PHOTOLYSIS OF ^{13}C-METHYLCOBALAMIN. DIRECT DETECTION OF GENERATED FORMALDEHYDE BY USING ^{13}C-LABELING[†]

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Abstract- Photolysis of methylcobalamin was followed by UV, CD and ^{1}H-NMR spectroscopic methods. The direct detection of formaldehyde as a photolysis product was achieved by ^{13}C-NMR using ^{13}C-methylcobalamin, prepared from vitamin B_{12} and ^{13}C-methyl iodide, as a substrate.

INTRODUCTION

We have reported the biosynthesis of vitamin B_{12} (cyanocobalamin), which is a precursor of coenzymes such as methylcobalamin, hydroxocobalamin and 5'-deoxyadenosylcobalamin (Figure 1).[1] Methylcobalamin is required in the metabolism of methionine, methane and acetic acid. The biosynthesis of methionine is catalyzed by methionine synthase with methylcobalamin as a coenzyme. It is known that the methyl group of methionine is transferred from N^5-methyltetrahydrofolic acid *via* methylcobalamin.[2] Although methylcobalamin is used in medical care, it is decomposed by light, generating hydroxocobalamin.[3] Alkylcobalamins are known to be unstable on irradiation with light, undergoing homolytic cleavage of the carbon-cobalt bond to afford the corresponding alkyl radical and vitamin B_{12r}.[4] 5'-Deoxyadenosylcobalamin is decomposed by light to vitamin B_{12r} and 5'-adenosyl radical, which are oxidized to hydroxocobalamin and adenosine-5'-aldehyde in the presence of oxygen.[5] Photolysis of methylcobalamin proceeds smoothly to afford hydroxocobalamin and formaldehyde in the presence of oxygen.[6] The resulting formaldehyde has been determined by several groups, but they employed indirect methods, i. e., the dimedone method[6a,b] or the chromotropic acid

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[†]Dedicated to Professor Koji Nakanishi on the occasion of his 75th birthday

method.[6c]   So, we investigated the photolysis of methylcobalamin by UV, CD, [1]H-
and ^{13}C-NMR, and acheaved direct detection of the labeled formaldehyde
generated from ^{13}C-labeled methylcobalamin.

L = CN: vitamin B$_{12}$
L = Me: methylcobalamin
L = ^{13}C-Me: ^{13}C-methylcobalamin
L = OH: hydroxocobalamin

