, s), 3.73 (1.4H, s), 3.63 (2H, br), 3.48 (3.2H, s), 3.46 (2.8H, s), 1.48 (4H, s), 1.45 (5H, s), 1.39 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.86, 169.69, 169.51, 169.43, 169.35, 168.25, 167.89, 154.06, 143.75, 141.97, 141.84, 133.37, 133.13, 126.19, 125.97, 122.44, 122.21, 121.23, 121.11, 82.35, 81.92, 81.28, 63.18, 52.41, 52.17, 52.07, 51.69, 51.59, 51.52, 51.46, 47.99, 47.42, 47.21, 28.00, 27.84.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{29}H_{38}N_2NaO_{11}^+$: 613.2368. Found 613.2369.

Anal. Calcd. for $C_{29}H_{38}N_2O_{11}$; C, 58.97; H, 6.49; N, 4.74. Found C, 58.93; H, 6.44; N, 4.61.



79

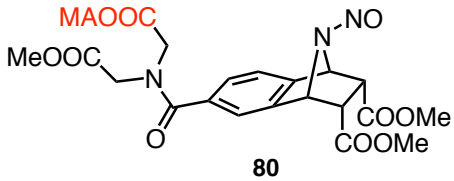
Compound **78** (274.3 mg, 0.4644 mmol) was dissolved in TFA (3.0 mL) and the mixture was stirred for 2 h at room temperature. Then TFA was removed by evaporation. Sodium nitrite (138.8 mg, 2.0116 mmol), formaldehyde solution 37% (49.8 mg) and PBS pH 6.4 (9.3 mL) was added into the mixture and stirred for 23 h at room temperature. The mixture was acidified with HCl 1M until pH 3, extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 2:1 to 1:1) to give **79** (63.4 mg, 30%) as yellow solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 8.42 (1H, br), 7.62-7.26 (3H, m), 6.23 (1H, br), 5.99 (1H, br), 4.44-4.39 (1H, m), 4.19-4.03 (3H, m), 3.77 (1.5H, s), 3.75 (1.5H, s), 3.68 (1H, br), 3.50 (7H, br)

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 172.34, 172.21, 171.93, 169.56, 169.48, 168.69, 168.62, 168.52, 168.41, 141.91, 140.64, 140.07, 138.67, 133.80, 133.57, 126.97, 126.92, 123.54, 123.24, 123.07, 122.79, 122.37, 121.93, 121.61, 64.36, 59.02, 52.55, 52.39, 52.19, 52.14, 51.98, 51.79, 51.56, 48.65, 48.23, 48.01, 47.86, 47.59, 44.65, 44.24, 44.06

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{20}H_{20}N_3O_{10}^-$: 462.1154. Found 462.1179.

Mp: 87-89 °C.



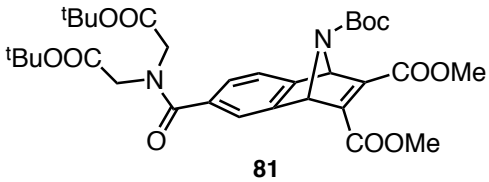
80

To a solution of compound **79** (76.1 mg, 0.1642 mmol) in 3.2 mL of acetonitrile was added DIEA (84.2 μL, 0.4750 mmol) and bromomethyl acetate (48.3 μL, 0.4925 mmol). The mixture was stirred at room temperature for 24 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:EtOAc = 2:1 to 1:1) to give **80** (47.1 mg, 54%) as yellow foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 7.60-7.39 (3H, m), 6.23 (1H, brs), 5.98 (1H, brs), 5.84-5.78 (1H, m), 5.47-5.29 (1H, m), 4.47 (1H, m), 4.19-4.06 (3H, m), 3.77 (1.5H, m), 3.75 (1.5H, s), 3.68 (0.5H, brs), 3.61 (0.5H, brs), 3.51 (7H, brs), 2.16-2.07 (3H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.58, 171.55, 170.19, 169.56, 169.49, 169.45, 169.36, 169.27, 168.60, 168.53, 168.42, 168.19, 167.84, 141.81, 140.51, 140.10, 138.68, 133.92, 127.13, 127.03, 123.34, 122.87, 122.34, 121.84, 87.88, 87.50, 86.83, 86.72, 79.92, 79.48, 64.45, 64.31, 59.09, 52.48, 52.33, 52.12, 52.00, 51.58, 51.52, 48.72, 48.19, 47.42, 44.69, 44.13, 20.92, 20.86, 20.72, 20.64.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{23}H_{25}N_3NaO_{12}^+$: 558.1330. Found 558.1329.



