Supplemental Data

Trans-Nitrosylation Directs TRPA1 Selectivity in *N*-Nitrosamine Activators

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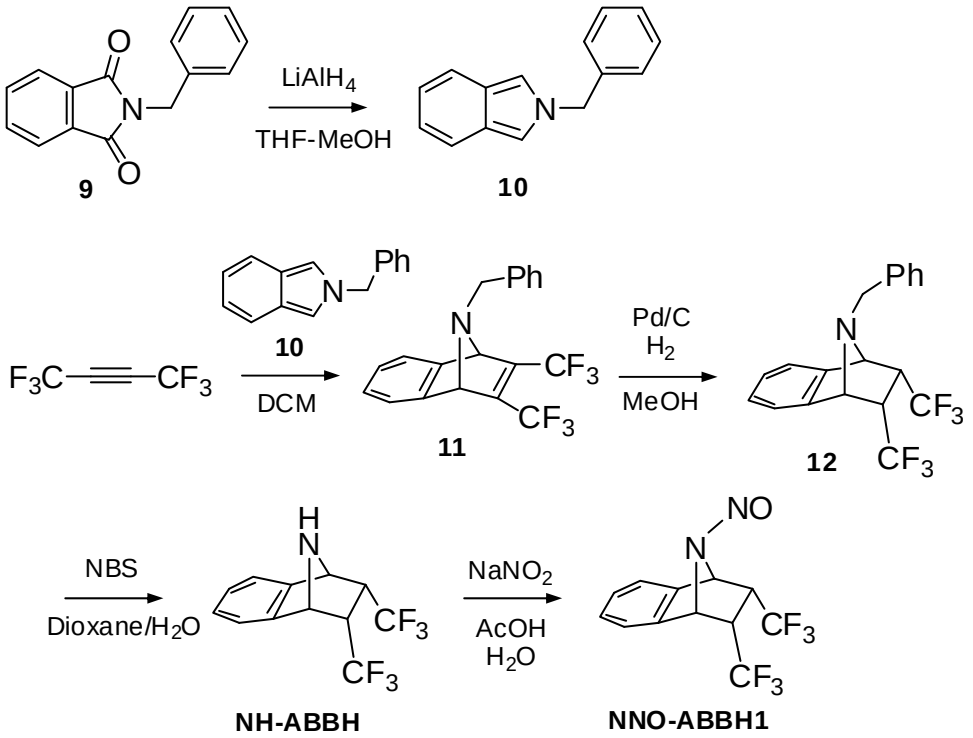
Molecular Pharmacology

Supplemental Materials and Methods

General Methods of Synthesis

All the melting points (Mp) were measured with a Yanaco Micro Melting Point Apparatus and are uncorrected. Proton (400 MHz) NMR and carbon (100 MHz) NMR spectra were measured on a Bruker Avance 400 NMR spectrometer with TMS as an internal reference in $CDCl_3$ as the solvent, unless otherwise specified. Chemical shifts (δ) are shown in ppm. Coupling constants are given in Hz. High-resolution mass spectrometry was obtained by electron-spray ionization time-of-flight mass spectra (ESI-TOF MS, ESI^+ or ESI^-) were recorded on a microTOF05, Bruker Daltonics. The combustion analyses were carried out in the microanalytical laboratory of Graduate School of Pharmaceutical Sciences, the University of Tokyo.

Synthesis of *N*-nitroso-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane (NNO-ABBH1)



