Proton Nuclear Magnetic Resonance (^{1}H-NMR) Signal Assignment of Vitamin B_{12} Based on Normal Two-Dimensional NMR and Feeding Experiments

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Proton nuclear magnetic resonance (^{1}H-NMR) signal assignment of cyanocobalamin (vitamin B_{12}) was achieved by the normal two-dimensional NMR method. ^{1}H–^{1}H correlation spectroscopy (COSY) of vitamin B_{12} showed correlation networks of dimethyl benzimidazole, ribose, isopropanolamine, and corrin ring proton peaks. ^{1}H–^{1}H nuclear Overhauser effect (NOE) spectroscopy (NOESY) allowed assignment of the benzimidazole protons. For assignment of methyl and methylene protons of the corrin ring, ^{1}H–^{13}C COSY measurements were made on ^{13}C-enriched vitamin B_{12}, isolated after feeding experiments with [$^{13}CH_3$]methionine, [2-^{13}C]5-aminolevulinic acid (ALA), and [3-^{13}C]ALA.

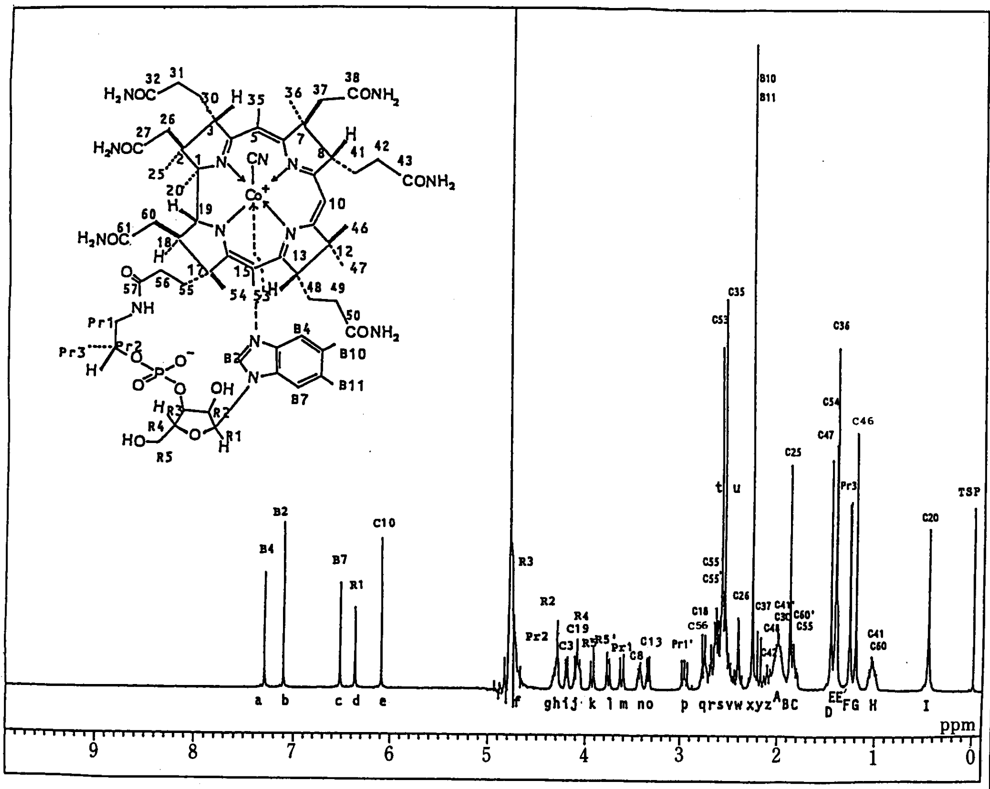
Keywords vitamin B_{12}; ^{1}H–^{1}H COSY; ^{1}H–^{1}H NOESY; ^{1}H–^{13}C COSY; incorporation; biosynthesis; [$^{13}CH_3$]methionine; [2-^{13}C]ALA; [3-^{13}C]ALA