81

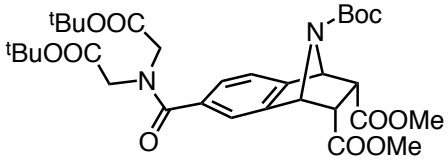
To a solution of **24** (272.3 mg, 0.6750 mmol), **71** (333.3 mg, 1.3587 mmol), and DIEA (185 μL, 2.0210 mmol) in DMF (5.2 mL), $HOBt.H_2O$ (181.1 mg, 1.3403 mmol) and DIC (208 μL, 1.3515 mmol) were added and stirred at 50 °C for 24 h. After the reaction was completed, water was added into the mixture, extracted with EtOAc, washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 5:1) to give **81** (358.3 mg, 84%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.45 (1H, s), 7.40 (1H, d, *J*=7.6 Hz), 7.14 (1H, d, *J*=7.6 Hz), 5.73 (2H, br), 4.14 (2H, s), 3.91 (2H, s), 3.78 (3H, s), 3.77 (3H, s), 1.47 (9H, s), 1.43 (9H, s), 1.36 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.25, 167.97, 167.65, 162.46, 162.03, 153.81, 150.36, 150.20, 147.47, 146.04, 132.78, 124.74, 121.57, 120.36, 82.23, 81.65, 81.54, 68.44, 52.15, 48.11, 27.76, 27.70, 27.60.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{32}H_{42}N_2NaO_{11}^+$: 653.2681. Found 653.2682.

Anal. Calcd. for $C_{32}H_{42}N_2O_{11}$; C, 60.94; H, 6.71; N, 4.44. Found C, 60.90; H, 6.79; N, 4.47.



82

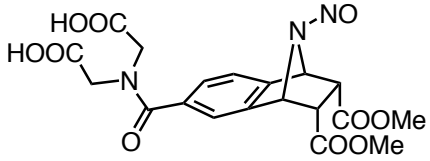
To a solution of **81** (180.3 mg, 0.2859 mmol) in MeOH (15 mL), 10% Pd-C (37.8 mg) was added and stirred vigorously under H_2 for 8 h at rt. Pd-C was removed by filtration through Celite® and washed with MeOH. The solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:acetone = 3:1) to give **82** (164.6 mg, 91%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.43 (1H, s), 7.37 (1H, d, *J*=7.6 Hz), 7.31 (1H, dd, *J*=1.6 Hz, 7.6 Hz), 5.29 (1H, s), 5.25 (1H, s), 4.16 (2H, d, *J*=11.6 Hz), 3.95 (2H, d, *J*=3.2 Hz), 3.64 (2H, s), 3.48 (6H, s), 1.49 (9H, s), 1.46 (9H, s), 1.40 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.66, 169.36, 169.25, 168.25, 167.92, 153.99, 143.49, 141.88, 133.45, 125.94, 122.36, 120.91, 82.15, 81.71, 81.17, 63.17, 52.33, 51.60, 51.46, 48.15, 47.06, 27.94, 27.78

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{32}H_{44}N_2NaO_{11}^+$: 655.2837. Found 655.2819.

Anal. Calcd. for $C_{32}H_{44}N_2O_{11}$; C, 60.75; H, 7.01; N, 4.43. Found C, 60.65; H, 6.86; N, 4.38.



