Attenuated Desensitization of β-Adrenergic Receptor by Water-Soluble N-Nitrosamines That Induce S-Nitrosylation Without NO Release

Noriko Makita,* Yoji Kabasawa,* Yuko Otani, Firman, Junichiro Sato, Makiko Hashimoto, Michio Nakaya, Hiroaki Nishihara, Masaomi Nangaku, Hitoshi Kurose, Tomohiko Ohwada, Taroh Iiri

Rationale: The clinical problem of loss of β-adrenergic receptor (β-AR) response, both in the pathogenesis of heart failure and during therapeutic application of β-agonists, is attributable, at least in part, to desensitization, internalization, and downregulation of the receptors. In the regulation of β-AR signaling, G protein–coupled receptor kinase 2 (GRK2) primarily phosphorylates agonist-occupied β-ARs, and this modification promotes desensitization, internalization, and downregulation of β-ARs. It has been demonstrated that GRK2 is inhibited by its S-nitrosylation. However, compounds that induce S-nitrosylation, such as S-nitrosoglutathione, simultaneously generate NO, which has been demonstrated to operate for cardiovascular protection.

Objective: We examine whether S-nitrosylation without NO generation inhibits desensitization of β2-AR by GRK2.

Methods and Results: We have developed water-soluble N-nitrosamines that have S-nitrosylating activity but lack NO-generating activity. These compounds, at least partly, rescue β-AR from desensitization in HEK293 cells expressing FLAG-tagged human β2-AR and in rat cardiac myocytes. They inhibit isoproterenol-dependent phosphorylation and internalization of β2-AR. Indeed, they nitrosylate GRK2 in vitro and in cells, and their S-nitrosylation of GRK2 likely underlies their inhibition of β2-AR desensitization.

Conclusions: Compounds that induce S-nitrosylation without NO release inhibit GRK2 and attenuate β2-AR desensitization. Developing water-soluble drugs that specifically induce S-nitrosylation may be a promising therapeutic strategy for heart failure. (Circ Res. 2013;112:327-334.)

Key Words: adrenergic receptor ■ desensitization ■ G protein–coupled receptor kinases ■ nitrosamines ■ S-nitrosylation

G protein–coupled receptors (GPCRs) comprise a huge family of receptors that mediate many of the cell–cell communication pathways in human tissues.1-3 β-adrenergic receptor (β-AR) is a prototype GPCR, which plays a critical role in the regulation of cardiovascular4-6 and pulmonary functions,7 as well as other biological processes. The physiological control mechanisms of β-AR signaling include desensitization, internalization, and downregulation.8 On the contrary, loss of β-AR response is a serious problem associated with the pathogenesis of heart failure and also with the therapeutic use of β-agonists9,10 and is thought to be linked to morbidity and mortality10 in the clinical settings,8 probably arising from heart failure11 and asthma.7,12,13 It has been shown that GPCR kinases (GRKs) interact with the agonist-activated form of GPCRs to initiate receptor phosphorylation, which results in a profound impairment of receptor.14-16 In the regulation of β2-AR signaling, GPCR kinase 2 (GRK2) plays a key role in phosphorylating agonist-occupied β2-ARs and promoting their desensitization, internalization, and downregulation.17-19 A recent study indicated that the catalytic activity of GRK2 for phosphorylation of β2-AR is controlled by S-nitrosylation,18 that is, S-nitrosylation of GRK2 reduced the phosphorylation activity. S-nitrosoglutathione (GSNO) was demonstrated to inhibit β2-AR desensitization on β-agonist (isoproterenol [ISO]) stimulation.18,19

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and was suggested to be an exogenous (and possibly also endogenous) transnitrosylation agent, reactive at cysteine residues of GRK2. Cysteine-based S-nitrosothiols, such as GSNO and S-nitrosocysteine, are highly water soluble but are unstable and prone to decompose in aerated solutions as a result of their weak S-N(O) bond (Figure 2). GSNO releases NO radical in water through homolytic bond cleavage of the S-N(O) bond, and also GSNO is readily reduced by single-electron reductants to release NO. Therefore, new classes of drugs that target GRK2 are desirable. Here, we have synthesized a new class of nitrosylation agent that is designed to target GRK2 through transnitrosylation to avoid reductive, homolytic chemistry. Although endogenous N-nitrosamines are thought to play similar physiological roles to S-nitrosothiols, their nitrosylation reactivities and their contributions to biological events have been little understood. In this study, we aimed to generate water-soluble N-nitrosamines (WNNOs) of 7-azabicyclo[2.2.1]heptanes, which resemble conformationally constrained N-nitrosoproline derivatives (Figure 1). We found that the resulting compounds were soluble and stable in water, and they exhibit nitrosylation reactivity to thiols, which could be used as functional mimics of GSNO in terms of S-nitrosylation but not in terms of direct generation of NO. We also examined the ability of these WNNOs to block desensitization of β2-AR signaling on agonist (ISO) stimulation.

Methods

Biological Studies

cAMP Assay

cAMP accumulation in intact cells was assayed as described previously. Briefly, for measuring intracellular cAMP, HEK293 cells or rat cardiac myocytes in 24-well plates prelabeled with [3H]adenine (0.5–2 μCi/well for 24 hours) were washed once with HEPES-buffered DMEM and incubated (37°C for 30 minutes) in the same medium containing 1 mmol/L 3-isobutyl 1-methylxanthine with or without ISO and WNNOs. The reaction was terminated by aspiration and by the immediate addition of ice-cold 5% trichloroacetic acid with 1 mmol/L cAMP/ATP (0.5 mL/well). [3H]cAMP and [3H]ATP were separated on AG 40W-X4 Dowex and alumina columns, and the data are presented as the ratio of [3H]cAMP to [3H]cAMP plus [3H]ATP, as described previously.

Cell Surface ELISA

The cell surface expression of exogenous receptors in HEK293 cells stably expressing FLAG-tagged human β2-AR was quantified by ELISA. One day before the assay, cells were seeded into 12-well plates. At 20 hours before the assay, cells were pretreated with 30 μmol/L WNNO7 or GSNO, and 2 hours or 30 minutes before the assay, the cells were pretreated with 10 μmol/L ISO. At the assay, the cells were washed twice with ice-cold 1% BSA-PBS placed on ice for 5 minutes and incubated in an anti-FLAG antibody solution (1:4000) diluted in 1% BSA-PBS for 1 hour at 4°C. This was followed by 2 further washes in 1% BSA-PBS. The cells were next fixed at 4°C for 15 minutes in 4% paraformaldehyde-PBS and again washed twice by 1% BSA-PBS and incubated in anti-mouse horseradish peroxidase–conjugated secondary antibody solution (1:12000) diluted in 1% BSA-PBS for 1 hour at 4°C. This was followed by 2 washes in 1% BSA-PBS and incubated in anti-mouse horseradish peroxidase–conjugated secondary antibody solution (1:12000) diluted in 1% BSA-PBS for 1 hour at room temperature. This was followed by 2 washes in 1% BSA-PBS for 20 minutes and a final wash in PBS. Finally, the cells were treated with substrate (o-phenylenediamine dihydrochloride; Sigma, St. Louis, MO) for 5 minutes at room temperature. This reaction was stopped by the addition of the non-standard Abbreviations and Acronyms
of an equivalent volume of 2.5 N aqueous HCl. The absorption levels were then read at 492 nm using a plate reader (Biorad, Hercules, CA).