Compound 10: To a suspension of $LiAlH_4$ (3.500 g, 92.3 mmol) in dry THF (75 ml) under argon atomospher, a solution of MeOH (6.5 ml) in THF (75 ml) was added over 30 min at 0°C, followed by cooling of the whole to –78°C (in an acetone-dry ice bath). To this suspension, *N*-benzylphthalimide **9** (7.023 g, 29.6 mmol) was added in three portions at –78°C, and the mixture was stirred at this temperature for 30 min. Then the mixture was stirred at 0°C for 30 min. A solution of sodium sulfate (1.0 g) in water (9 ml) was added, and the resultant inorganic salt was filtered by suction, and the precipitate was washed with acetone. The combined organic layer was dried over sodium sulfate, and the organic solvent was evaporated below 40°C. To the obtained residue, EtOH (20 ml) was added, and the whole was cooled to –28°C overnight. The precipitate was filtered and washed with cold EtOH. Evaporation of the solvent in vacuum gave **10** (3.4028 g, 16.42 mmol, 56% yield) (Ohwada et al., 2001). ^{1}H-NMR ($CDCl_3$): δ = 7.52 (2H, d, d, *J* = 6.8 Hz, 3.2 Hz), 7.31 (3H, m), 7.14 (3H, m), 6.92 (2H, d, d, *J* = 6.4 Hz, 2.8 Hz), 5.36 (2H, s).

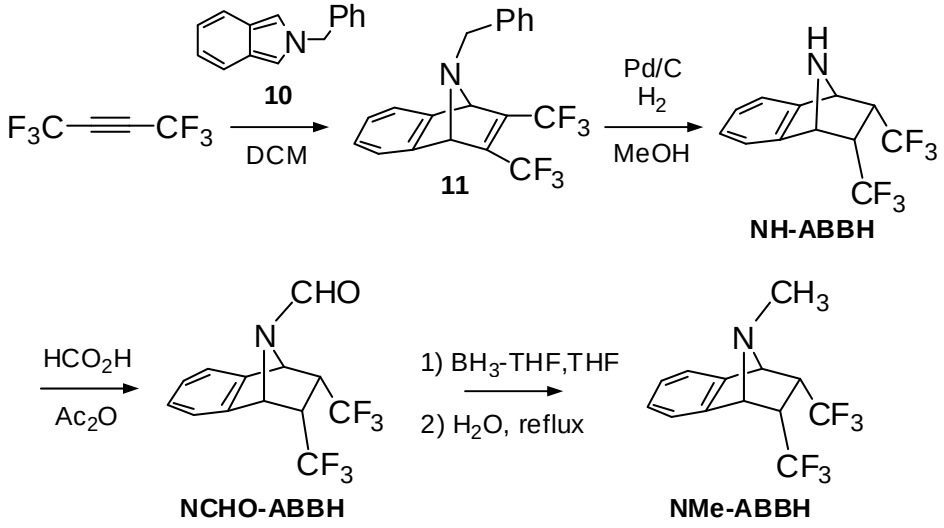
Compound 11: To a solution of hexafluoro-2-butyne (750 mg, 4.16 mmol) in CH_2Cl_2 (10 ml), stirred at –78°C, isoindoline **10** (958.3 mg, 4.62 mmol) in CH_2Cl_2 (5 ml) was added. The mixture was stirred for 4 h at –20°C and the whole was poured into water and extracted with CH_2Cl_2. The combined organic phase was dried over sodium sulfate. The solution was concentrated under reduced pressure to give the residue (1670.5 mg), which was purified by open chromatography (silica gel, *n*-hexane:CH_2Cl_2 = 4:1) to afford **11** (1062.6 mg, 2.87 mmol, 69% yield) as yellow oil. ^{1}H-NMR ($CDCl_3$): δ = 7.11–7.37 (9H, m), 4.88 (2H, s), 3.65 (2H, brs). ^{13}C-NMR ($CDCl_3$): δ = 145.29, 136.61, 129.15, 128.69, 128.69, 127.77, 126.34, 122.44 (broad m), 121.50 (q, *J* = 272 Hz), 72.1, 53.38. ESI-TOF ($[M+H]^+$): Calcd. for $C_{19}H_{14}F_6N^+$, 370.1025. Found: 370.1046.