Introduction

We have been interested in the biosynthesis of vitamin B_{12}. For our studies[1] on the origins of the hetero atoms (nitrogen, oxygen and protons) of vitamin B_{12}, we required a complete ^{1}H signal assignment of cyanocobalamin (vitamin B_{12}). A complete ^{13}C signal assignment was presented by Hogenkamp *et al.* in 1982.[2] In the case of the proton nuclear magnetic resonance (^{1}H-NMR) spectrum,[3] many peaks remain unresolved, owing to the complexity of overlapping peaks in the upfield region. In 1986 Bax *et al.* proposed signal assignments of all protons of (5′-deoxyadenosyl)cobalamin (coenzyme B_{12}).[4] They used new types of two-dimensional (2D) NMR techniques, such as 2D homonuclear Hartmann–Hahn (HOHAHA), 2D spin-locked nuclear Overhauser effect (NOE), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) methods. These techniques are attractive, but are restricted in use at present. We used normal 2D methods, namely ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{1}H NOESY, and ^{1}H–^{13}C COSY. These methods are well-known and are sufficiently effective for the ^{1}H-assignment of vitamin B_{12}, if specific carbons are labeled with ^{13}C. We previously obtained ^{13}C-enriched vitamin B_{12} during the course of investigations on the biosynthesis of corrinoids.[5,6] By making use of them, we were able to make signal assignments for all protons of vitamin B_{12}.

Results and Discussion

Figure 1 shows the 400 MHz ^{1}H-NMR spectrum (ho-



peak	δ (ppm)		assignment
a	7.28	1H	B4
b	7.09	1H	B2
c	6.51	1H	B7
d	6.36	1H	R1
e	6.09	1H	C10
f	4.74	1H	R3
g	4.32	1H	Pr2
h	4.28	1H	R2
i	4.19	1H	C3
j	4.08	2H	C19, R4
k	3.93	1H	R5
l	3.76	1H	R5'
m	3.61	1H	Pr1
n	3.43	1H	C8
o	3.34	1H	C13
p	2.96	1H	Pr1'
q	2.79	2H	C18, C56
r	2.69	1H	C55'
s	2.65	3H	C49
t	2.59	3H	C53
u	2.55	3H	C35
v	2.53	2H	C31
w	2.44	2H	C26
x	2.26	6H	B10, B11
y	2.20	1H	C37
z	2.14	1H	C42
A	2.05	5H	C48, C30, C41
B	1.87	3H	C25
C	1.84	2H	C55, C60'
D	1.45	3H	C47
E	1.41	3H	C54
E'	1.39	3H	C36
F	1.26	3H	Pr3
G	1.20	3H	C46
H	1.03	2H	C41, C60
I	0.46	3H	C20

Fig. 1. ^{1}H-NMR Spectrum of Vitamin B_{12} (400 MHz, D_2O, TSP) and Complete ^{1}H-NMR Signal Assignment of Vitamin B_{12}