**Phosphorylation Assay of Human β2-AR Using Autoradiography**

Phosphorylation of exogenous receptors in HEK293 cells stably expressing FLAG-tagged human β2-AR was quantified by autoradiography. At 1 day before the assay, the cells were seeded into 60-mm dishes. At 20 hours before performing the assay, the cells were pretreated with 100 μmol/L WNN07 or GSNO, and 4 hours before the assay, the medium was changed into phosphate-free medium supplemented with [32P]orthophosphoric acid (100 μCi/mL) and 1% fetal bovine serum with or without 100 μmol/L WNN07 or GSNO. At the assay, the cells were stimulated with 10 μmol/L ISO for 10 minutes and put on ice. The cells were then washed twice with ice-cold PBS and lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 100 mmol/L NaCl, 2 mmol/L Na-orthovanadate, 10 mmol/L Na-PPi, 100 mmol/L NaF, 1 mmol/L dithiothreitol, protease inhibitors, and 1% sodium dodecyl sulfate) on ice by gently shaking each for 5 minutes. After 30 minutes, cell lysates were collected in tube and centrifuged at 20 000 g for 10 minutes at 4°C. Supernatants were incubated with 2 μg monoclonal anti-Flag antibody (Sigma) for 1 hour at 4°C and absorbed to protein G plus Sepharose (Santa Cruz) for 1 hour at 4°C. Bound complexes were washed 3× with immunoprecipitation buffer (50 mmol/L Tris-HCl [pH 7.4], 100 mmol/L NaCl, 2 mmol/L Na-orthovanadate, 10 mmol/L Na-PPi, 100 mmol/L NaF, 1 mmol/L dithiothreitol, protease inhibitors, 1% NP-40, and 0.5% sodium dodecyl sulfate). Proteins were separated by boiling with gel loading buffer and then subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue (R-250) and dried. 32P incorporation was detected by autoradiography using image scanner.

**Biotin Switch Assay**

The biotin switch assay was performed, as described,14 with the following modifications. For in vitro experiments, cells overexpressing FLAG-tagged GRK2 (wild type or C340S) were blocked and biotinylated, followed by separation of GSNO, WNNO6, or WNNO7 for indicated times at room temperature. The final concentrations of WNNO7 and the thiol were 5 μmol/L WNNO (1500 μmol/L), 25 μmol/L, respectively. The sample was prepared in a UV cuvette, and the absorbance at 570 nm was measured. The absorbance increased time-dependently.

**Griess Assay**

A 10% aqueous solution of sulfanilamide (1 g) in distilled water (10 mL) and phosphoric acid (4 mL) and a 1% aqueous solution of N-(1-naphthyl)ethylenediamine dihydrochloride (100 mg) in distilled water (10 mL) were prepared. To a mixture of equal volumes of the above 2 solutions, a solution of WNNO in water was added to obtain a final concentration of WNNO of 25, 50, or 100 μmol/L in water. After 5 hours at 37°C, the absorbance at 570 nm was measured. The absorption increased time-dependently.

**Kinetic Studies by Means of UV-Visible Spectroscopy**

The UV-visible spectra were recorded on JASCO V-550 spectrometer. The bandwidth was 2.0 nm, the scan speed was 200 nm/min, and the data acquisition interval was 1 nm. Spectroscopic grade chloroform was purchased and used without further purification. PBS buffer (pH 7.4) was also purchased from Wako Pure Chemicals (Japan). We estimated the kinetic constants of the transnitrosylation reaction of some of the WNNOs with triphenylmethylthiol in chloroform. The second-order rate constants for WNN07 and WNN06 in chloroform were obtained under conditions where the concentrations of the N-nitrosamine and the thiol are the same, that is, a single-component approximation of a second-order reaction. That is, to a solution of WNN07 (1500 μL, 10 mmol/L) in chloroform (1200 μL), a solution of thiol (300 μL, 50 mmol/L) was mixed at the specified temperature. The final concentrations of WNN07 and the thiol were 5 mmol/L, respectively. The sample was prepared in a UV cuvette, and
the spectrum was monitored at 37°C. The initial appearance of the S-nitrosotriphenylmethylthiol was monitored by following the optical density at 603 nm. An expanded Methods section is available in the Online Data Supplement.

Results

Successful Synthesis of Water-Soluble and Stable Compounds Inducing S-Nitrosylation Without NO Release

To confirm that S-nitrosylation without NO generation can rescue β-AR from desensitization and to obtain practically useful drugs, we have developed a series of water-soluble compounds that may induce S-nitrosylation and yet do not generate NO. We synthesized a series of ethylene glycol–containing molecules (WNNOs; Figure 1), which exhibited different strengths of the N-NO bond, as estimated by Griess assay (diazo-coupling–based dye formation assay under acidic conditions) in 100% H2O (Figure 1). The evaluation of water solubility of WNNO6 and WNNO6a-b (see Online Table I) indicated that the triethylene glycol unit affords adequate water solubility. The single carboxylic acid functionality of compound WNNO5 (Figure 1) did not significantly improve the aqueous solubility (2 mmol/L; Online Table I). We thus synthesized the triethylene glycol–appended analogs WNNO7 and WNNO8 (Figure 1; Online Schemes I and II) and examined their water solubility (Online Table I). The triethylene glycol–conjugated N-nitroso α-proline WNNO9 (Figure 1) was also synthesized as a model compound of endogenous water-soluble nitrosamines. These triethylene glycol–substituted compounds (WNNO7-9) showed good water solubility (>50 mmol/L; Online Table I). All these WNNOs are stable in 100% water, and the 1H NMR spectra in D2O did not change at ambient temperature for at least a week. To estimate the N-NO bond strength, we performed Griess assays in 100% water (Figure 1). The present Griess assay results showed that the introduction of the triethylene glycol moiety has little effect on the N-NO bond reactivity. That is, the order of attenuation of the N-NO bond strength of WNNOs in 100% water is WNNO7 > WNNO6 > WNNO8 > WNNO9, as judged from the amount of dye formation in the Griess assay. This trend corresponds well to the S-nitrosylation reactivities (Figure 3). Furthermore, these WNNOs do not release NO radical by themselves under neutral aqueous conditions in the absence
and presence of a thiol (glutathione; Figure 2). These results are in sharp contrast to the fact that GSNO releases NO radical in water spontaneously, particularly in the presence of excess glutathione (thiol form; Figure 2).31

S-nitrosylation reactivities of the WNNOs were evaluated with triphenylmethylthiol as a model thiol in CHCl₃. The pegylated WNNOs are soluble in both water and CHCl₃, but triphenylmethylthiol is insoluble in water, whereas GSNO is insoluble in CHCl₃, and is known to be unstable in the presence of excess glutathione (thiol form) in water (Figure 2).31 The nitrosylation reactivities can be represented in terms of the initial rates of S-nitrosylation of triphenylmethylthiol by WNNOs in CHCl₃, to give S-nitrosotriphenylmethylthiol (Figure 3 and Online Figure I). The S-nitrosylation reactions of WNNO6, WNNO7, and WNNO8 were detected at 37°C, whereas that of WNNO9 was not. The increasing order of the transnitrosylation reactivities, estimated from the initial rates of the nitrosylation reaction is WNNO7>WNNO6>WNNO8>>>WNNO9. Unfortunately, the nitrosylation activity of GSNO cannot be measured under the similar conditions. Nevertheless, WNNO7 could be used as functional mimics of GSNO at least partially in terms of the nitrosylation activity of GSNO cannot be measured under the similar conditions. Nevertheless, WNNO7 could be used as functional mimics of GSNO at least partially in terms of S-nitrosylation but not in terms of direct generation of NO.

Inhibition of Desensitization of β₂-AR on Agonist (ISO) Stimulation by Application of WNNOs