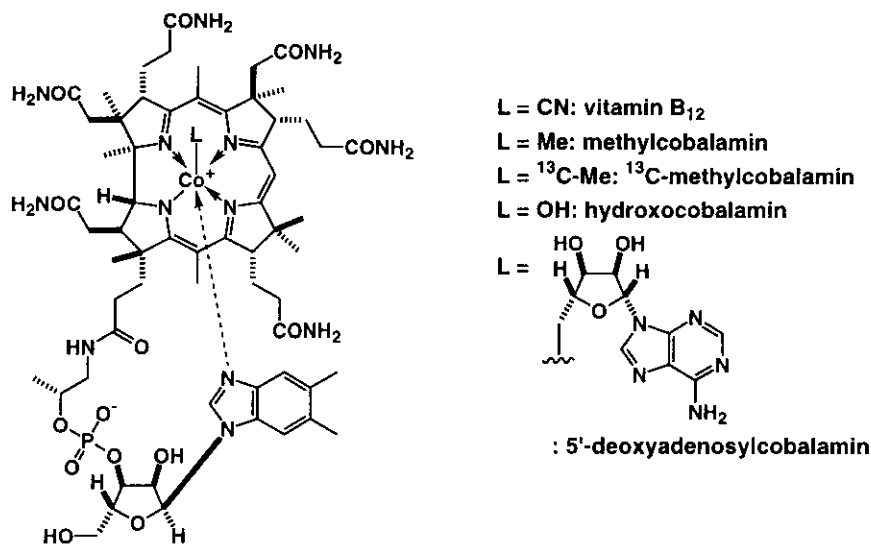: 5'-deoxyadenosylcobalamin

Figure 1

RESULTS AND DISCUSSION

Photolysis of methylcobalamin was performed by irradiation with a 100 W
incandescent lamp at a distance of 45 cm.   The UV spectra were recorded at one-
minute intervals for a 0.05 mg/mL solution (Figure 2).   Methylcobalamin λ_{max} at
267, 343 and 522 nm decreased with time, and λ_{max} at 273, 352 and 529 nm,
assigned to hydroxocobalamin on the basis of comparison with the spectrum of
an authentic sample, increased.   Methylcobalamin was completely converted to
hydroxocobalamin by irradiation with light for 15 min.   Similarly, CD spectra
was measured at 2-min intervals at the concentration of 0.1 mg/mL (Figure 3).
Although the sample-to-light source separation was only 30 cm, complete
conversion required 26 min.   The positive Cotton effects at 485 and 384 nm and
negative Cotton effects at 556 and 320 nm of methylcobalamin were decreased.
The negative Cotton effect at 426 nm and the positive Cotton effects at 351 and
273 nm were increased.   The final spectrum coincided with that of authentic
hydroxocobalamin.

NMR spectroscopy is more powerful than UV and CD spectroscopy for the
identification of chemical structures, and for investigating the course of chemical
and enzymatic transformations.   The ^{1}H-NMR results obtained in this study are
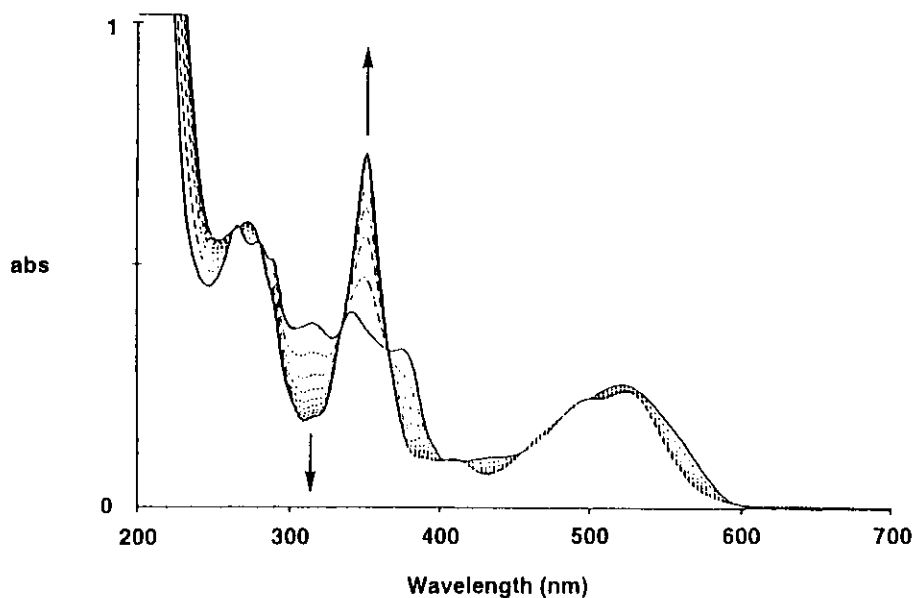shown in Figure 4.   A 4.0 mg/mL solution of methylcobalamin in D$_2$O was

Figure 2   Time-course UV spectra of photolysis of methylcobalamin. Arrows indicate variations of absorbance with time.
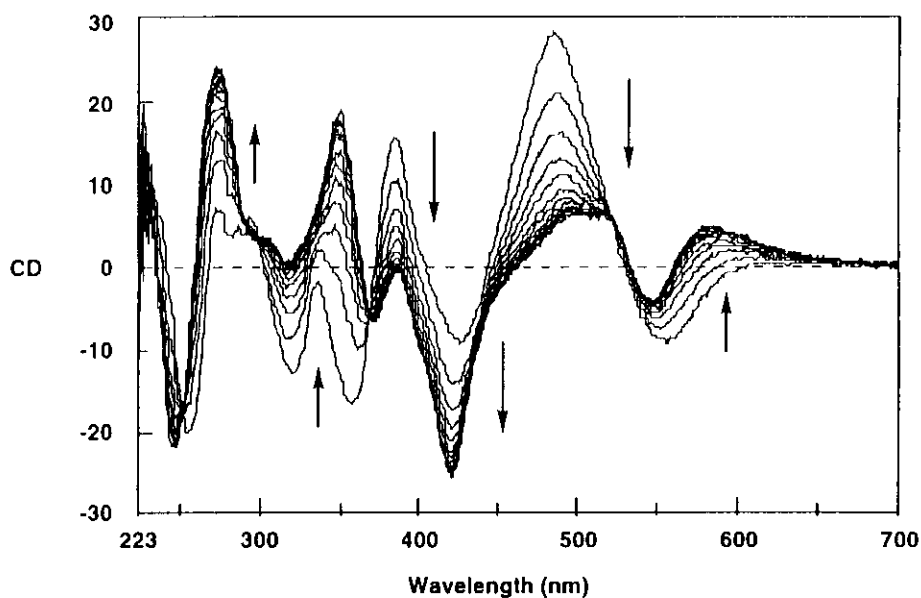