Compound 12: The alkene derivative **11** (1000.1 mg, 2.70 mmol) was hydrogenated in MeOH (60 ml) over 10% Pd-C (200.3 mg) at room temperature (rt) for 8 h. Pd-C was removed by filtration with a celite pad, and the solvent was evaporated to afford crude **12** (1026.1 mg), which was purified by open chromatography (silica gel, *n*-hexane:CH_2Cl_2 = 3:1) to afford **12** (456.1 mg, 1.23 mmol, 47% yield) as white solid. Colorless cubes. Mp: 85–87°C (recrystallized from CH_2Cl_2/*n*-hexane). ^{1}H-NMR ($CDCl_3$): δ = 7.14–7.41 (9H, m), 4.37 (2H, s), 3.51–3.53 (2H, m), 3.25 (2H, s). ^{13}C-NMR ($CDCl_3$): δ = 141.15, 136.02, 128.72, 128.53, 127.50, 127.43, 124.68 (q, *J* = 278 Hz), 66.59, 50.90, 46.77 (m). ESI-TOF ($[M+H]^+$): Calcd. for $C_{19}H_{16}F_6N^+$, 372.1220. Found: 372.1181. Anal. Calcd. for $C_{19}H_{15}F_6N$: C, 61.46; H, 4.07; N, 3.77. Found: C, 61.71; H, 4.15; N, 3.61.

NH-ABBH: To a solution of **12** (400.1 mg, 1.072 mmol) in dioxane/H_2O (4.0 ml/3.0 ml), NBS (229.6 mg, 1.296 mmol) was added. The mixture was stirred at rt for 17 h. The residue obtained after evaporation was treated with 1 M NaOH aq. (20 ml), and the free amine liberated was extracted with ether (2 × 50 ml). The combined organic phase was dried over sodium sulfate. The solution was concentrated under reduced pressure to give the residue (203.1 mg), which was purified by open chromatography (silica gel, $CHCl_3$:MeOH = 10:1) to afford **NH-ABBH** (190.3 mg, 0.67 mmol, 63% yield) as colorless solid. The compound was identical with the amine obtained by the direct hydrogenation.

NNO-ABBH1: To a solution of **NH-ABBH** (100.1 mg, 0.268 mmol) in dioxane/H_2O (2.0 ml/1.0 ml), NBS (57.4 mg, 0.324 mmol) was added. The mixture was stirred at rt for 17 h. The residue obtained after evaporation was added AcOH/H_2O (1.4 ml/1.4 ml) and stirred at 0°C and $NaNO_2$ (22.4 mg. 0.324 mmol) in H_2O (1.4 ml) was added. After stirred for 3 h at 0°C the whole was poured into water and extracted with $CHCl_3$. The combined organic phase was dried over sodium sulfate. The solution was concentrated under reduced pressure to give the residue (136.1 mg), which was purified by open chromatography (silica gel, *n*-hexane:ethyl acetate = 5:1) to afford **NNO-ABBH1** (49.5 mg, 0.159 mmol, 59% yield) as yellow solid. Mp: 53–54°C (recrystallized from CH_2Cl_2/*n*-hexane). ^{1}H-NMR ($CDCl_3$): δ = 7.37–7.51 (4H, m), 6.30 (1H, brs), 5.98 (1H, brs), 3.49 (1H, brs), 3.27 (1H, brs). ^{13}C-NMR ($CDCl_3$): δ = 138.71, 137.39, 128.73 (C × 2), 123.53 (q, *J* = 270 Hz, CF_3 × 2), 123.31, 122.65, 63.65, 58.17, 47.98 (m), 43.41 (m). ESI-TOF ($[M+H]^+$): Calcd. for $C_{12}H_8F_6N_2ONa^+$, 333.0450. Found: 333.0433. Anal. Calcd. for $C_{12}H_8F_6N_2O$: C, 47.99; H, 4.91; N, 17.06. Found: C, 47.83; H, 4.70; N, 17.06.