The groups of Lefkowitz and Stamler recently reported that GSNO can inhibit desensitization of β₂-AR, at least in part, by nitrosylating and thereby inactivating GRK2, which plays a key role in desensitization of β₂-AR.18 However, GSNO also generates NO, which might have an additional effect on β₂-AR desensitization.32,33 Therefore, we examined whether our WNNOs could block desensitization/ internalization of β₂-AR on agonist (ISO) stimulation in HEK293 cells stably overexpressing human β₂-AR. Preincubation of the HEK cells with WNNO6 or WNNO7 for 12 hours, followed by administration of ISO (1 μmol/L) for 30 minutes, resulted in an increase in cAMP accumulation, which suggested that WNNOs might inhibit desensitization of β₂-AR (Figure 4A).18 Increasing the preincubation time with WNNO6 and WNNO7 from 1 hour to 24 hours caused a further increase in cAMP accumulation on ISO stimulation (Figure 4B). The increase in cAMP accumulation (ie, increase in β₂-AR signaling) by WNNO6 and WNNO7 was dose-dependent (Figure 4A). When we incubated the cells with water-insoluble N-nitrosamines with the aid of cosolvent dimethyl sulfoxide (<1%), the resultant changes of cAMP on ISO stimulation were no longer reproducible, although the protocol was unchanged (data not shown). The reason for this is probably that dimethyl sulfoxide perturbed the cell membranes during the preincubation period. The N-nitrosoproline mimic WNNO9 did not show any effect on cAMP production. The order of magnitude of the increase of cAMP in the presence of WNNOs, that is, WNNO7>WNNO6>>>WNNO9, under the dimethyl sulfoxide–free conditions (Figure 4A and 4B) corresponds well to the order of S-nitrosylation reactivities of the WNNOs, as estimated in terms of the reaction of the WNNOs with triphenylmethylthiol (Figure 3). This observation is consistent with the hypothesis that S-nitrosylation, probably of GRK2, is involved in the inhibition of desensitization of agonist-activated β₂-AR.18 This conclusion is further supported by the fact that the N-nitrosoproline mimic WNNO9, which exhibited no S-nitrosylation reactivity (Online Figure I), had no effect on the cAMP accumulation (Figure 4A and 4B).
Potentiation of cAMP accumulation by GSNO was observed when cells were stimulated with ISO, whereas such potentiation was not observed when cells were stimulated with forskolin (a direct activator of adenylyl cyclase) or cholera toxin (a direct activator of Gs protein; Figure 4C). These results support the hypothesis that GSNO acts specifically on β2-AR but not on other downstream signaling molecules, such as Gs and adenylyl cyclase.

We next examined whether WNNO7, as well as GSNO, can indeed inhibit desensitization of β2-AR signaling. When HEK293 cells stably expressing β2-AR were preincubated with ISO (1 μmol/L) for 4 hours, the subsequent ISO-dependent cAMP accumulation was attenuated (see control data in Figure 5A), suggesting that desensitization of ISO-stimulated β2-AR indeed occurs in our intact cell system. This desensitization was inhibited when the cells were incubated with WNNO7 or GSNO (Figure 5A). The desensitization of β2-ARs and its inhibition by WNNO7 and GSNO were also observed in rat cardiac myocytes (Figure 5B).

**Inhibition of GRK-Dependent Phosphorylation and Downregulation of β2-ARs by Application of WNNOs**

We thus suspected that WNNO7, as well as GSNO, might inhibit desensitization of β2-ARs by inhibiting GRK-dependent phosphorylation and downregulation of β2-ARs. Indeed, ISO-dependent phosphorylation of β2-AR was inhibited when the cells were incubated with WNNO7 or GSNO (Figure 6A). Furthermore, ISO-dependent internalization of β2-AR, assessed in terms of ISO-dependent decrease of cell surface β2-AR measured with ELISA assay, was also inhibited by WNNO7 or GSNO (Figure 6B). It is of note that neither GSNO nor WNNO7 increased cell surface expression of β2-AR at the resting state (Figure 6B), indicating that increase in ISO-dependent cAMP accumulation induced by these agents (Figures 4 and 5) does not result from the increase in β2-AR expression at the resting state. Taken together, these results are consistent with the idea that WNNO7, as well as GSNO, can inhibit GRK-dependent desensitization of β2-ARs.

**S-Nitrosylation of GRK2 by Application of WNNOs**

Finally, we investigated whether WNNO7, as well as GSNO, might nitrosylate GRK2. Indeed, WNNO7 and GSNO nitrosylated FLAG-tagged GRK2 in vitro (Figure 7A, left) and in intact HEK293 cells (Figure 7B, right), whereas WNNO6 did not significantly. In vitro S-nitrosylation of FLAG-tagged GRK2 by WNNO7 was almost inhibited by C340S mutation (Figure 7B), suggesting that C340 is the S-nitrosylation site of GRK2 as previously reported.Using HEK293 cells transiently transfected with β2-AR/GRK2-wild type and incubated with small interfering RNA for endogenous GRK2 showed a large
inhibition of desensitization after treatment with WNNO7 or GSNO, whereas cells expressing GRK2-C340S do not do so (Figure 7C). These data suggest that S-nitrosylation of GRK2 at C340 is likely to underlie the rescue of β2-AR from desensitization at least in part.

Discussion

In our present study, we have developed water-soluble compounds that specifically induce S-nitrosylation. We have found that our WNNOs are indeed stable in water and have S-nitrosylation activity. These WNNOs do not release NO by themselves under neutral aqueous conditions (Figure 2). These WNNOs rescued β2-AR signaling after agonist stimulation in a comparable manner with GSNO in HEK293 cells expressing β2-AR and in rat cardiac myocytes. The S-nitrosylation reactivity with a model thiol, triphenylmethylthiol, coincided well with the magnitude of the rescue of agonist-activated β2-AR signaling, evaluated in terms of cAMP potentiation. This finding supports the hypothesis that S-nitrosylation is involved in the inhibition of the desensitization of β2-AR, and the net nitrosylation activities of WNNO7 are comparable with that of GSNO. These compounds also inhibited ISO-dependent phosphorylation and internalization of β2-AR in HEK293 cells stably expressing FLAG-tagged human β2-AR. Indeed, these compounds nitrosylated GRK2 at C340 and inhibited GRK2-dependent desensitization of β2-AR.

Our findings indicate that a strategy of targeting GRK2 with compounds having nitrosylation activity may be a promising approach to block endogenous and exogenous agonist-induced desensitization of β2-AR in the pathogenic and clinical settings.11,14,15 Our compounds are expected to be useful biological tools for further studies on agonist-induced desensitization of β2-AR. Furthermore, there is considerable theoretical and experimental evidence that nitrogen-pyrimidized N-nitroso 7-azabicyclo[2.2.1]heptane derivatives (such as WNNOs) are not mutagenic,24 and if this is confirmed, our compounds might also be potential candidates for clinical application.

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Disclosures

None.

References

Our compounds nitrosylate GRK2 in vitro and in intact cells. We have successfully developed water-soluble compounds that specifically induce S-nitrosylation but do not generate NO.

**What Is Known?**

- Desensitization of β-adrenergic receptors (β-ARs) underlies the pathogenesis of heart failure and resistance to β-agonists.
- G protein–coupled receptor kinase 2 (GRK2) plays an essential role in β-AR desensitization, at least in part, and is upregulated in heart failure.
- Although it has been shown that S-nitrosylation of GRK2 inhibits GRK2 activity, a potential problem in concluding this is that compounds that induce S-nitrosylation, such as S-nitrosoglutathione, also generate NO, which by itself operates for cardiovascular protection.

**What New Information Does This Article Contribute?**

- Our compounds likely inhibit GRK2 activity and thereby specifically inhibit β-AR desensitization.

GRK2 may be a potential therapeutic target because GRK2 is critically involved in the desensitization of β-ARs, which underlies heart failure and resistance to β-agonists. Inhibition of GRK2 by genetic means has been shown to rescue heart failure in animal models. Although it has been shown that S-nitrosylation of GRK2 inhibits GRK2 activity, a potential problem is that compounds that induce S-nitrosylation also generate NO, which itself operates for cardiovascular protection. In our present study, we have successfully developed water-soluble compounds that induce nitrosylation but do not generate NO. Our compounds nitrosylate GRK2 in vitro and in intact cells, likely inhibit GRK2 activity, and attenuate β-AR desensitization. Our compounds and their relatives may serve as useful biological tools for future studies and potential therapeutics for clinical application.
Attenuated Desensitization of β-Adrenergic Receptor by Water-Soluble N-Nitrosamines That Induce S-Nitrosylation Without NO Release

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Water-soluble Nitrogen-Pyramidal N-Nitrosamines Mimic the S-Transnitrosylation Reactivity of S-Nitrosoglutathione and Block Desensitization of Agonist-Stimulated β2-Adrenergic Receptors

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Online Scheme I. Synthesis of 6a, 6b and WNNO6 (6c).
Online Scheme II. Synthesis of WNNO8

Online Scheme III. Synthesis of WNNO9
### Online Table I. Water Solubility of N-Nitrosamines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solubility (in D$_2$O, 23 °C)</th>
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<td><img src="image" alt="Structure 9" /></td>
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Online Figure I. UV-Visible Spectral Changes of S-Transnitrosylation of WNNO6 and WNNO8. No Spectral Change in the case of WNNO9.
Experimental Procedure for Chemistry

1. General Methods for Synthesis

All the reagents were commercially available and used without further purification, unless otherwise noted. All the NMR data 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 ($\delta$) are reported in ppm with respect to an internal tetramethylsilane ($\delta=$0 ppm) or undeuterated residual solvent (i.e. CHCl$_3$ ($\delta$=7.265 ppm)). Coupling constants are given in hertz. Coupling patterns are indicated as followed: m=multiplet, d=doublet, s=singlet, br=broad. 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 and flash column chromatography were carried out on silica gel, silica gel 60N (100-210 $\mu$m) and silica gel 60N (40-50 $\mu$m), respectively (Kanto Chemicals, Japan). All the melting points were measured with a Yanaco Micro Melting Point Apparatus and are uncorrected. Microwave heating was carried out on a Biotage Initiator system (400W). Combustion analyses were carried out in the microanalysis laboratory of this faculty in Graduate School of Pharmaceutical Sciences.