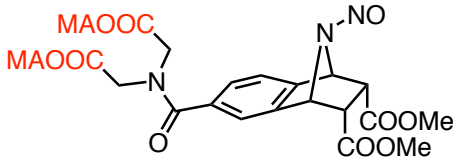
83

Compound **82** (164.6 mg, 0.2602 mmol) was dissolved in TFA (1.0 mL) and the mixture was stirred for 2 h at room temperature. Then TFA was removed by evaporation. Sodium nitrite (72.7 mg, 1.0536 mmol), formaldehyde solution 37% (43.5 mg) and PBS pH 6.4 (5.2 mL) was added into the mixture and stirred for 17 h at room temperature. The mixture was extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified with flash column chromatography ($CHCl_3$:MeOH = 10:1) to give **83** (101.0 mg, 86%) as yellow foam solid.

^{1}H-NMR (400 MHz, MeOD): δ (ppm) = 7.60 (1H, brs), 7.49 (1H, brs), 7.36 (1H, brs), 6.39 (1H, brs), 6.07 (1H, brs), 4.33-4.21 (2H, m), 4.09 (2H, brs), 3.88 (1H, brs), 3.61 (1H, brs), 3.51 (6H, brs).

^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 174.24, 172.31, 172.05, 170.52, 170.38, 143.86, 142.56, 142.25, 140.94, 135.53, 127.34, 124.84, 124.37, 122.89, 122.44, 65.78, 60.34, 52.83, 52.54, 52.42, 48.86, 45.89, 45.81, 45.52, 45.41.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{19}H_{18}N_3O_{10}^-$: 448.0998. Found 448.0993.



84

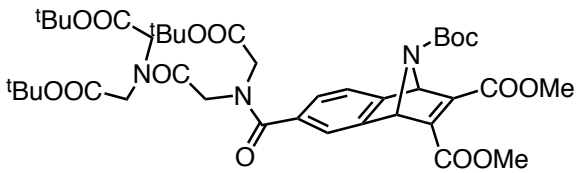
To a solution of compound **83** (156.4 mg, 0.3480 mmol) in 6 mL of acetonitrile was added *N,N*-diisopropylethylamine (238 μL, 1.3921 mmol) and bromomethyl acetate (273 μL, 2.7839 mmol). The mixture was stirred at room temperature for 12 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 2:1 to 1:1) to give **84** (105.9 mg, 51%) as yellow foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 7.58 (0.5H, brs), 7.47 (1H, brs), 7.38 (1.5 H, brs), 6.23 (1H, brs), 5.97 (1H, brs), 5.83-5.68 (3H, m), 5.36-5.28 (1H, m), 4.43 (0.5H, brs), 4.39 (0.5H, brs), 4.24-4.06 (3H, m), 3.78-3.59 (1.5H, m), 3.50 (6H, brs), 4.06 (0.5H, brs), 2.12 (3H, s), 2.10 (3H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.47, 170.11, 170.08, 169.47, 169.36, 168.54, 168.34, 167.97, 167.68, 141.85, 140.53, 140.04, 138.66, 133.58, 127.01, 123.30, 122.78, 122.23, 121.75, 87.80, 87.41, 86.69, 86.58, 79.86, 79.41, 64.37, 64.16, 58.99, 52.05, 52.00, 51.42, 48.64, 48.05, 47.39, 44.63, 44.02, 20.82, 20.78, 20.62, 20.55.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{25}H_{27}N_3NaO_{14}^+$: 616.1385. Found 616.1369.

Anal. Calcd. for $C_{17}H_{23}NO_6$; C, 50.59; H, 4.59; N, 7.08. Found C, 50.38; H, 4.72; N, 6.81.