Synthesis of *N*-H-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane (NH-ABBH), *N*-formyl-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane (NCHO-ABBH), and *N*-methyl-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane (NMe-ABBH)



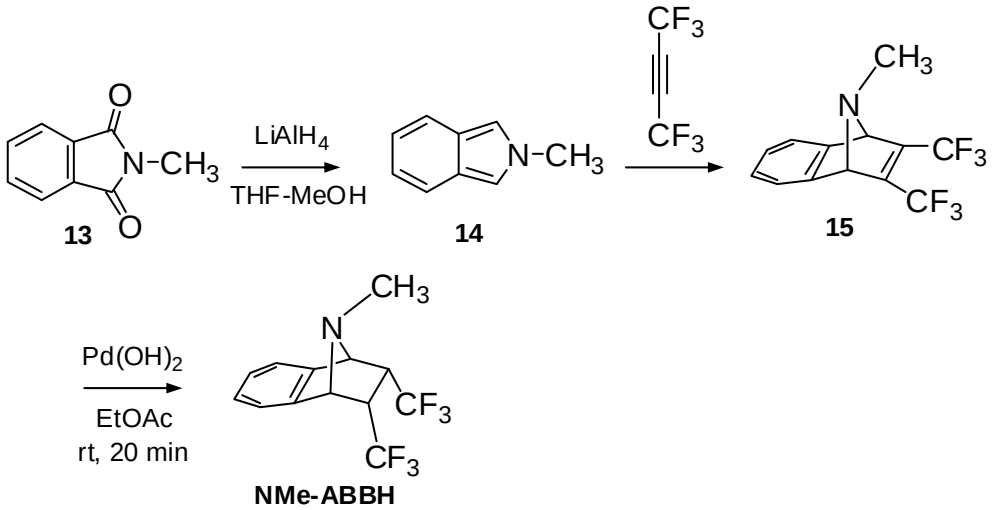
Direct hydrogenation of Diels-Alder adduct 11 to amine NH-ABBH: A solution of the olefin **11** (1.354 g) in 10 ml of methanol was hydrogenated at rt over 10% wet Pd/carbon (277.6 mg, 20% wet) for 100 h. The whole was filtrated with a celite pad and the Pd-carbon was washed with toluene (40 ml × 2). The combine organic solvent was evaporated to give an off-white powder (952.5 mg), which was flash-column chromatographed (ethyl acetate:*n*-hexane = 2:1) to give the amine **NH-ABBH** (831.2 mg, 81% yield). Coreless cubes. Mp: 124–128°C. ^{1}H-NMR ($CDCl_3$): δ = 7.350 (2H, m), 7.209 (2H, m), 4.719 (2H, s), 3.486 (2H, brs), 2.845 (1H, brs). ^{13}C-NMR ($CDCl_3$): δ = 144.82, 127.20, 124.72 (q, J = 277 Hz), 122.11, 63.07, 46.22 (m). ESI-TOF ([M+H$^+$]): Calcd. for $C_{12}H_{10}F_6N^+$, 282.07119. Found: 282.07095. Anal. Calcd. for $C_{12}H_9F_6N$: C, 51.26; H, 3.23; N, 4.98. Found: C, 51.07; H, 3.52; N, 5.26. The amine, **NH-ABBH**, obtained in this method was used for biological study.

NCHO-ABBH: To a pre-cooled solution of the amine **NH-ABBH** (280.3 mg) in 2 ml of formic acid, acetic anhydride (2 ml) was added at 0°C (in an ice-water bath). The whole was stirred at 0°C for 30 min, and the cooling bath was removed to allow the reaction mixture warm to rt. The whole was stirred at rt for 20 h. The whole was added dropwise to an ice-cooled aqueous saturated $NaHCO_3$ solution to neutralize the mixture. The whole was extracted with CH_2Cl_2 (80 ml × 2), and the organic layer was washed with brine (30 ml × 2), dried over magnesium sulfate. Evaporation of the solvent gave the residue (281.8 mg) as a white powder. The crude product was flash-column chromatographed (ethyl acetate:*n*-hexane = 2:1) to give the formamide (**NCHO-ABBH**), 277.1 mg (90% yield) as a white powder. Mp: 128–131°C (recrystallized from CH_2Cl_2/*n*-hexane). Colorless rods. ESI-TOF ([M+Na]$^+$): Calcd. for $C_{13}H_9F_6NO$+Na, 332.04805. Found: 322.04679. Anal. Calcd. for $C_{13}H_9F_6NO$: C, 50.50; H, 2.93; N, 4.53. Found: C, 50.38; H, 3.23; N, 4.76. ^{1}H-NMR ($CDCl_3$): δ = 8.035 (1H, s), 7.417 (2H, brs), 7.298 (2H, m), 5.732 (1H, brs), 5.372 (1H, brs), 3.499 (2H, brs). ^{13}C-NMR ($CDCl_3$): δ = 157.05, 140.48 (quartet), 140.12 (quartet), 127.94 (C × 2), 123.73 (quartet, J = 277.0 Hz, CF_3 × 2), 122.68, 121.85, 60.68, 57.72 (broad), 47.79 (quartet, J = 31 Hz), 45.65 (quartet, J = 27 Hz).