2. Synthesis

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\begin{align*}
\text{NH} \quad \text{O} & \quad \text{O} \\
\text{Cl} & \quad \text{OMe} \\
\text{N} & \quad \text{O} \\
\text{Me} & \quad \text{N} \quad \text{O} \\
\text{OH} & \quad \text{O} \\
\end{align*}
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**Compound 11:** To a solution of maleimide 10 (968.8 mg, 10.0 mmol) and N-methylmorphorine (1.32 mL, 12.0 mmol) in AcOEt (50 mL), methyl chlorocarbonate (1.56 mL, 20.2 mmol) was added at 0 °C. After 2.5 hrs stirring, the precipitate was filtered off, washed with AcOEt, the filtrate and washings were combined, and the whole was washed with brine, dried over sodium sulfate, and the solvent was evaporated. The residue was purified by flash column chromatography (1:2 ethyl acetate : $n$-hexane) to afford 11 (1.0445 g, 67%) as a white solid. Colorless plates (recrystallized from CH$_2$Cl$_2$ / $n$-hexane).
Mp: 60.5-62 °C. $^1$H-NMR (400 MHz, CDCl$_3$): δ = 6.84 (2H, s), 3.96 (3H, s). $^{13}$C-NMR (100 MHz, CDCl$_3$): δ (ppm) = 165.57, 148.04, 135.23, 54.25. HRMS (ESI$^+$,[M+Na]$^+$): Calcd. for C$_6$H$_5$NNaO$_4^+$, 178.0111. Found: 178.0112. Anal. Calcd. for C$_6$H$_5$NO$_4$: C, 46.46; H, 3.25; N, 9.03. Found: C, 46.32; H, 3.30; N, 8.95.

**Compound 12:** To a solution of γ-aminobutylic acid (GABA) (113.0 mg, 1.1 mmol) in saturated aqueous NaHCO$_3$ (5 mL), 11 (155 mg, 1.0 mmol) was added with rapid stirring at 0 °C in an ice-water bath. The resulting solution was stirred at 0 °C for 30 min, then the cooling bath was removed and the whole was allowed to warm to rt over 90 min. The reaction mixture was acidified with 2M aqueous HCl (pH=2) and the whole was extracted with chloroform and the combined organic layer was dried over Na$_2$SO$_4$. Evaporation of the solvent gave 12 as a white solid (109.8 mg, 0.60 mmol, 60 %). Colorless plates (recrystallized from acetone/ n-hexane). Mp: 88-90°C. $^1$H-NMR (CDCl$_3$): δ = 6.71 (2H, s), 3.60 (2H, t, $J = 16.0$ Hz), 2.83 (2H, t, $J = 8.0$ Hz), 1.90-1.97 (2H, m). $^1$H-NMR (DMSO-d$_6$): δ = 12.10 (1H, s), 6.99 (2H, s), 3.42 (2H, t, $J = 6.8$ Hz), 2.20 (2H, t, $J = 7.2$ Hz), 1.71 (2H, q, $J = 7.2$ Hz). $^{13}$C-NMR (CDCl$_3$): δ = 171.2, 134.2, 59.1, 52.2, 34.3, 31.2. $^{13}$C-NMR (DMSO-d$_6$): δ (ppm) = 173.77, 171.11, 134.46, 36.55, 30.82, 23.38. HRMS (ESI-TOF, [M-H]$^-$): Calcd. 182.0459; Found: 182.0453. Anal. Calcd. for C$_8$H$_9$NO$_4$: C, 52.46 ; H, 4.95 ; N, 7.65. Found: C, 52.37; H, 4.86; N, 7.52.

**Compound 17:** A solution of a mixture of TosMIC (5.8565 g, 30 mmol) and fumaronitrile 16 (2.3464 g, 30 mmol) in Et$_2$O-DMSO (90 mL/45 mL) was added to a
suspension of NaH (60% in oil, 1.4403 g, 36 mmol) in Et₂O (60 mL) at 0 °C. The mixture was refluxed for 30 min. The whole was poured into water and extracted with Et₂O. The combined organic phase was dried over Na₂SO₄. The solution was concentrated under reduced pressure to give the residue which was purified by flash column chromatography (silica gel, hexane/acetone = 3/1) to afford 17 (2.2489 g, 19.2 mmol, 64% yield) as white solid. White solid. Mp: 233.0–234.5 °C (recrystallized from acetone/ n-hexane). ¹H-NMR (DMSO-d₆): δ = 12.66 (1H, s), 7.87 (2H, s). ¹³C-NMR (100 MHz, DMSO-d₆): δ (ppm) = 129.33, 113.95, 94.07. Anal. Caled. for C₆H₃N₃: C, 61.54; H, 2.58; N, 35.88. Found: C, 61.78; H, 2.85; N, 35.61.

**Compound 18:** To a solution of 17 (1.3996 g, 11.4 mmol) in DMF (24 mL), NaH (60% in oil, 557.8 mg, 13.9 mmol) was added at 0 °C. After 10 min, BnBr (1.6 mL, 13.7 mmol) was added to the above suspension. The mixture was heated at 50 °C for 10 min with stirring. The whole was poured into water and extracted with AcOEt. The combined organic phase was washed with brine and dried over Na₂SO₄. The solution was concentrated under reduced pressure to give the residue, which was purified by flash column chromatography (silica gel, hexane/AcOEt= 3/1 to 1/1) to afford 18 (2.3218 g, 98% yield) as colorless solid. Colorless cubes. Mp: 153.5–155°C (recrystallized from CH₂Cl₂/n-hexane). ¹H-NMR (DMSO-d₆): δ = 8.01 (2H, s), 7.41–7.29 (5H, m), 5.25 (2H, s). ¹³C-NMR (DMSO-d₆): δ (ppm) = 136.19, 131.54, 128.87, 128.28, 127.71, 113.43, 94.53, 53.42. Anal. Caled. for C₁₃H₉N₃: C, 75.35; H, 4.38; N, 20.28. Found: C, 75.39; H, 4.56; N, 20.21.

**Compound 19:** To a solution of 18 (1.0094 g, 4.9 mmol) in CH₂Cl₂ (30 mL) was added DIBAL-H (1M in toluene, 9.8 mL, 9.8 mmol). The reaction mixture was stirred at 0°C for 30 min. After 30 min stirring at 0°C, water (2 mL) was added into the mixture and continued to stir for 10 min at 0°C. Then silica gel (5 g) was added into the mixture and the mixture was stirred for another 30 min. Then anhydrous Na₂SO₄ (8 g) was added into the mixture, stirred for another 5 min, filtered through a Celite pad and the pad was washed with acetone. The filtrate was concentrated under reduced pressure to give the residue, which was purified by flash column chromatography (n-hexane/acetone = 3/1) to afford 19 (870.7 mg, 84% yield) as colorless solid. Colorless plates (recrystallized from

**Compound 20:** To a solution of 19 (746.4 mg, 3.5004 mmol) in EtOH (26.6 mL), glycine ethyl ester hydrochloride (755.8 mg, 5.4148 mmol), diethylamine (0.724 mL, 7.0024 mmol) was added. The mixture was refluxed for 20 min and the whole was poured into water and extracted with AcOEt. The combined organic phase was washed with brine and dried over Na₂SO₄. The solution was concentrated under reduced pressure to give the residue, which was purified by open column chromatography (n-hexane/AcOEt = 3/2) to afford 20 (944.0 mg, 96% yield) as orange solid. Orange solid. Mp: 109-110°C (recrystallized from EtOAc/n-hexane).