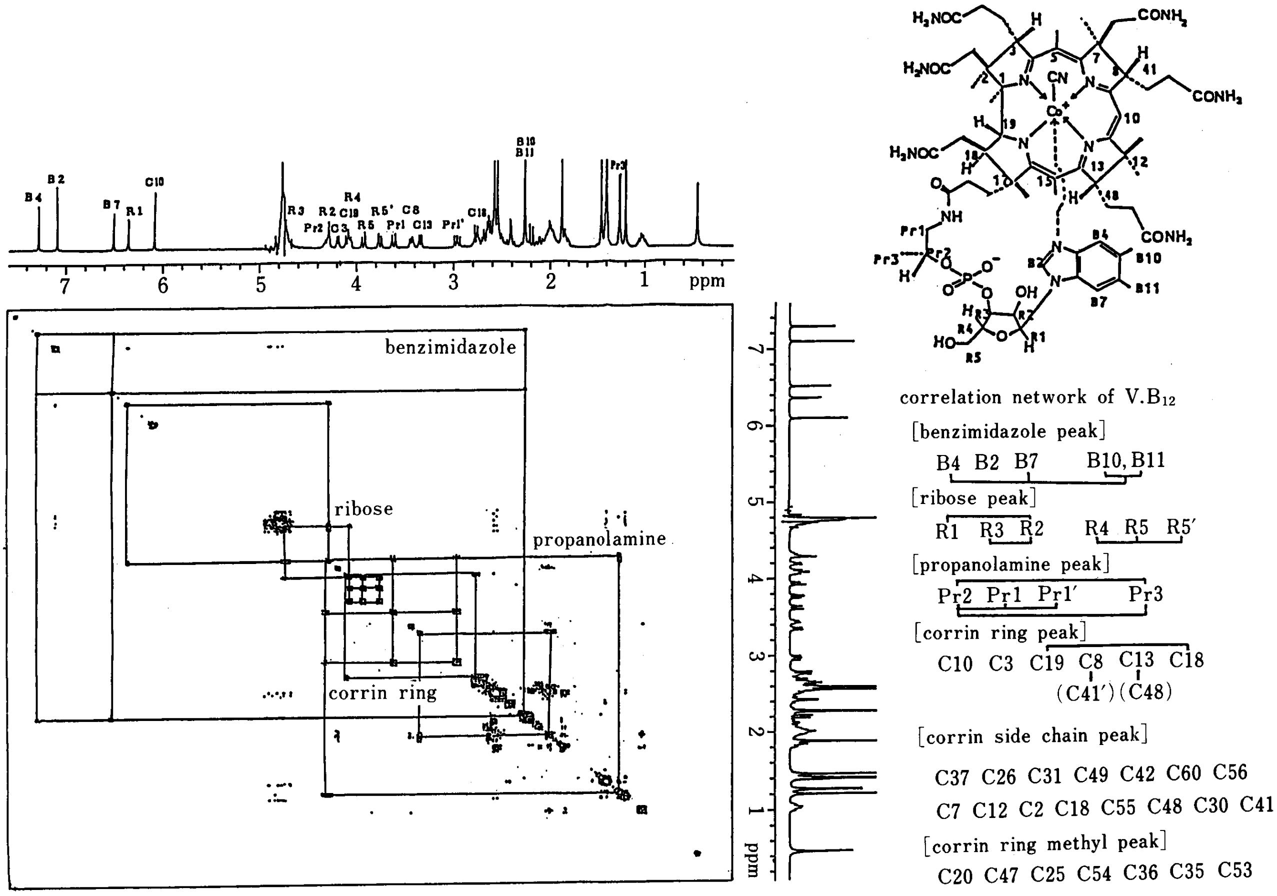


Fig. 2. ^1H–^1H COSY Spectrum of Vitamin B_{12}

mogate mode, D_2O, sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)-propionate (TSP)), and the result of the signal assignment of vitamin B_{12}. The large peak due to water was diminished. Thirty-six peaks (from a to I), belonging to vitamin B_{12} were seen. The numbers of protons were based on the integral intensity of each peak. Assignment was conducted as follows.

First, the ^1H–^1H COSY spectrum was measured (Fig. 2). It shows three clear groups of correlation networks. They should belong to dimethylbenzimidazole, ribose, and propanolamine. The intense singlet at 2.26 ppm (peak x) has already been assigned to the B_{10} and B_{11} methyl protons.[4] Thus, the group to which this signal belongs must be the benzimidazole group. Peaks a and c correlate with peak x. Thus, these signals are due to the B_4 and B_7 protons. These two assignments were confirmed by measurement of the 2D-NOESY spectrum (Fig. 3). Peak d can be assigned to the R_1 proton. Among conjugate region peaks (a—e), a—c belong to benzimidazole, while e shows no correlation to other peaks and can be assigned to the C_{10} proton. In the 2D-NOESY spectrum, peak c shows NOE connectivity with peak d (R_1), while peak a shows no connectivity. Thus, peak a was assigned to the B_4 proton, and peak c to the B_7 proton, considering the space distance to R_1. This peak connectivity between c and d is peculiar to NOESY, as COSY shows no correlation. In the COSY network, peak d (R_1) shows connectivity with peak h (R_2), which correlate with peak f (R_3), which correlates with peak j (R_4), which in turn shows connectivity with peaks k (R_5) and l ($R_{5'}$).

In the same way, the propanolamine protons were assigned. The characteristic doublet of peak F was assigned to the Pr_3 methyl protons. Peak F (Pr_3) correlates strongly with peak g (Pr_2), which correlates with peaks m (Pr_1) and p ($Pr_{1'}$).