Figure 3 Time-course CD spectra. Arrows indicate variations of CD with time.
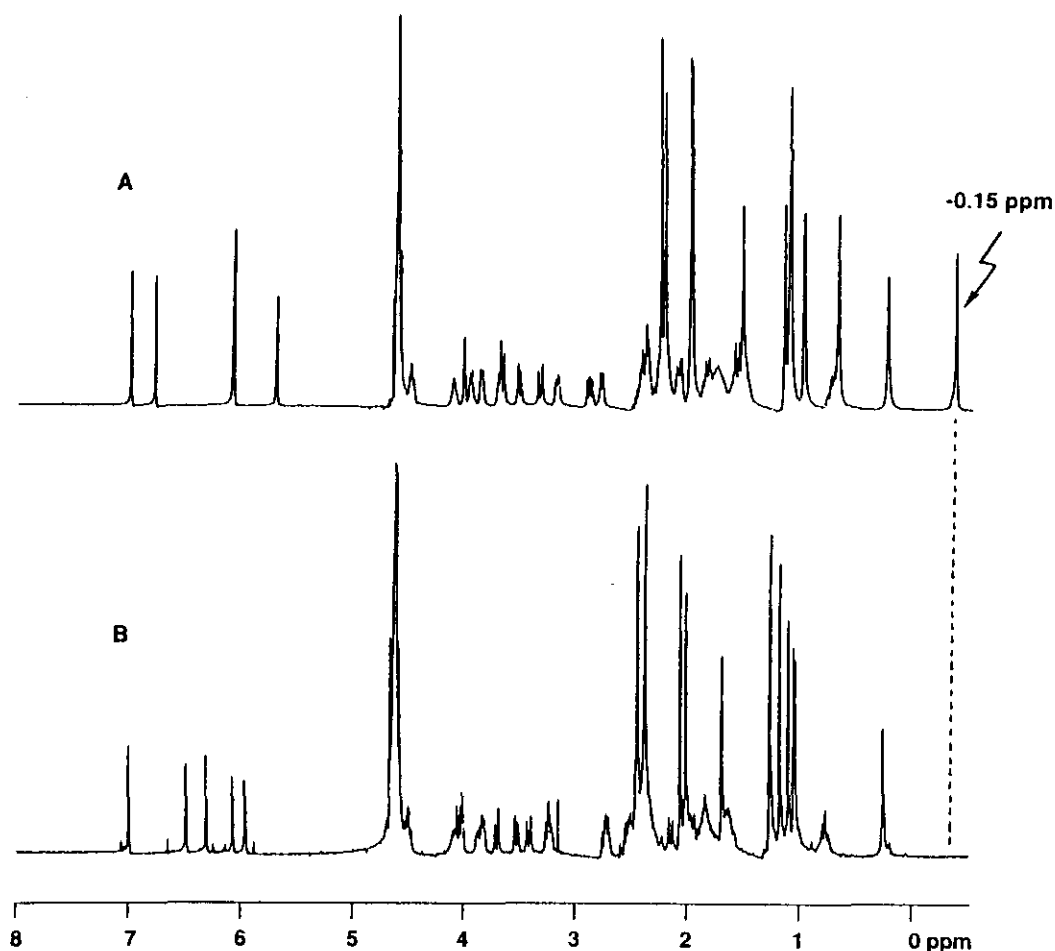
Figure 4 ^{1}H-NMR spectra during photolysis of methylcobalamin. (A) Before light irradiation. (B) After irradiation for 20 h.

photolysized for 20 h.   In the initial spectrum of methylcobalamin, the signal corresponding to the methyl group on cobalt was observed at -0.15 ppm.   After 20 h, this signal had disappeared and other signals assigned to hydroxocobalamin were observed.