¹H-NMR (CDCl₃): δ = 9.06 (1H, s), 8.36 (1H, s), 7.39 – 7.32 (5H, m), 7.19 – 7.17 (2H, m), 5.42 (2H, s), 4.46 (2H, q, J = 7.2 Hz), 1.44 (3H, t, J = 7.2 Hz). ¹³C-NMR (CDCl₃): δ (ppm) = 166.82, 146.24, 136.23, 135.67, 129.12, 128.65, 127.58, 124.43, 121.98, 118.32, 114.25, 114.09, 61.22, 55.45, 14.51. HRMS (ESI⁺, [M+Na]⁺): Calcd. for C₁₇H₁₆N₂NaO₂⁺, 303.1104. Found: 303.1068. Anal. Calcd. for C₁₇H₁₆N₂O₂: C, 72.84; H, 5.75; N, 9.99. Found: C, 72.64; H, 5.82; N, 9.83.

**Compound 21:** To a solution of 12 (1.0212 g, 3.6 mmol) in CH₂Cl₂ (14 mL), 20 (739.6 mg, 4.04 mmol) was added at rt. The mixture was stirred at rt for 12 hrs, and the whole was concentrated under reduced pressure to give the residue, which was purified by flash column chromatography (CHCl₃/MeOH = 100/1) to afford 21 (1.5696 g, 93% yield) as white solid. Colorless cubes. Mp: 134-136.5°C (recrystallized from acetone/n-hexane).

¹H-NMR (CDCl₃): 8.67 (1H, s), 8.09 (1H, s), 7.28-7.32 (3H, m), 7.13-7.15 (2H, m), 4.67-4.72 (2H, m), 4.43-4.49 (2H, m), 3.76-3.81 (2H, m), 3.29 (2H, s), 2.96-3.00 (2H, t, J = 8.0 Hz), 1.90-1.94 (2H, m), 1.43 (3H, t, J = 8.0 Hz), 1.06-1.13 (2H, m). ¹H-NMR (DMSO-d₆): δ = 11.94 (1H, s), 8.56 (1H, s), 7.94 (1H, s), 7.35 – 7.23 (5H, m), 4.78 – 4.76 (2H, m), 4.32 (2H, q, J = 7.2 Hz), 3.92 – 3.91 (2H, m), 3.32 (2H, s), 2.80 (2H, td, J = 6.8
Hz, 2.4 Hz), 1.76 (2H, t, J = 7.2 Hz), 1.31 (3H, J = 7.2 Hz), 0.81 – 0.73 (2H, m). $^{13}$C-NMR (CDCl$_3$) δ = 170.5, 174.6, 174.5, 164.5, 151.5, 148.3, 144.1, 139.7, 136.8, 128.8, 128.6, 127.9, 127.9, 120.3, 66.4, 64.8, 62.3, 52.4, 46.6, 46.3, 37.4, 30.4, 22.1, 14.3. $^{13}$C-NMR (DMSO-d$_6$): δ (ppm) = 175.33, 174.54, 174.50, 164.52, 151.46, 148.41, 144.12, 139.65, 136.89, 128.73, 128.56, 127.84, 120.25, 66.47, 64.82, 62.23, 52.40, 46.67, 46.35, 37.41, 30.58, 22.08, 14.27. HRMS (ESI-TOF, [M-H]–): Calcd. for C$_{25}$H$_{24}$N$_3$O$_6$: 462.1671. Found: 462.1691. Anal. Calcd. for C$_{25}$H$_{25}$N$_3$O$_6$; C, 64.79; H, 5.44; N, 9.07. Found: C, 64.71; H, 5.46; N, 8.89.

**Compound 22**: To a solution of 21 (100 mg, 0.215 mmol) in dioxane/H$_2$O (1.0 mL / 1.0 mL), NBS (45.9 mg, 0.258 mmol) was added. The mixture was stirred at rt for 17 hr. To the residue, obtained after evaporation, was added AcOH/H$_2$O (1.0 mL/1.0 mL) and stirred at 0 °C and a solution of NaNO$_2$ (17.9 mg, 0.258 mmol) in H$_2$O (1.0 mL) was added. After stirred for 6 hr at 0°C the whole was poured into water and extracted with CHCl$_3$. The combined organic phase was dried over Na$_2$SO$_4$. The solution was concentrated under reduced pressure to give the residue (80.3 mg), which was purified by open column chromatography (silica gel, CHCl$_3$/MeOH = 50/1) to afford 22 (40.3 mg, 0.117 mmol, 54% yield) as pale yellow solid.

**Another nitrosation reaction conditions**: To a solution of compound 135 (462.3 mg, 0.9974 mmol) in mixture of dioxane (5 mL) and water (2 mL) was added NBS (258.8 mg, 1.2074 mmol) and the whole was stirred at rt for 24 hrs. After dioxane was evaporated, sodium nitrite (277.6 mg, 4.0232 mmol), paraformaldehyde (64.2 mg) and PBS (pH 6.4, 20 mL) was added and the whole was stirred at rt for 17 hrs. The mixture was acidified with 1N HCl solution, extracted with EtOAc, and the organic phase was washed with brine, dried over anhydrous Na$_2$SO$_4$, and evaporated. The residue obtained was purified with flash column chromatography (CHCl$_3$ : MeOH = 50 : 1) to give 22 (149.2 mg, 37%)

Pale yellow cubes. Decomposition: 165 °C (recrystallized from CHCl$_3$). $^1$H-NMR (CDCl$_3$): δ = 10.40 (1H, s), 8.76 (1H, s), 8.136 (1H, s), 6.53 (1H, brs), 6.31 (1H, brs), 4.40 (2H, q, J = 7.2 Hz), 3.88 (2H, brs), 2.98 (2H, t, J = 7.2 Hz), 1.95 (2H, t, J = 7.2 Hz), 1.37 (3H, t, J = 7.2 Hz), 1.11 (2H, q, J = 7.2 Hz). $^{13}$C-NMR (CDCl$_3$): δ = 76.38, 172.40, 172.37, 163.70, 148.64, 148.18, 142.64, 137.31, 118.56, 62.48, 58.03, 56.43, 47.17, 44.21, 37.79,

**7 (WNNO7):** To a stirred solution of **22** (137.0 mg, 0.315 mmol), triethylene glycol (180 mg, 1.32 mmol) and DMAP (2 mg, 0.016 mmol) in CH₂Cl₂ (5.0 mL), EDC (78.2 mg, 0.409 mmol) was added at 0°C. After stirred for 17 h at rt, the whole was poured into water and extracted with AcOEt. The combined organic phase was dried over Na₂SO₄. The solution was concentrated under reduced pressure to give the residue (137.3 mg), which was purified by open column chromatography (silica gel, CHCl₃/MeOH = 100/1) to afford **7 (WNNO7)** (124.3 mg, 0.232 mmol, 74% yield) as yellow oil. ¹H-NMR (CDCl₃): δ = 8.75 (1H, s), 8.15 (1H, s), 6.34 (2H, brs), 4.44-4.49 (2H, q), 3.59-3.73 (12H, m), 2.99 (2H, t, J = 8.0 Hz), 1.96-2.06 (2H, m), 1.45 (3H, t, J = 8.0 Hz), 1.11-1.23 (2H, m). ¹³C-NMR (CDCl₃): 173.1, 172.3, 128.9, 72.5, 69.1, 64.0, 63.6, 61.8, 58.5. HRMS (ESI-TOF, [M+Na]⁺): Calcd. for C₂₄H₃₀N₄O₁₀Na⁺: 557.1854. Found: 557.1843.

**Compound 29:** To a precooled suspension of GABA (γ-aminobutyric acid) (1030 mg, 10 mmol) in MeOH (14.7 mL) at 0 °C, SOCl₂ (1442 µl) was carefully added in a dropwise manner with stirring. The resultant mixture was then warmed to 40 °C. After 3.5 h, the solvent was removed by rotary evaporation to afford **29** (1530 mg, 10 mmol, 100%) as a
white solid. White powder. Mp. 122-124 °C (recrystallized from MeOH). $^1$H-NMR (D$_2$O): $\delta$ = 3.70 (3H, s), 3.03 (2H, $\tau$, $J$ = 7.6 Hz), 2.52 (2H, $\tau$, $J$ = 7.2 Hz), 1.96 (2H, m).

