Supplementary Information

for

In situ analysis of [8-^{13}C-7-^{15}N]-double-labelled theophylline by triple resonance NMR technique

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1. Synthesis

General. The reagents and solvents were purchased from standard suppliers and used without further purification. The NMR spectra were measured using a Bruker Avance III spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) and a JEOL JNM-ECA 600 (60 MHz for ^{15}N). DMSO (2.62 ppm), DMSO (39.6 ppm), and NH_4NO_3 (30 ppm) were used as standards for ^{1}H, ^{13}C, and ^{15}N measurements, respectively. Mass spectra (MS) were measured using a JEOL JMS-HX110A (FAB).

Synthesis of 2. $Na^{15}NO_2$ (1.05 g, 15.0 mmol, 1.26 eq) in water (4 mL) was added dropwise to a solution of 6-amino-1,3-dimethyluracil (1.85 g, 11.9 mmol) in acetic acid (5 mL) and water (5 mL). After stirring for 2 h at 80 °C, the mixture was stirred for an additional 1 h at room temperature, and kept at 4 °C overnight. The precipitates were filtered, washed with cold water, and dried to give **2** as a purple solid (yield = 99%): ^{1}H NMR (DMSO-d_6, 400 MHz) δ = 3.39 (s, 3H), 3.36 (s, 3H); ^{15}N NMR (DMSO-d_6, 60 MHz) δ = 684.1; HRMS (FAB): *m*/*z* calc. for $C_6H_8O_3N_3{}^{15}N_1$ [M^+] = 185.0561, found = 185.0568.

Synthesis of [8-^{13}C-7-^{15}N]-theophylline. Synthesized **2** (1.11 g, 6.0 mmol) was dissolved in 14% aqueous ammonia solution (20 mL), and the resulting solution was stirred for 30 min at 70 °C then cooled

to 50 °C, and then $Na_2S_2O_4$ (3.13 g, 18.0 mmol) was added slowly. After stirring until the solution turned yellow, the resulting solution was stirred for an additional 0.5 h at room temperature. Half of the solvent volume was evaporated, and the solution was kept at 4 °C for 1 h. Precipitates were collected by filtration and dried. The precipitates were redissolved in acetic acid (5 mL) containing $H^{13}C(OCH_2CH_3)_3$ (500 mg, 3.35 mmol, 1.15 eq relative to **2**), and the solution was stirred at 100 °C overnight. After cooling to room temperature, the solvent was removed *in vacuo*, and the resulting residue was purified using silica gel column chromatography (eluent: CH_3OH:$CHCl_3$ = 5:95) to give **3** as a white solid (yield = 29%): ^{1}H NMR (DMSO-d_6, 400 MHz) δ = 8.14 (dd, J = 211.6 and 8.4 Hz, 1H), 3.55 (s, 3H), 3.35 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ = 140.5 (d, J = 10 Hz); ^{15}N NMR (DMSO-d_6, 60 MHz) δ = 169.0; HRMS (FAB): m/z calc. for $C_6{}^{13}C_1H_8N_3{}^{15}N_1O_2$ [M^+] = 182.0646, found = 182.0650.

2. NMR analyses

General. NMR spectra in Fig. 2 and Fig. 3 were acquired at 298 K on a Bruker Avance III (400 MHz) without CryoProbe and Avance 700 (700 MHz) spectrometer equipped with a 5 mm TCI CryoProbe, respectively. One-dimensional triple resonance spectra were obtained by using a 1D HCN pulse sequence (Figure S1). Parameters for detection of theophylline were as follows: transmitter offsets of C and N = 142 and 154 ppm, respectively; the delay intervals $1/4^1J_{CH}$ and $1/4^1J_{CN}$ = 1.18 and 25.5 ms, respectively. Data processing and analysis were performed using the Topspin 2.1 (Bruker Biospin, Karlsruhe, Germany).