85

To a solution of **24** (353.1 mg, 0.8753 mmol), **76** (473.8 mg, 1.1376 mmol), and DIEA (240 μL, 2.6219 mmol) in DMF (7 mL), $HOBt.H_2O$ (237.2 mg, 1.7555 mmol) and DIC (270 μL, 1.7543 mmol) were added and stirred at 50 °C for 22 h. After the reaction was completed, water was added into the mixture, extracted with EtOAc, washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was

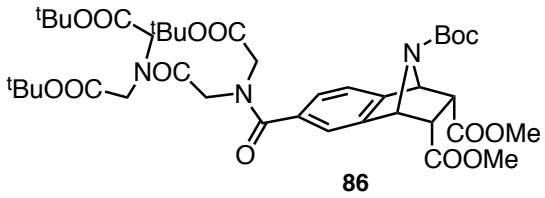
purified with flash column chromatography (*n*-hexane:EtOAc = 3:1 to 2:1) to give **85** (568.7 mg, 81%) as pale yellow foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the amide bond was present in 2:3 ratio. δ (ppm) = 7.54 (0.4H, s), 7.49 (0.6H, s), 7.43-7.39 (1H, m), 7.23 (0.4H, dd, *J*=7.6 Hz, 1.2 Hz), 7.15 (0.6H, d, *J*=7.6 Hz), 5.85-5.74 (2H, m), 4.39-4.00 (7H, m), 3.80-3.78 (6H, m), 3.74 (1H, s), 1.49 (9H, s), 1.48 (3.6H, s), 1.46 (5.4H, s), 1.43 (5.4H, s), 1.39 (3.6H, s), 1.38 (5.4H, s), 1.35 (3.6H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.62, 171.37, 168.86, 168.67, 168.56, 167.93, 167.64, 167.23, 162.79, 162.25, 154.07, 151.58, 150.62, 150.33, 147.75, 147.58, 146.21, 146.06, 133.10, 132.55, 125.80, 124.94, 121.81, 121.08, 120.70, 82.98, 82.36, 82.09, 82.02, 81.84, 81.71, 68.69, 52.42, 52.36, 51.98, 51.01, 50.53, 50.29, 49.64, 49.14, 48.30, 46.50, 28.07, 28.03, 28.00, 27.94, 27.88, 27.80.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{40}H_{55}N_3NaO_{14}^+$: 824.3576. Found 824.3575.

Anal. Calcd. for $C_{40}H_{55}N_3O_{14}$; C, 59.91; H, 6.91; N, 5.24. Found C, 59.89; H, 7.03; N, 5.04.



86

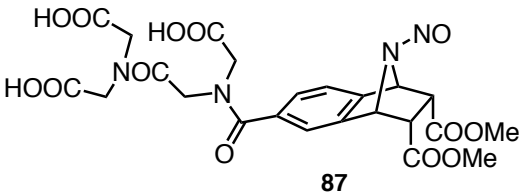
To a solution of **85** (568.7 mg, 0.7092 mmol) in MeOH (40 mL), 10% Pd-C (108.3 mg) was added and stirred vigorously under H_2 for 7.5 h at rt. Pd-C was removed by filtration through Celite® and washed with MeOH. The solvent was evaporated and the residue obtained was purified with flash column chromatography (*n*-hexane:EtOAc = 3:1 to 2:1) to give **86** (318.2 mg, 56%) as white foam solid, and *exo-endo* **86** (103.3 mg, 18%) as white foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the amide bond was present in 2:3 ratio. δ (ppm) = 7.42 (1H, s), 7.37 (1H, d, *J*=3.6 Hz), 7.33-7.29 (1H, m), 5.29 (2H, brs), 4.49-3.74 (8H, m), 3.63 (2H, br), 3.49 (3H, s), 3.48 (3H, s), 1.48 (9H, s), 1.46 (9H, s), 1.40 (9H, s), 1.39 (9H, s).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.72, 171.61, 169.53, 169.37, 169.17, 168.98, 168.60, 168.58, 168.39, 167.83, 167.58, 167.46, 167.39, 153.99, 153.90, 143.38, 143.23, 141.82, 133.48, 133.43, 126.09, 125.95, 122.40, 121.08, 120.74, 82.74, 82.59, 81.94, 81.80, 81.65, 81.44, 81.12, 81.04, 63.18, 51.82, 51.62, 51.55, 51.42, 50.84, 50.41, 50.18, 49.45, 49.00, 48.05, 47.02, 46.26, 27.93, 27.86, 27.81, 27.73

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{40}H_{57}N_3NaO_{14}^+$: 826.3733. Found 826.3728.