**Compound 31:** To a stirred solution of GABA methyl ester hydrochloride 29 (284.7 mg, 2.04 mmol) in dry toluene (75 mL) and Et$_3$N (2458 ml, 10 mmol) was added a solution of succinic anhydride 30 (980.6 mg, 10 mmol) in dry toluene (25 mL) in a dropwise manner at rt. During the addition, an exothermic reaction occurred with instantaneous formation of white precipitates. After the addition was complete, the resulting suspension was stirred for an additional 1 h and then ZnCl$_2$ (1360 mg, 10 mmol) was added in one portion. The resulting mixture was heated at 80 °C, and then a solution of hexamethyldisilazane (HMDS) (2676 mg, 15 mmol) in dry toluene (25 mL) was added slowly, and then the mixture was refluxed for an additional 19 h. The reaction mixture was cooled to rt and poured into 0.5 N aqueous HCl (120 mL). The aqueous phase was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were washed successively with 30 mL of saturated NaHCO$_3$ and brine and dried over anhydrous Na$_2$SO$_4$. The solution was concentrated under reduced pressure to give the residue, which was purified by flash column chromatography (silica gel, hexane/EtOAc = 3/1) to afford 31 (1439.9 mg, 7.30 mmol, 73% yield) as white powder. White powder. Mp. 56.5-57.5 °C. $^1$H-NMR (CDCl$_3$): $\delta$ = 6.66 (2H, s), 3.62 (3H, s) 3.53 (2H, $\tau$, $J$ = 7.2 Hz), 2.28 (2H, $\tau$, $J$ = 7.2 Hz), 1.88 (2H, m). HRMS (ESI-TOF, [M+H]$^+$): Calcd. for C$_9$H$_{12}$NO$_4$+: 198.0761. Found: 198.0753.

**Compound 15-Me**

To a solution of 14 (1471.6 mg, 7.1 mmol) in CH$_2$Cl$_2$ (32 mL) a solution of 31 (1400 mg, 7.1 mmol) in CH$_2$Cl$_2$ (4.3 mL) was added over 5 min at 0°C. The mixture was stirred at 0°C for 2.5 h. The residue, obtained after evaporation of the solvent, was chromatographed (n-hexane : AcOEt = 3 : 1) to afforded 15-Me (2640.6 mg, 6.528 mmol 91.9%) as a white solid. Colorless cubes. Mp: 62-63°C (recrystallized from CH$_2$Cl$_2$). $^1$H-NMR (CDCl$_3$): $\delta$ = 7.33-7.18 (9H, m), 4.55 (2H, m), 3.67 (2H, q, $J$ = 7.2 Hz), 3.63 (3H, s), 3.33 (2H, s), 2.92 (2H, $\tau$, $J$ = 8.0 Hz), 1.89 (2H, $\tau$, $J$ = 8.0 Hz), 1.03 (2H, m). $^{13}$C-NMR (CDCl$_3$) $\delta$ = 177.3, 175.6, 140.3, 137.7, 128.8, 128.5, 128.0, 127.5, 123.7, 66.8. 66.3, 52.3, 47.2, 37.2, 31.1,
22.3. Anal. Calcd. for C_{24}H_{24}N_{2}O_{4}: C, 71.27; H, 5.98; N, 6.93. Found: C, 71.27; H, 6.19; N, 6.93.

**Compound 15:**

(method A) To a solution of 14 (1471.6mg, 7.1 mmol) in CH_{2}Cl_{2} (32 mL) a solution of 12 (1400 mg, 7.1 mmol) in CH_{2}Cl_{2} (4.3 mL) was added over 5 min at 0 °C. The mixture was stirred at rt for 22 h. The residue, obtained after evaporation of the solvent, was column-chromatographed (CHCl_{3} : MeOH = 100 : 1) to afford 15 (2640.6 mg, 6.528 mmol 91.9%) as a white solid.

(method B) To a solution of 15-Me (50 mg, 0.124 mmol) in MeOH:THF (2:1, 1.5 mL) was added 2% aqueous NaOH (20 mL) and the whole was stirred 2 hr at ambient temperature. The solution was poured into 2 M aqueous HCl (0.5 mL) and H_{2}O (2.0 mL) and the whole was extracted with CH_{2}Cl_{2} (3 × 20 mL). The combined organic phase was dried over Na_{2}SO_{4}. The solution was concentrated under reduced pressure to give the residue, which was purified by flash column chromatography (silica gel, hexane/EtOAc=3/1) to afford 15 (24.7 mg, 0.063 mmol, 51% yield) as a solid. Colorless plates. Mp: 127-128 °C (recrystallized from CH_{2}Cl_{2}). \(^1\)H-NMR (CDCl_{3}): \(\delta = 7.33-7.18\) (9H, m), 4.57 (2H, m), 3.70 (2H, q, \(J = 7.2\) Hz), 3.33 (2H, s), 2.96 (2H, t, \(J = 8.0\) Hz), 1.92 (2H, t, \(J = 8.0\) Hz), 1.07 (2H, m). \(^{13}\)C-NMR (CDCl_{3}): \(\delta = 177.3, 175.6, 140.3, 137.7, 128.8, 128.5, 128.0, 127.5, 123.7, 66.8, 52.3, 47.2, 37.2, 31.1, 22.3\). Anal. Calcd. for C_{24}H_{24}N_{2}O_{4}: C, 71.27; H, 5.98; N, 6.93; O, 15.82. Found: C, 71.19; H, 7.00; N, 16.01.
Compound 5-M: To a solution of the adduct 15-Me (150 g, 0.370 mmol) in 1.7 mL of dioxane, a solution of NBS (79.5 mg, 4.44 mmol) in 0.7 mL of H_2O was added over 2 min at ambient temperature. The mixture was stirred at rt for 24 hr. To the residue obtained after evaporation was added AcOH / H_2O (0.9 mL / 1.5 mL). To this solution, a solution NaNO_2 (34.5 mg, 0.481 mmol, 1.3 eq.) in H_2O (0.9 mL) was added with stirring at 0°C. After stirred for 2 h at 0°C, the whole was poured into water and extracted with AcOEt. The combined organic phase was dried over Na_2SO_4. The solvent was evaporated to give the residue, which was purified by flash column chromatography (silica gel, hexane/EtOAc = 3/1) to afford 5-Me (125.7 mg, 0.367 mmol, 99.2% yield) as yellow solid. Yellow cubes. Mp: 130-132°C (decomp.) (recrystallized from CH_2Cl_2/n-hexane). ^1H-NMR (CDCl_3): δ =7.33-7.18 (9H, m), 6.13-6.40 (2H, d), 4.55 (2H, m), 3.50-3.78 (2H, d), 3.64 (3H, s), 2.97 (2H, t, J = 8.0 Hz), 1.92 (2H, t, J = 8.0 Hz), 1.06 (2H, m). ^13C-NMR (CDCl_3): δ = 177.0, 173.0, 128.9, 122.8, 122.1, 66.4, 63.3, 58.3, 48.2, 44.8, 37.6, 30.8, 22.1. HRMS (ESI-TOF, [M+Na]^+): Calcd. for C_{17}H_{17}N_3NaO_5^+: 366.1060. Found: 366.1024. Anal. Calcd. for C_{17}H_{17}N_3O_5: C, 59.47; H, 4.99; N, 12.24. Found: C, 59.19; H, 5.00; N, 12.04.

Compound 33:
To a stirred solution of sodium hydride (60% dispersion in mineral oil, 2.94 g, 1.1 eq) in THF (134 mL) was added triethyleneglycol (8.80 mL, 67 mmol) dropwise at 0°C. After stirring at 0°C for 5 min, tert-butyldimethylsilyl chloride (12.0 g, 1.2 eq) was added and
the stirring was continued for 1 h at rt. The reaction mixture was quenched with water, and the whole was extracted with ethyl acetate. The organic layer was washed with saturated aqueous ammonium chloride and brine and dried over magnesium sulfate. The solvent was evaporated to give the residue, which was purified by flush column chromatography (neat n-hexane then 50% ethyl acetate in n-hexane) to afford 33 (9.7 g, 55%) as a colorless oil. 