To determine the fate of the cleaved methyl group, ^{13}C-labeled methylcobalamin was photolysized and the ^{13}C-NMR spectrum was measured.   Direct detection of the formaldehyde was achieved.   Preparation of ^{13}C-methylcobalamin was carried out in the dark as follows.   Vitamin B_{12} was reduced with sodium borohydride to vitamin B_{12s}, and the resulting cyanide ion was removed from the reaction system as a precipitate with Fe^{2+}.   Finally, vitamin B_{12s} was alkylated with ^{13}C-methyl iodide to give ^{13}C-methylcobalamin.   Photolysis of ^{13}C-methylcobalamin was performed under the same conditions as used for the ^{1}H-NMR study.   The signal of the ^{13}C-methyl carbon on cobalt was observed at

9.0 ppm in the ^{13}C-NMR. After irradiation with light for 20 h, the signal was no longer detected and another signal at 84.6 ppm was observed. When air in the NMR tube was replaced as thoroughly as possible with nitrogen, the disappearance time of the ^{13}C-methyl carbon signal was extended to 10 days. The signal at 84.6 ppm of the resulting product was determined to be due by hydrated ^{13}C-formaldehyde by comparison with the spectrum of diluted formalin[7] (Figure 5). Weissbach *et al.* isolated radioactive methanol and formaldehyde after photolysis of ^{14}C-methylcobalamin.[6d] In our study, no signal corresponding to ^{13}C-methanol was detected in the presence or absence of oxygen. The photo-degradation of methylcobalamin was slow in the absence of oxygen, because the resulting methyl radical and vitamin B_{12r} regenerate the carbon-cobalt bond in the absence of a radical trapper such as oxygen, homocysteine or benzoquinone.[8] Therefore it was considered that the ^{13}C-formaldehyde was generated by the oxidation of the formed ^{13}C-methyl radical with traces of oxygen present as contaminant.
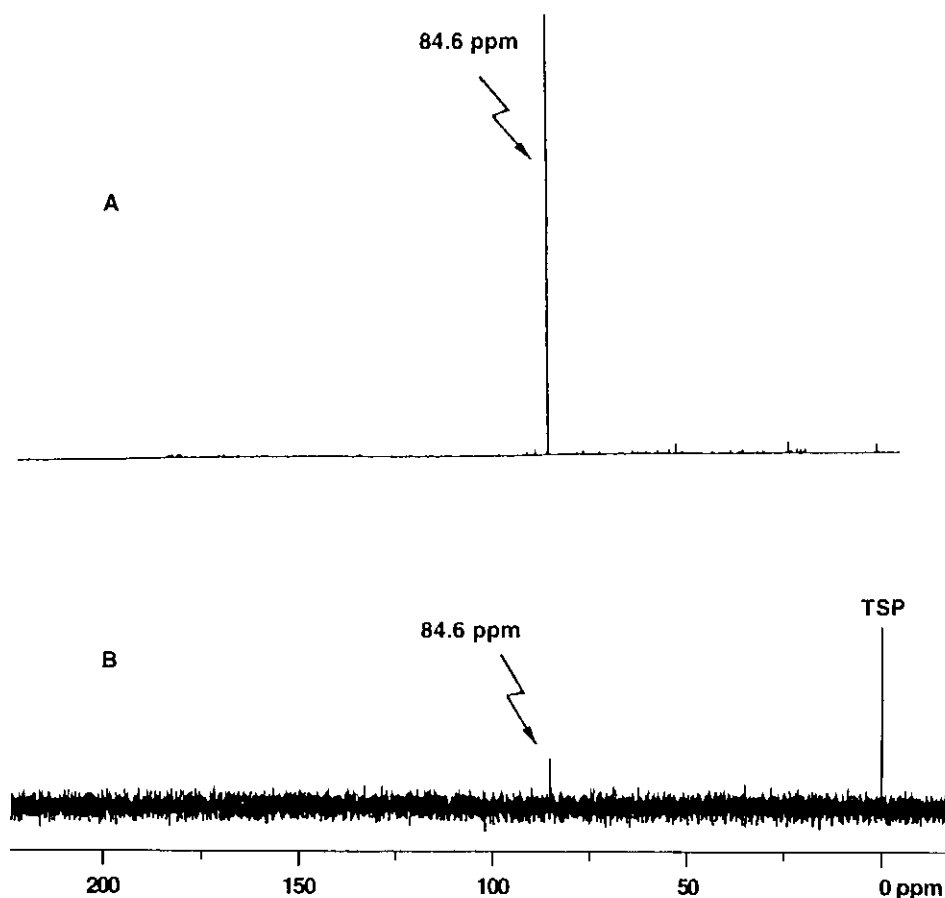