NMe-ABBH: To a solution of *N*-formamide **NCHO-ABBH** (150.6 mg) in dry THF (1 ml), BH_3-THF solution in THF (0.98 mmol/ml) (5.9 ml, 10 equiv.) was added over 2

min at 0°C (an ice-water bath). After stirring at 0°C for 10 min, the whole was heated at reflux for 1 h. After cooling to rt, 2 ml of water was added slowly. The whole was heated at reflux for 2 h. The whole was diluted with water (40 ml), and the solvent was evaporated. The residue was extracted with methylene chloride (80 ml), washed with brine (60 ml), and the organic phase was dried over sodium sulfate. The solvent was evaporated to give the residue (132.5 mg), which was flash-column chromatographed (ethyl acetate:*n*-hexane = 1:2) to give the *N*-methyl derivative **NMe-ABBH** (116.6 mg, 81% yield) as an off-white solid. Colorless rods. Mp: 79–81°C. ESI-TOF ($[M+H]^+$): Calcd. for $C_{13}H_{11}F_6N$+H, 296.08685. Found: 296.08659. Anal. Calcd. for $C_{13}H_{11}F_6N$: C, 52.89; H, 3.76; N, 4.74. Found: C, 52.73; H, 3.93; N, 4.95. ^{1}H-NMR ($CDCl_3$): δ = 7.378 (2H, m), 7.257 (2H, m), 4.304 (2H, s), 3.507 (2H, m), 1.996 (3H, s). ^{13}C-NMR ($CDCl_3$): δ = 140.75 (s), 127.40 (s), 122.88 (broad m), 121.53 (quartet, *J* = 271 Hz), 74.36 (s), 35.93 (s). The *N*-methyl amine, **NMe-ABBH**, obtained in this method and in the following method was used for biological study. There was no difference in biological activity.

Another Synthesis of NMe-ABBH



Compound 14: To a suspension of $LiAlH_4$ (1.1403 g) in dry THF (24 ml) under argon atmosphere, a solution of MeOH (2 ml) in THF (24 ml) was added over 16 min at 0°C, followed by cooling of the whole to –74°C (in an acetone-dry ice bath). To this suspension, *N*-methylphthalimide **13** (1.6177 g, 10.0 mmol) was added in portions at –74°C, and the mixture was stirred at this temperature for 40 min. Then the mixture was