$^1$H NMR (CDCl$_3$) $\delta$ = 3.75 (t, $J = 4.8$ Hz, 2 H), 3.72-3.68 (m, 2H), 3.66-3.62 (m, 4H), 3.60-3.58 (m, 2H), 3.55 (t, $J = 5.2$ Hz, 2H), 2.42 (br, 1H), 0.88 (s, 9H), 0.05 (s, 6H).

$^{13}$C-NMR (CDCl$_3$): $\delta$ = 72.69, 72.49, 70.79, 79.48, 62.69, 25.92, 18.36, -5.30. HRMS (ESI-TOF, [M+Na]$^+$): Calcd. for C$_{12}$H$_{28}$NaO$_4$Si$^+$: 287.1649. Found: 287.1643.

**Compound 34**

To a solution of 15 (120.0 mg, 0.307 mmol) in CH$_2$Cl$_2$ (1.9 mL), DMAP (3.8 mg, 0.031 mmol 0.15eq.) and 33 (105 mg, 0.399 mmol, 4.0eq.) was added and the whole was stirred at 0 °C. After 10 min, EDC (76.5 mg, 0.399 mmol, 1.3eq.) was added to the mixture and the whole was stirred for 17 hrs at rt. The solution was poured into water (2.0 mL) and the whole was extracted with CH$_2$Cl$_2$ (3 × 20 mL). The combined organic phase was dried over Na$_2$SO$_4$. The solution was concentrated under reduced pressure. The residue was purified with open column chromatography (50% ethyl acetate in n-hexane) to afford 34 (253.8 mg, 0.300 mmol) as colorless oil. $^1$H-NMR (CDCl$_3$): $\delta =$ 7.33-7.18 (9H, m), 4.55 (2H, m), 3.75 (2H, m), 3.65 (8H, m), 3.55 (2H, t), 3.34 (2H,s) 2.91 (2H, t, $J = 7.2$ Hz), 1.90 (2H, t, $J = 7.2$ Hz), 1.05 (2H, m), 0.89 (9H,s), 0.05 (6H, s).

Upon elimination of the benzyl group, multiple by-products were formed partially because cleavage of the peg group.

**Compound 5:**

To a solution of 15 (160 g, 0.409 mmol) in 2.0 mL of dioxane, a solution of NBS (80 mg, 0.40 mmol) in 1.0 mL of H$_2$O was added over 2 min at ambient temperature. The mixture was stirred at rt for 24 hr. To the residue obtained after evaporation was added AcOH/H$_2$O
(1.0 mL / 1.5 mL) and the whole was stirred at 0°C. To this solution, a solution of NaNO₂ (45.0 mg, 0.665 mmol) in H₂O (0.9 mL) was added. After stirring for 3 h at 0°C, the whole was poured into water and the pH was adjusted to 8 with saturated aqueous NaHCO₃. The aqueous layer was acidified with 2M aqueous HCl, and the whole was extracted with AcOEt. The combined organic phase was dried over Na₂SO₄. The solution was concentrated under reduced pressure to afford 5 (80.2 mg, 0.2435 mmol, 60% yield) as yellow solid. Yellow needles. Mp: 115-117°C (recrystallized from MeOH). ¹H-NMR (CDCl₃): δ 7.30-7.40 (4H, m), 6.23 (2H, d, J = 10.4 Hz), 3.76-3.80 (2H, d, J = 10.6 Hz), 3.01 (2H, t, J = 8.0 Hz), 1.96 (2H, t, J = 8.0 Hz), 1.09 (2H, m). ¹³C-NMR (CDCl₃): δ = 176.97, 172.99, 128.89, 122.78, 122.11, 63.28, 58.28, 48.23, 44.79, 37.61, 30.80, 22.13. HRMS (ESI-TOF, [M-H]⁻): Calcd. for C₁₆H₁₅N₃O₅: 328.0939. Found: 328.0956. Anal. Calcd. for C₁₆H₁₅N₃O₅+0.5H₂O; C, 56.80; H, 4.77; N, 12.42. Found: C, 56.60; H, 4.80; N, 12.27.

**Compound 6a**

To a solution of 5 (100 mg, 0.33 mmol), ethylenegrycol (37.2 mg, 0.60 mmol) and DMAP (4 mg, 0.032 mmol) in CH₂Cl₂ (3.0 mL) was added EDC (75.3 mg, 0.394 mmol) with stirring at 0°C. After stirring for 19 h at rt, the whole was poured into water and was extracted with AcOEt. The combined organic phase was dried over Na₂SO₄. The solution was concentrated under reduced pressure to give the residue (121.3 mg), which was purified by open column chromatography (silica gel, CHCl₃/MeOH = 100/1) to afford 6a (44.8 mg, 0.120 mmol, 39% yield) as yellow solid. Yellow cubes. Mp: 53-54°C. ¹H-NMR (CDCl₃): δ =7.30-7.40 (4H, m), 6.23 (2H, d, J = 10.4 Hz), 4.19 (2H, t, J = 8.0 Hz), 3.50-3.83 (4H, m), 2.96 (2H, t, J = 8.0 Hz), 2.41 (1H, brs), 1.96 (2H, t, J = 8.0 Hz), 1.07-1.15 (2H, m); ¹³C-NMR (CDCl₃): δ = 172.14, 172.23, 128.87, 122.75, 122.21, 66.25, 63.72, 61.04, 58.22, 48.21, 44.89, 37.10, 31.00, 22.23. HRMS (ESI-TOF, [M+Na]⁺): Calcd.
for C\textsubscript{18}H\textsubscript{19}N\textsubscript{3}NaO\textsubscript{6}: 396.1166. Found: 396.1158. Anal. Calcd. for C\textsubscript{18}H\textsubscript{19}N\textsubscript{3}O\textsubscript{6}+0.2H\textsubscript{2}O; C, 57.35; H, 5.19; N, 11.15. Found: C, 57.64; H, 5.20; N, 10.80.

**Compound 6b:**
To a solution of 5 (100 mg, 0.33 mmol), diethylene glycol (63.6 mg, 0.60 mmol) and DMAP (4 mg, 0.032 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (3.0 mL), EDC (75.3 mg, 0.394 mmol) was added at 0°C with stirring. After stirring for 19 h at rt, the whole was poured into water and the whole was extracted with AcOH. The combined organic phase was dried over Na\textsubscript{2}SO\textsubscript{4}. The solution was concentrated under reduced pressure to give the residue (129.8 mg), which was purified by open column chromatography (silica gel, CHCl\textsubscript{3}/MeOH = 100/1) to afford 6b (54.3 mg, 0.130 mmol, 42% yield) as yellow solid. Yellow powder. M.p.: 66-67 °C. 
\textsuperscript{1}H-NMR (CDCl\textsubscript{3}): \( \delta =7.30-7.40 \) (4H, m), 6.26 (2H, d, \( J = 10.6 \) Hz), 4.29 (2H, t, \( J = 8.0 \) Hz), 3.60-3.77 (8H, m), 2.99 (2H, t, \( J = 8.0 \) Hz), 1.96 (2H, t, \( J = 8.0 \) Hz), 1.09-1.16 (2H, m).
\textsuperscript{13}C-NMR (CDCl\textsubscript{3}): \( \delta = 173.14, 172.30, 128.93, 122.67, 122.30, 72.48, 69.11, 63.72, 63.56, 61.81, 58.12, 48.23, 45.89, 37.73, 31.14, 22.32. \)
HRMS (ESI-TOF, \([\text{M+Na}]^+\)): Calcd. for C\textsubscript{20}H\textsubscript{23}N\textsubscript{3}O\textsubscript{7}Na\textsuperscript{+}: 440.1428. Found: 440.1401.
Anal. Calcd. for C\textsubscript{20}H\textsubscript{23}N\textsubscript{3}O\textsubscript{7}Na; C, 57.55; H, 5.55; N, 10.07. Found: C, 57.33; H, 5.50; N, 9.77.