Figure 5 ^{13}C-NMR spectra during methylcobalamin photolysis. (A) After irradiation for 10 days. (B) The spectrum of authentic formalin.

EXPERIMENTAL

Materials   Methyl iodide (99% atom ^{13}C) was purchased from CIL Inc.   Other reagents were purchased from Aldrich, Wako Pure Chemicals (Tokyo), Tokyo Chemical Industry Co., Ltd. (Tokyo) and Kanto Chemicals (Tokyo).

Instruments   UV and CD spectra were recorded on a JASCO UVDEC-610C spectrophotometer and a JASCO J-720WI spectrometer, respectively. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GSX-400 Fourier-transform spectrometer (400 MHz and 100 MHz).   Chemical shifts are given downfield from the signal of HDO at 4.7 ppm in the case of ^{1}H-NMR and from sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 (TSP) at 0 ppm as an internal standard in the case of ^{13}C-NMR.   Fast atom bombardment MS spectra (FABMS) were recorded on a JEOL DX-302 spectrometer equipped with a JMA DA5000 data system.   The matrix was m-nitrobenzyl alcohol (mNBA).

Preparation of ^{13}C-Methylcobalamin   Sodium borohydride (60 mg, 1.5 mmol) in dry ethanol (20 mL) was added dropwise to a stirred solution of cyanocobalamin (200 mg, 0.15 mmol) in water (20 mL) at 0 °C under an argon atmosphere in the dark, and the reaction mixture was stirred for 20 min at rt .   A solution of iron sulfate heptahydrate (50 mg, 0.18 mmol) in water (2.0 mL) was added. The mixture was stirred for 5 min, and then ^{13}C-methyl iodide (99.0% atom ^{13}C, 0.25 mL, 3.99 mmol) was added dropwise to it.   Stirring was continued for 2 h, then the reaction mixture was filtered through Celite under an argon atmosphere in the dark, and evaporated.   The residue was extracted with a 1 : 1 mixture (20 mL) of chloroform and phenol.   The organic layer was diluted with ether (200 mL) and extracted with water (20 mL).   The aqueous layer was washed with chloroform and ether, and evaporated.   The crude product was crystallized from a 1 : 8 mixture of water and acetone to give methylcobalamin as red needles (161 mg) in 80% yield. ^{13}C-Methylcobalamin ^{1}H-NMR (400 MHz, D$_2$O) δ: -0.28 (3H, d, $J_{CH} = 138.6$ Hz, ^{13}CH$_3$).   FABMS (mNBA) m/z: 1345 (M$^+$).

Photolysis of Methylcobalamin

UV Spectra   Methylcobalamin (0.2 mg) was dissolved in distilled water (4 mL) in a UV cell.   The solution was irradiated with a 100 W incandescent lamp at a distance of 45 cm and the spectra were recorded at one-minute intervals.   The scanning width was from 200 nm to 900 nm.   Methylcobalamin λ_{max} (H$_2$O): 267, 343, 522 nm.   Hydroxocobalamin λ_{max}: 273, 352, 529 nm.