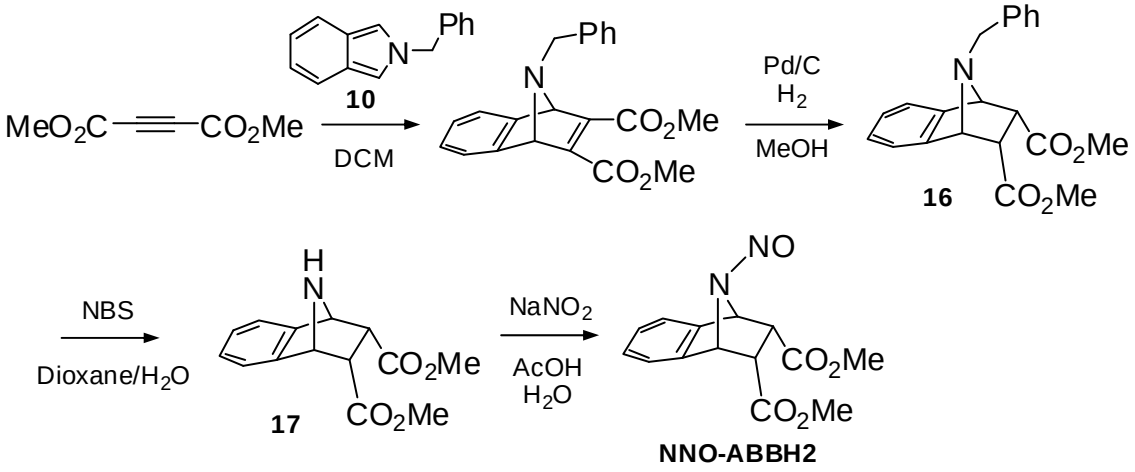
stirred at 0°C for 45 min. A saturated aqueous solution of sodium sulfate (8 ml) was added, and the resultant inorganic salt was filtered by suction, and the precipitate was washed with acetone. The combined organic layer was dried over magnesium sulfate, and the organic solvent was evaporated at 30°C to give the residue **14** (yellow solid, 1.1544 g, 88% yield). This intermediate is rather unstable, and it was used with further purification. ^{1}H-NMR ($CDCl_3$): δ = 7.500 (2H, m), 7.040 (2H, s), 6.904 (2H, m), 3.987 (3H, s).

Compound 15: To a solution of hexafluoro-2-butyne (3.5867 g) in dry CH_2Cl_2 (4 ml), stirred at –65°C, isoindoline, prepared from *N*-methylphthalimide **14** (806.1 mg), in dry CH_2Cl_2 (4 ml) was added. The mixture was stirred for 35 min at –65°C and at –40°C for 35 min, and the organic solvent was evaporated to give the red-colored oil, which was flash-column chromatographed (silica gel, *n*-hexane:ethyl acetate = 4:1) to afford **15** (450.7 mg, 31% yield) as yellow oil. The product was further flash-column chromatographed (CH_2Cl_2) to give colorless cubes. Mp: 44–45.5°C. ^{1}H-NMR ($CDCl_3$): δ = 7.370 (2H, m), 7.084 (2H, m), 4.811 (2H, s), 2.283 (3H, brs). ^{13}C-NMR ($CDCl_3$, 40°C): δ = 145.27 (broad m), 126.33 (s), 122.88 (broad m), 124.66 (quartet, *J* = 278 Hz, CF_3), 124.47, 68.78 (s), 46.67 (s), 34.08. ESI-TOF ($[M+H]^+$): Calcd. for $C_{13}H_9F_6N$+H, 294.0712. Found: 294.0735. Anal. Calcd. for $C_{13}H_9F_6N$: C, 53.25; H, 3.09; N, 4.78. Found: C, 53.07; H, 3.21; N, 5.01.

NMe-ABBH: The olefin **15** (66.7 mg) was hydrogenated in ethyl acetate (1 ml) at rt over $Pd(OH)_2$ (27.7 mg) with H_2 (at atmosphere pressure) for 20 min. The whole was filtrated through a celite pad, and the celite pad was washed with EtOAc (59 ml). The organic solvent was evaporated to give a solid off-white solid (66.8 mg, 99.4% yield) of **NMe-ABBH**. Mp: 77.5–79.5°C. Colorless cubes. ^{1}H-NMR ($CDCl_3$): δ = 7.363 (2H, m), 7.242 (2H, m), 4.290 (2H, s), 3.491 (2H, m), 1.980 (3H, s). ^{13}C-NMR ($CDCl_3$): δ = 140.75 (s), 127.40 (s), 122.88 (broad m), 121.53 (quartet, *J* = 271 Hz), 74.36 (s), 35.93 (s). ESI-TOF, ($[M+H]^+$): Calcd. for $C_{13}H_{11}F_6N$+H, 296.08685. Found: 296.08691. Anal. Calcd. for $C_{13}H_{11}F_6N$+0.2 H_2O: C, 52.25; H, 3.85; N, 4.69. Found: C, 52.29; H, 3.70; N, 4.97. The *N*-methyl amine, **NMe-ABBH**, obtained in this method was also used for biological study. There was no difference in biological activity of the relevant amines obtained in this method and in the above-mentioned method.