Anal. Calcd. for $C_{40}H_{57}N_3O_{14}$; C, 59.76; H, 7.15; N, 5.23. Found C, 59.79; H, 7.13; N, 5.20.



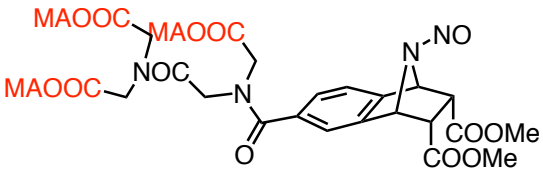
87

Compound **86** (279.1 mg, 0.3472 mmol) was dissolved in TFA (2.0 mL) and the mixture was stirred for 2 h at room temperature. Then TFA was removed by evaporation. Sodium nitrite (96.3 mg, 1.3956 mmol), formaldehyde solution 37% (51.7 mg) and PBS pH 6.4 (7 mL) was added into the mixture and stirred for 17 h at room temperature. The mixture was extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give **87** (114.6 mg, 58%) as yellow foam solid.

^{1}H-NMR (400 MHz, MeOD): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 7.60 (1H, brs), 7.49 (1H, brs), 7.35 (1H, brs), 6.39 (1H, brs), 6.08 (1H, brs), 4.52-3.97 (8H, m), 3.90-3.62 (2H, m), 3.54 (6H, brs).

^{13}C-NMR (100 MHz, MeOD): δ (ppm) = 174.13, 172.51, 172.29, 172.24, 171.97, 171.88, 171.61, 170.91, 170.74, 170.47, 170.37, 143.73, 142.34, 140.95, 135.57, 135.22, 127.56, 127.36, 124.81, 124.33, 123.22, 122.74, 122.35, 65.76, 60.32, 52.79, 52.66, 52.60, 52.46, 52.41, 50.69, 50.62, 50.28, 46.02, 45.54, 45.27.

HRMS (ESI-TOF, $[M-H]^-$): Calcd. for $C_{23}H_{23}N_4O_{13}^-$: 563.1267. Found 563.1286.



88

To a solution of compound **87** (86.5 mg, 0.1532 mmol) in 3 mL of acetonitrile was added DIEA (157 μL, 0.9196 mmol) and bromomethyl acetate (180 μL, 1.8386 mmol). The mixture was stirred at room temperature for 22 h and the solvent was evaporated. The residue was purified with flash column chromatography (*n*-hexane:acetone = 2:1 to 1:1) to give **88** (39.3 mg, 33%) as orange foam solid.

^{1}H-NMR (400 MHz, $CDCl_3$): isomer due to the N-NO bond was present in 1:1 ratio. δ (ppm) = 7.53-7.37 (3H, m), 6.23 (1H, brs), 6.00 (1H, brs), 5.81-5.76 (3H, m), 5.66 (1H, brs), 5.47-5.41 (1H, m), 5.37-5.29 (1H, m), 4.48-4.07 (7H, m), 3.90 (1H, brs), 3.67 (1H, brs), 3.51 (7H, brs), 2.13-2.07 (9H, m).

^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) = 171.39, 171.34, 170.19, 170.13, 169.52, 169.43, 169.40, 169.30, 169.15, 169.08, 168.80, 168.49, 168.44, 168.34, 167.59, 167.47, 167.33, 167.25, 141.61, 140.22, 138.90, 133.95, 127.00, 123.70, 123.31, 123.20, 122.80, 122.02, 121.59, 79.76, 79.70, 79.48, 79.31, 64.27, 58.95, 52.10, 51.17, 50.85, 49.75, 49.29, 48.66, 48.29, 48.10, 47.65, 46.39, 44.65, 44.11, 20.86, 20.68, 20.64, 20.58, 20.50.

HRMS (ESI-TOF, $[M+Na]^+$): Calcd. for $C_{32}H_{36}N_4NaO_{19}^+$: 803.1866. Found 803.1867.