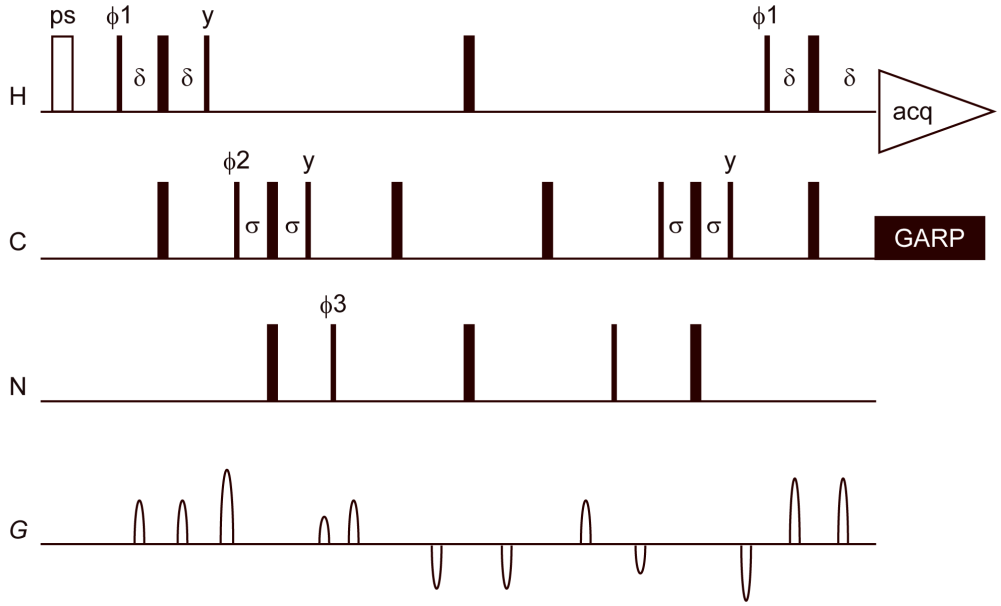

Figure S1. The pulse scheme of the one-dimensional ^{1}H-^{13}C-^{15}N triple-resonance NMR experiment used in this study. The narrow and broad filled bars represent the 90° and 180° pulses, respectively. All pulses have phase = x unless otherwise

indicated. The delay intervals are set to; δ = 1.18 ms, σ = 25.5 ms for detection of H8 of the ^{13}C,^{15}N-labelled theophylline. PS denotes a pre-saturation pulse (1.5 ms) used for water suppression. The phase cycle is ϕ1 = *x*, -*x*; ϕ2 =2(*x*), 2(-*x*); ϕ3 = 4(*y*), 4(-*y*) and receiver = 2(*y*), 4(-*y*), 2(*y*). During the detection period, ^{13}C GARP decoupling is used. All gradients were applied along the *z* axis.

Preparation of mouse liver lysate. The liver tissues of female C57BL/6J mice (Shimizu Laboratory Supplies Co. Ltd., Kyoto, Japan) weighing approx. 15 g were harvested and homogenized (1:2, w/v) in 20 mM Tris–HCl (pH 8.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol, using a Qiagen TissueLyser. The homogenate was then centrifuged at 25,000 g for 1 h at 4 °C. The supernatant fluid was collected and used as the liver lysate.

NMR measurements in a mixture containing amino acids. 0.2 mM [8-^{13}C-7-^{15}N]-theophylline was dissolved in D_2O (500 μL) containing 42.875 mM amino acid mix (amino acid standard, Sigma-Aldrich, USA) and was subjected to NMR analysis (256 scans).

NMR measurements in a mixture containing mouse liver lysate. 0.2 mM [8-^{13}C-7-^{15}N]-theophylline was dissolved in 500 μL of Tris–HCl buffer (10 mM, pH 8.0) containing 0.5 mM EDTA, 0.5 mM 2-mercaptoethanol, 2 mM dithiothreitol, and 10%(v/v) crude liver lysate. The mixture was lyophilized to dryness, dissolved in D_2O (500 μL) and subjected to NMR analysis (256 scans).