CD Spectra   Aqueous solution of methylcobalamin (0.1 mg/mL) was placed in a CD cell (cell length 1 cm) and irradiated with a 100 W incandescent lamp at a distance of 30 cm.   CD spectra of the solution were measured at 2-min intervals. The scanning width was from 223 nm to 700 nm.   Methylcobalamin CD λ_{ext} nm ($\Delta\varepsilon$, H$_2$O) 557 (-3.79), 485 (11.55), 425 (-3.72), 384 (6.29), 357 (-6.76), 336

(-0.74), 321 (-5.27), 294 (2.51), 273 (2.93), 254 (-8.16). Hydroxocobalamin 584 (1.94), 548 (-1.83), 511 (2.65), 421 (-10.29), 387 (-0.20), 372 (-2.65), 351 (7,74), 316 (0.14), 273 (10.01), 248 (-8.64).

^{1}H-NMR A 0.5 mL aliquot of methylcobalamin solution in D_2O (4.0 mg/mL) was placed in a 5 mmφ NMR tube, and irradiated with a 100 W incandescent lamp at a distance of 45 cm for 20 h, when it was completely converted into hydroxocobalamin. The spectrum was recorded under the following conditions: 45° pulse angle, 4205.2 Hz spectrum width, 32K data points, an acquisition time of 3.896 s, and a pulse delay of 2.500 s. An exponential line narrowing of 0.10 Hz was used prior to Fourier transformation. Methylcobalamin ^{1}H-NMR (400 MHz, D_2O) δ: -0.15 (3H, s), 0.33 (3H, s), 0.73-0.90 (2H, m), 0.77 (3H, s), 1.08 (3H, d, J = 6.2 Hz), 1.20 (3H, s), 1.21 (3H, s), 1.25 (3H, s), 1.55-2.60 (21H, m), 1.62 (3H, s), 2.07 (3H, s) 2.08 (3H, s), 2.30 (3H, s), 2.34 (3H, s), 2.87 (1H, d, J = 10.5 Hz), 2.97 (1H, dd, J = 7.8, 14.4 Hz), 3.26 (1H, dd, J = 4.9, 10.8 Hz), 3.42 (1H, m), 3.60 (1H, dd, J = 3.9, 12.3 Hz), 3.73-3.80 (2H, m), 3.94 (1H, m), 4.04 (1H, m), 4.10 (1H, m), 4.20 (1H, m), 4.57 (1H, m), 5.76 (1H, s), 6.13 (1H, s), 6.14 (1H, d, J = 3.1 Hz), 6.83 (1H, s), 7.05 (1H, s). Hydroxocobalamin ^{1}H-NMR (400 MHz, D_2O) δ: 0.39 (3H, s), 0.74-0.93 (2H, m), 1.12 (3H, d, J = 5.9 Hz), 1.22 (3H, s), 1.25 (3H, s), 1.35 (3H, s), 1.37 (3H, s), 1.64-2.18 (9H, m), 1.79 (3H, s) 2.08 (3H, s), 2.14 (3H, s), 2.26-2.60 (11H, m), 2.50 (3H, s), 2.56 (3H, s), 2.77 (1H, m), 2.87 (1H, m), 3.33 (1H, m), 3.40-3.54 (2H, m), 3.58 (1H, m), 3.76 (1H, m), 3.88 (1H, m), 4.04-4.20 (4H, m), 4.56 (1H, m), 6.11 (1H, s), 6.16 (1H, s), 6.32 (1H, s), 6.38 (1H, s), 7.03 (1H, s).

Photolysis of ^{13}C-Methylcobalamin in the Absence of Oxygen A 0.5 mL aliquot of methylcobalamin solution (4.0 mg/mL) in degassed D_2O was placed in a 5 mmφ NMR tube under a nitrogen atmosphere. The solution was irradiated with a 100 W incandescent lamp at a distance of 45 cm for 10 days, when methylcobalamin was completely converted into hydroxocobalamin. The spectrum showed that the methyl group of ^{13}C-methylcobalamin was converted to ^{13}C-formaldehyde hydrate and hydroxocobalamin. The spectrum was recorded under the following conditions: 45° pulse angle, 24038.5 Hz spectrum width, 32K data points, an acquisition time of 0.682 s, and a pulse delay of 2.500 s. An exponential line narrowing of 1.00 Hz was used prior to Fourier transformation. ^{13}C-Methylcobalamin δ: 9.0 (^{13}CH$_3$). ^{13}C-Formaldehyde hydrate δ: 84.6.

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7.   Formalin (37% formaldehyde, 7-13% methanol, Wako Pure Chemicals) was diluted with D_2O.

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