The five remaining peaks (i, j, n, o and q) of the midfield region are due to corrin ring protons (C_3, C_8, C_{13}, C_{18} and C_{19}). From ^1H–^{13}C COSY measurement of ^{13}C-labeled vitamin B_{12}, peak q is due to the C_{18} proton, which correlates with peak j (C_{19}), while peak H is due to the C_{41} and C_{60} protons, which correlate with peak n (C_8), and peak t is due to C_{53}, which correlates with peak r (C_{13}). The left peak i is due to the C_3 proton.

Upfield region peaks are those of corrin methyl protons and corrin ring side-chain methylene protons. To assign these peaks, ^1H–^{13}C COSY spectra were measured on various kinds of ^{13}C-enriched vitamin B_{12}.

Figure 4 shows the ^1H–^{13}C COSY spectrum of ^{13}C-enriched vitamin B_{12}, which was isolated after incorporation of [$^{13}CH_3$]methionine. It has been demonstrated that the methyl group of methionine is incorporated at C_{20}, C_{47}, C_{25}, C_{54}, C_{36}, C_{35} and C_{53}.[7] It is established that the C_{47} methyl group is derived from methionine,[7] and C_{46} is derived from 5-aminolevulinic acid (ALA). Based on the correspondence of ^{13}C-NMR signal to ^1H-NMR signals, all methyl protons of methyl groups derived from methionine were assigned in the ^1H-NMR spectrum.

To clarify the assignment of methylene protons attached to the corrin ring, the ^1H–^{13}C COSY of [2-^{13}C]ALA-incorporated vitamin B_{12} was measured (Fig. 5). The correspondence of eight carbon peaks in the ^{13}C-NMR