Synthesis of *N*-nitroso-2-exo,3-exo-dimethoxycarbonyl-7-azabenzobicyclo[2.2.1]heptane (NNO-ABBH2)

Synthesis of **NNO-ABBH2** was carried out as described previously (Ohwada et al., 2001) with a few modifications.



NNO-ABBH2: The crude amine compound **17** was dissolved in a mixture of 8 ml of acetic acid and 18 ml of water. To this solution, an aqueous solution of $NaNO_2$ (417.0 mg, 6.04 mmol) in 10 ml of water was added over 5 min at 0°C and the whole was stirred at 0°C for 3 h. The whole was extracted with $CHCl_3$, and the organic layer was washed with saturated aqueous Na_2CO_3 and brine, and dried over sodium sulfate. The solvent was evaporated to give brown oil, which was open column-chromatographed (*n*-hexane:ethyl acetate = 4:1) to give **NNO-ABBH2** as a brown solid (674.0 mg, 61% yield from **16**). The brown solid was recrystallized from CH_2Cl_2/*n*-hexane to give a yellow solid (123.0 mg). Mp: 103.0–104.0°C. ESI-TOF ($[M+Na]^+$): Calcd. for $C_{14}H_{14}N_2O_5Na$, 313.0800. Found: 313.0800. Anal. Calcd. for $C_{14}H_{14}N_2O_5$: C, 57.93; H, 4.86; N, 9.65. Found: C, 57.86; H, 4.85; N, 9.57. ^{1}H-NMR ($CDCl_3$): δ = 7.492–7.477 (1H, m), 7.382–7.368 (1H, m), 7.300–7.260 (2H, m), 6.194 (1H, brs), 5.992 (1H, brs), 3.672–3.648 (1H, m), 3.549 (3H, s), 3.519 (3H, s), 3.549–3.457 (1H, m).

Synthesis of *N*-nitroso-2-exo-methoxycarbonyl-7-azabenzobicyclo[2.2.1]heptane (NNO-ABBH3)

Synthesis of **NNO-ABBH3** was carried out as described previously (Yanagimoto et al., 2007).

Synthesis of *N*-nitroso-7-azabenzobicyclo[2.2.1]heptane (NNO-ABBH4)

Synthesis of **NNO-ABBH4** was carried out as described previously (Yanagimoto et al., 2007).

Synthesis of *N*-nitroso-2-exo-methoxycarbonyl-7-azabicyclo[2.2.1]heptane (NNO-ABBH5)

Synthesis of **NNO-ABBH5** was carried out as described previously (Karaki et al., 2012).

Supplemental References

Karaki F, Kabasawa Y, Yanagimoto T, Umeda N, Firman, Urano Y, Nagano T, Otani Y, and Ohwada T (2012) Visible-light-triggered release of nitric oxide from N-pyramidal nitrosamines. *Chem Eur J* **18**:1127–1141.

Ohwada T, Miura M, Tanaka H, Sakamoto S, Yamaguchi K, Ikeda H, and Inagaki S (2001) Structural features of aliphatic *N*-nitrosamines of 7-azabicyclo[2.2.1]heptanes that facilitate N–NO bond cleavage. *J Am Chem Soc* **123**:10164–10172.

Yanagimoto T, Toyota T, Matsuki N, Makino Y, Uchiyama S, and Ohwada T (2007) Transnitrosation of thiols from aliphatic *N*-nitrosamines: *S*-nitrosation and indirect generation of nitric oxide. *J Am Chem Soc* **129**:736–737.