5.2. Photo Cleavage of N-NO Bonds –DAF-2 Assays–

Fluorescence spectroscopic studies were performed on a Perkin Elmer LS55. Photo-irradiation experiments in cuvettes were carried out in a monochromator unit (Bunko-Keiki Co., Ltd., Japan) equipped with a 500 W xenon lamp (Usio Inc., Japan) as a light source. The light intensity was measured with a Nova Display (OPHIR Japan Ltd.).
A 5 μL aliquot of 5 mM DMSO stock solution of DAF-2 and a 40 μL aliquot of 6.25 mM DMSO stock solution of the nitrosamine were diluted by adding 2455 μL PBS buffer (Nippon Gene Co., Ltd., Japan, pH=7.4) in a 1.0 cm x 1.0 cm quartz cuvette: the final concentrations were 10 μM for DAF-2 and 100 μM for nitrosamine, in PBS containing 1.8 % DMSO. The cuvette containing the prepared solution was held on a cell holder placed in the light path of a monochromator, and illuminated at 420 nm (±20 nm, 2.3 mW/cm^2 at 420 nm) for a specified period. After each irradiation, the fluorescence spectrum was recorded in the range from 450 nm to 650 nm, with 492 nm excitation. The set-up parameters were as follows: excitation slit: 5.0 nm, emission slit: 1.0 nm, scan speed: 200 nm/min, photomultiplier voltage: auto.

The fluorescent intensity of DAF-2-triazol (DAF-2T) was converted to the concentration of NO on the basis of the comparison of DAF-2-T fluorescent intensity of compound **2** and other nitrosamines. NO concentration of compound **2** has been determined with the same condition by calibration curve method[27] and was set as the standard nitrosamine.

5.3. Photo Cleavage of N-NO Bonds in Cells –Piccell Assays–

NOC7 was dissolved in PBS (pH 7.4) with the final concentration is 100 nM as positive control in Piccell. Nitrosamines solutions for incubation (10 mM) were prepared in PBS (pH 7.4) that contains 1.8% of DMSO. Incubation of nitrosamines in Piccell was done for 1 hour and imaging was done directly for non-washing experiments.

For washing experiments, after incubation the Piccell culture medium was replaced with a Hank's balanced salt solution supplemented with 20 mM HEPES pH 7.4 as washing and was repeated for 6 or 7 times. The cells were imaged at 25 ^{o}C on Carl Zeiss Axiovert 200 microscope with a cooled CCD camera CoolSNAP HQ (Roper Scientific Inc, Tucson, AZ), controlled by MetaFluor (Universal Imaging, West Chester, PA). Upon excitation of cyan fluorescent protein (CFP) at 440±15 nm, fluorescence images were obtained through a 480±15 nm filter for CFP and 535±12.5 nm filter for yellow fluorescent protein (YFP) with a 40x oil immersion objective (Carl Zeiss, Jena, Germany). All filters and dichroic mirror (455DRLP) were obtained from Omega Optical (Brattleboro, VT).

The nitrosamines were uncaged by photolysis (440±10 nm) at a diameter of 20 μm and was achieved by inserting a pinhole, the diameter of which is 100 μm, at the field diaphragm of the fluorescence microscope. After the uncaging, CFP/YFP fluorescence resonance energy transfer (FRET) was observed in Piccell at 480±15 and 535+12.5 nm.

The NO release concentration in Piccell were calculated based on calibration curve of NO release in Piccell with diluted solution of NO gas in PBS at several concentration as reported by Sato, *et al*.[29]

5.4. S-transnitrosylation in Cuvette

A solution of nitrosamines in DMSO and a solution of triphenylmethanethiol in chloroform were prepared at 30 mM concentration freshly before measurement. From the stock solution, 250 μL of nitrosamines and 250 μL of triphenylmethanethiol was taken and diluted with 2 mL of chloroform. After mixed well, the solution was used for measurement at 37 ^{o}C (without irradiation) or 25 ^{o}C (with irradiation). The absorbance spectra were recorded on JASCO V-550 spectrometer. Bandwidth was 2.0 nm, scan speed was 200 nm/min, and data acquisition was 1 nm.

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