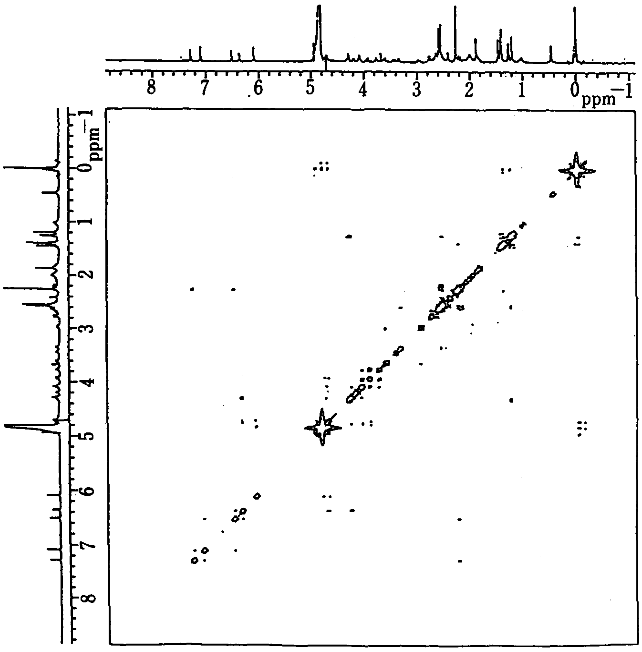


Fig. 3. ^1H–^1H NOESY Spectrum of Vitamin B_{12}

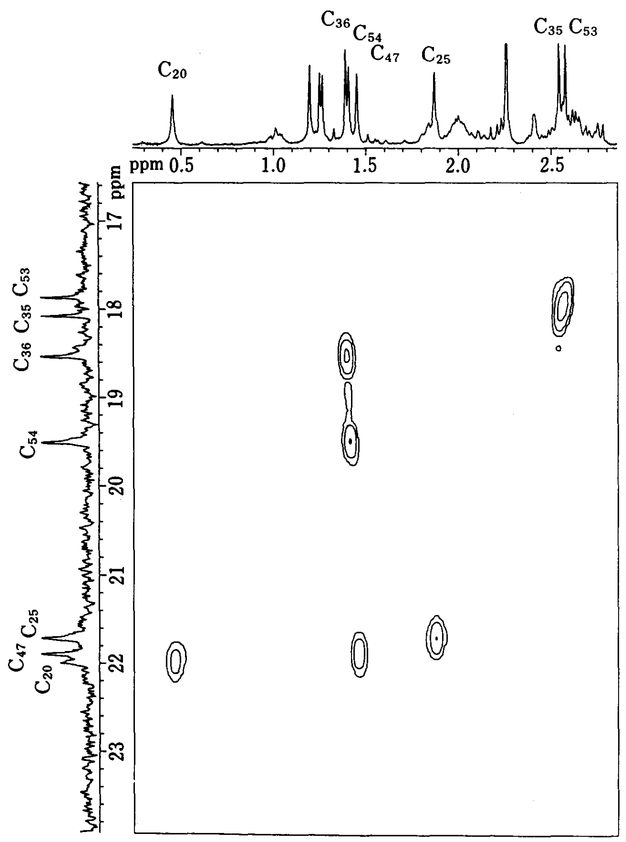


Fig. 4. ^1H–^{13}C COSY Spectrum of [$^{13}CH_3$]Methionine-Incorporated Vitamin B_{12}

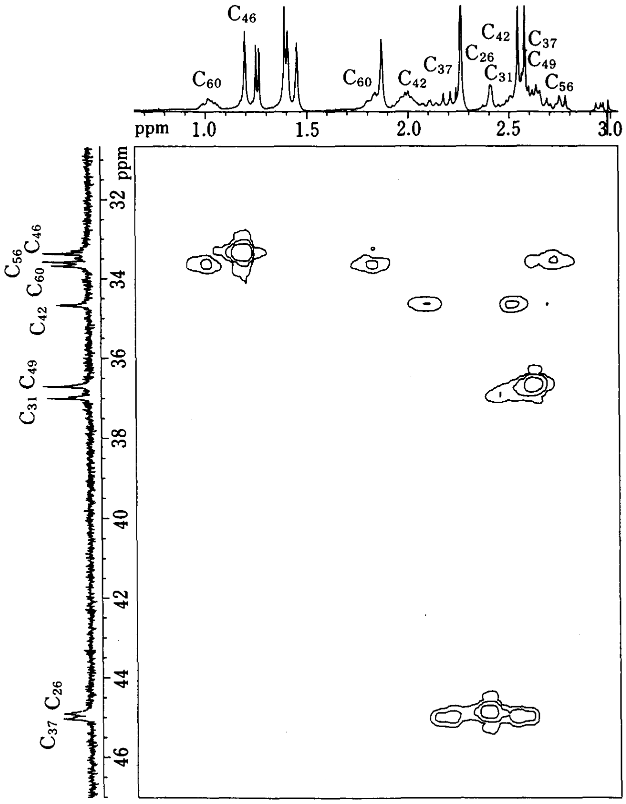


Fig. 5. ^1H–^{13}C COSY Spectrum of [2-^{13}C]ALA-Incorporated Vitamin B_{12}

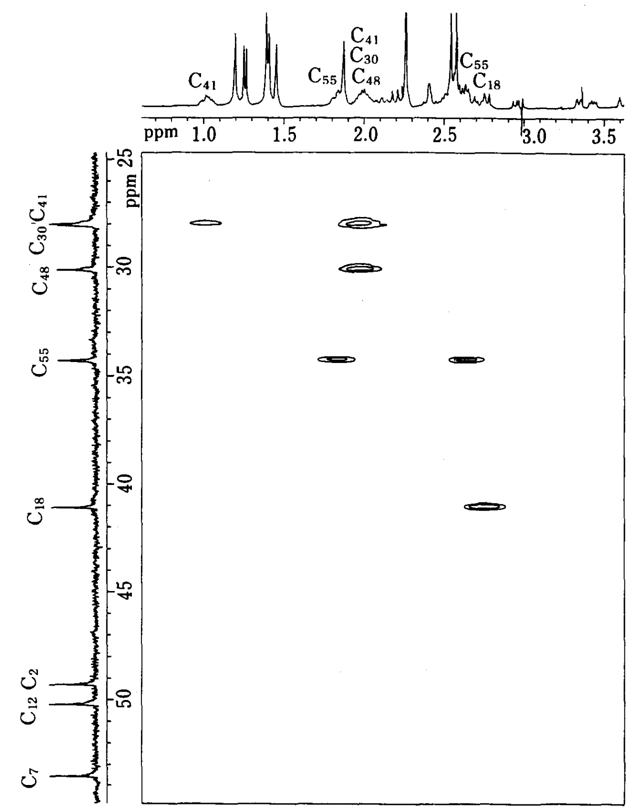


Fig. 6. ^1H–^{13}C COSY Spectrum of [3-^{13}C]ALA-Incorporated Vitamin B_{12}

(C_{37}, C_{26}, C_{31}, C_{49}, C_{42}, C_{60}, C_{56}, and C_{46}) to the ^1H-NMR peaks allowed assignment of the outer methylene protons of the corrin ring in the ^1H-NMR spectrum.