**Compound 6c:** To a solution of 5 (287 mg, 0.87 mmol), triethylene glycol (522.0 mg, 3.48 mmol) and DMAP (4 mg, 0.032 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (10.0 mL), EDC (216.5 mg, 1.133 mmol) was added at 0°C with stirring. After stirring for 10 h at rt, the whole was poured into water and the whole was extracted with AcOH. The combined organic phase was dried over Na\textsubscript{2}SO\textsubscript{4}. The solution was concentrated under reduced pressure to give the residue (400.3 mg), which was purified by open column chromatography (silica gel, CHCl\textsubscript{3}/MeOH = 100/1) to afford 6c (363.1 mg, 0.78 mmol, 91% yield) as yellow oil. Yellow oil; \textsuperscript{1}H-NMR (CDCl\textsubscript{3}): \( \delta =7.30-7.40 \) (4H, m), 6.26 (2H, d, \( J = 10.6 \) Hz), 4.20 (2H, t, \( J = 8.0 \) Hz), 3.00-4.21 (12H, m), 2.98 (2H, t, \( J = 8.0 \) Hz), 1.96 (2H, t, \( J = 8.0 \) Hz), 1.08 (2H, m).
\textsuperscript{13}C-NMR (CDCl\textsubscript{3}): \( \delta = 172.96, 172.22, 128.90, 122.74, 122.30, 72.47, 70.56, 70.35, 69.10, 63.47, 63.25, 61.76, 58.25, 48.13, 44.83, 37.69, 31.11, 22.28. \)
HRMS (ESI-TOF, \([\text{M+Na}]^+\)): Calcd. for C\textsubscript{22}H\textsubscript{27}N\textsubscript{3}O\textsubscript{8}Na\textsuperscript{+}: 484.1690; Found: 484.1663.

Anal. Calcd. for C\textsubscript{22}H\textsubscript{27}N\textsubscript{3}O\textsubscript{8}·0.7H\textsubscript{2}O; C, 55.74; H, 6.04; N, 8.86. Found: C, 55.64; H, 5.77; N, 8.56.
Compound 27: To a solution of 26 (579.1 mg, 2 mmol), TFA (4 mL) was added at 0 °C. The mixture was stirred at 0°C for 15 min. To the residue obtained after evaporation was added a solution of NaNO₂ (218.4 mg, 3.14 mmol) in AcOH/H₂O (3 mL/4 mL) at 0°C. After stirring at 0°C for 3 hr, the whole was poured into water and the whole was extracted with CHCl₃. The combined organic phase was dried over Na₂SO₄. The solution was concentrated under reduced pressure to give the residue (326.1 mg), which was purified by open column chromatography (silica gel, CHCl₃/MeOH = 100/1) to afford 27 (266.1 mg, 1.2 mmol, 61% yield) as colorless powder. Colorless powder. Mp: 105-106 °C. ¹H-NMR (CDCl₃): δ = 7.22-7.37 (4H, m), 6.04 (2H, t, J = 6.0 Hz), 3.28-3.46 (1H, d, J = 6.4 Hz), 2.28-2.48 (1H, d, J = 4.0 Hz), 1.74-1.96 (1H, m).

Compound 8: To a solution of 27 (160.4 mg, 0.733 mmol), triethyleneglycol (440.6 mg, 2.93 mmol) and DMAP (2 mg, 0.016 mmol) in CH₂Cl₂ (8.0 mL), EDC (182.8 mg, 0.95 mmol) was added at 0°C with stirring. After stirring for 18 h at rt, the whole was poured into water and the whole was extracted with CHCl₃. The combined organic phase was dried over Na₂SO₄. The solution was concentrated under reduced pressure to give the residue (332.4 mg), which was purified by open column chromatography (silica gel, CHCl₃/MeOH = 100/1) to afford 8 (206.1 mg, 0.58 mmol, 80% yield) as yellow oil. Yellow oil. ¹H-NMR (CDCl₃): δ = 7.23-7.41 (4H, m), 6.13 (1H, d, J = 9.2 Hz), 6.00 (1H, d, J = 9.2 Hz), 4.09-4.12 (2H, m), 3.64-3.76 (10H, m), 3.39 (1H, d, J = 7.2 Hz), 2.28-2.48 (2H, m), 1.81-2.03 (1H, m). ¹³C-NMR (CDCl₃) δ = 169.98, 142.83, 141.68, 139.12, 137.84, 128.27, 127.43, 122.55, 121.91, 120.52, 119.86, 72.51, 70.61, 70.37, 68.89, 64.20, 63.82, 63.05, 61.77, 58.21, 57.62, 45.10, 41.67, 32.04, 28.61. HRMS (ESI-TOF, [M+Na]⁺): Calcd. for C₁₇H₂₂N₂O₆Na⁺: 373.1370. Found: 373.1368.
Compound 29: The synthesis was carried out as described previously (reference 6). To a solution of L-proline 28 (576.6 mg, 5 mmol) and NaNO₂ in H₂O (4 mL), concentrated aqueous HCl (2.0 mL) was added at 0°C with stirring. The mixture was stirred at rt. After stirring for 15 h at rt, the whole was poured into water and the whole was extracted with tert-butyl methyl ether. The combined organic phase was dried over Na₂SO₄. The solution was concentrated under reduced pressure to give 29 (474.3 mg, 3.29 mmol, 65% yield) as pale yellow solid. Mp: 123.1-124.5 °C. ¹H-NMR (CDCl₃): δ = 7.88 (1H, brs), 5.31-5.34 (0.3H, m), 4.37-4.58 (2H, m), 3.66-3.72 (0.7H, m), 2.05-2.41 (4H, m). ¹³C-NMR (CDCl₃): 175.28, 173.36, 61.83, 58.00, 50.09, 45.77, 28.90, 27.59, 23.22, 21.12. HRMS (ESI-TOF, [M-H]⁻): Calcd. for C₅H₇N₂O₃⁻: 143.0462. Found: 143.0465.

Compound 9:

To a solution of 29 (288 mg, 2 mmol), triethylene glycol (600 mg, 4 mmol) and DMAP (4 mg, 0.032 mmol) in CH₂Cl₂ (18.0 mL), EDC (500.1 mg, 2.53 mmol) was added at 0°C with stirring. After stirring for 17 h at rt, the whole was poured into water and the whole was extracted with AcOEt. The combined organic phase was dried over Na₂SO₄. The solution was concentrated under reduced pressure to give the residue (400.3 mg), which was purified by open column chromatography (silica gel, CHCl₃/MeOH = 100/1) to afford 9 (312.3 mg, 1.13 mmol, 57% yield) as yellow oil. A mixture of the syn and anti isomers with respect to the N-NO bond. ¹H-NMR (CDCl₃): δ = 5.29-5.32 (0.4H, m), 4.25-4.40 (4.6H, m), 3.60-3.72 (10H, m), 2.05-2.51 (5H, m). ¹³C-NMR (CDCl₃) δ = 170.44, 168.62, 72.49, 70.61, 70.60, 70.32, 68.83, 68.78, 64.73, 64.40, 62.04, 61.74, 61.73, 58.11, 49.96, 45.68, 28.96, 27.76, 23.26, 21.14. HRMS (ESI-TOF, [M+Na]⁺): Calcd. for C₁₁H₂₀N₂O₆Na⁺: 299.1214. Found: 299.1232.
S-Nitrosoglutathion (GSNO)

The synthesis of GSNO was carried out as previously described (references 1 and 2). To a stirred ice-cold solution of glutathione (GSH) (1530 mg, 5 mmol) in water (8 mL) containing 2N HCl (2.5 mL) was added NaNO₂ (345.1 mg, 5 mmol). After 40 min at 0 °C the red-colored solution was quenched with acetone (10 mL) and the whole was stirred for a further 10 min. The precipitate was filtered off and then washed with ice-cold water 5 times, with acetone 3 times and with ether 3 times to afford S-nitrosoglutathion (GSNO) (302.5 mg, 0.89 mmol, 18%). Pink solid. Mp: 140-142 °C (decomp.); ¹H-NMR (400MHz/D₂O) : δ = 4.69 (1H, q, J = 8.0 Hz), 4.13-4.15 (1H, m), 3.96-4.03 (3H, m), 3.80 (1H, t, J = 8.0 Hz), 2.46 (1H, t, J = 16.0 Hz), 2.10-2.16 (1H, m).

References