^1H–^{13}C COSY of [3-^{13}C]ALA-derived vitamin B_{12} was also measured. In the ^{13}C-NMR eight carbon peaks appear (C_7, C_{12}, C_2, C_{18}, C_{55}, C_{48}, C_{30} and C_{41}). Of these, five peaks (C_{18}, C_{55}, C_{48}, C_{30} and C_{41}) show correlations with ^1H-NMR peaks. Thus, the inner methylene protons (C_{55}, C_{48}, C_{30} and C_{41}) and one corrin ring proton (C_{18}) were assigned in the ^1H-NMR spectrum. Figure 6 illustrates the above assignments.

The combination of biosynthetic feeding experiments and 2D-NMR measurement has enabled the ^{1}H-NMR signal assignment of vitamin B_{12}. For the NMR signal assignment of compounds which have large molecular weight and show poor solubility like vitamin B_{12}, the above technique seems to be quite effective.

Experimental

Each cyanocobalamin (vitamin B_{12}) was dissolved in 0.4 ml of D_2O (99.75%, Merck) (pH = 7.0). All experiments were performed by using a JEOL GSX-400 spectrometer at 27 °C. The ^{1}H and ^{13}C chemical shifts are referred to TSP (= 0 ppm) as an external standard in a capillary (D_2O solution).

^{1}H–^{1}H COSY Measurement of Vitamin B_{12} Commercial cyanocobalamin (Glaxo) was recrystallized from H_2O–acetone (1 : 7) and 5.0 mg of it was used for the measurement. The spectrum in Fig. 2 was obtained with a $2 \times 256 \times 1024$ data matrix, with eight scans per t_1 value. The delay time between scans was 0.660 s, and the total measuring time was 5.5 h.

^{1}H–^{1}H NOESY Measurement of Vitamin B_{12} The above solution was degassed and used for the measurement. The spectrum in Fig. 3 was obtained with a $2 \times 256 \times 2048$ data matrix, with 32 scans per t_1 value. The delay time between scans was 1.800 s, and the total measuring time was 3.5 h.

^{1}H–^{13}C COSY Measurement of Vitamin B_{12} (1) [^{13}CH$_3$]Methionine-Incorporated Vitamin B_{12} This was isolated from *Propionibacterium shermanii*, which was grown for 7 d in 3 l of casein culture under a nitrogen atmosphere, gathered, then fed with [^{13}CH$_3$]methionine (M.S.D, 90 mg, 90% atom ^{13}C) for 3 d. After the general procedure[7] (centrifugation, extraction with methanol containing KCN, phenol extraction, chromatography on SiO_2, recrystallization from water–acetone), 2.5 mg of ^{13}C-enriched vitamin B_{12} was obtained. It was dissolved in D_2O, and used for the measurement. The spectrum in Fig. 4 was obtained with a $2 \times 256 \times 4086$ data matrix size, with 920 scans per t_1 value. The delay time between scans was 1.000 s, and the total measuring time was 35.5 h.

(2) [2-^{13}C]ALA-Incorporated Vitamin B_{12} This was available in our laboratory[5]; 2.5 mg was used for the measurement. The spectrum in Fig. 6 was obtained with a $2 \times 128 \times 1024$ data matrix, with 560 scans per t_1 value. The delay time between scans was 1.000 s, and the total measuring time was 20.5 h.

(3) [3-^{13}C]ALA-Incorporated Vitamin B_{12} This was also available in our laboratory[6]; 3.0 mg was used for the measurement. The spectrum in Fig. 7 was obtained with a $2 \times 256 \times 2048$ data matrix, with 1000 scans per t_1 value. The delay time between scans was 0.300 s, and the total measuring time was 13.5 h.

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