74. Assignment of ^{15}N-NMR Resonances of Vitamin B_{12} Analogues by 2D-[^{15}N,^{1}H] Long-Range Correlation: Fully [^{15}N]-Labelled Co-β-cyano-5-hydroxybenzimidazoylcobamide (Factor III) and Derivatives

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(18.XII.92)

The ^{15}N-NMR spectra of vitamin B_{12} analogues obtained in fully ^{15}N-labelled form have been measured by direct and inverse (^{15}N,^{1}H) correlated spectroscopy. All resonances, except those of the NH_2 groups, have been assigned to individual N-atoms. The influences on δ(N) are analyzed and discussed which are caused by changing the β-face ligand from CN to H_2O or CH_3 and by switching the α-face ligand from the base-on to the base-off state. An implication of the correct resonance assignment on biosynthetic pathways is demonstrated.

1. Introduction. – The particular structure of corrinoids (*Fig. 1*) with their conjugated system extending from N(A) to N(D), the Co-atom in the center, and the nucleotide loop has after its discovery [1] also challenged NMR spectroscopists. Due to the large number of heteroatoms, it could be expected that the ^{1}H- and ^{13}C-NMR spectra show good dispersion in several spectral regions, which is indeed the case. Nevertheless, as the side chains of the corrin ring are structurally very similar (three acetamides and four propionamides), many resonances could initially only be assigned groupwise, *cf.* [2] for ^{1}H and [3] for ^{13}C. After the advent of high-field magnets and 2D-NMR techniques, complete assignments of ^{1}H- and ^{13}C-resonances of corrinoids have been published, *e.g.* [4–6]. The ^{31}P-atom [7] [8] and the ^{59}Co-atom [9] [10] have also been investigated by NMR spectroscopy. ^{15}N-NMR has been used to prove the biosynthetic origin of the corrin-N-atoms from δ-aminolevulinic acid and of one benzimidazole N-atom from riboflavine [11] or glycin [12].

Here, we report on the ^{15}N-NMR spectra of fully ^{15}N-labelled Co-β-cyano-5-hydroxybenzimidazoylcobamide, called factor III by *Friedrich* and *Bernhauer* [13][1]), and its derivatives **1**–**3**. Factor III differs from vitamin B_{12} in that it contains 5-hydroxy- instead of 5,6-dimethylbenzimidazole. Full assignment of all corrin, benzimidazole, and the NH-amide resonances is given, which helps to deduce the electron-distribution in the N(A) to N(D) chromophore. An implication of the correct resonance assignment on possible biosynthetic pathways will also be given.

[1]) Unfortunately, the same name, factor III, has been given later to 20-methylsirohydrochlorin [14].

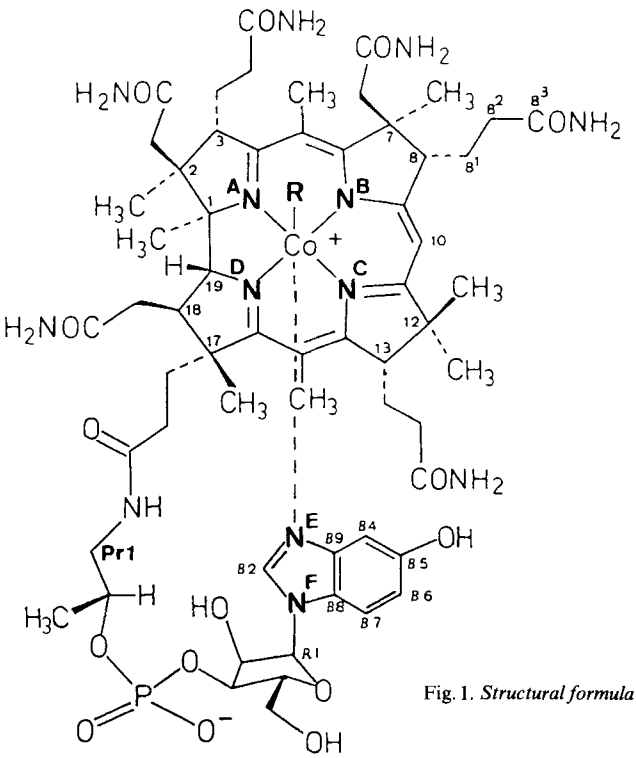


Fig. 1. *Structural formula of factor III*

2. Results. – The ^{15}N-NMR spectrum of factor III is shown in *Fig. 2*. Thirteen resonances for 13 labelled ^{15}N-atoms are detected, whereby the 7 amide resonances appear between −260 and −272 ppm relative to nitromethane. Out of these, the NH-amide was identified with an INEPT [15] spectrum where it displayed a ^1J(N,H) of 93 Hz. The remaining 6 signals between −160 and −220 ppm belong to the corrin and benzimidazole N-atoms, respectively. The assignment of individual N-atoms can be achieved by heteronuclear long-range [N–H] correlation, based on an unequivocal assignment of the protons coupling to ^{15}N.

By comparison of the ^{1}H-NMR spectra of unlabelled [16] and labelled factor III, some of the protons coupled to ^{15}N can easily be identified. For example, H–C(10) appears in labelled factor III as a *triplet* with two approximately equal ^3J(N,H) values of 4.5 Hz to N(B) and N(C), and H–C(B2) shows two ^2J(N,H) values of 5.2 and 7.5 Hz to N(E) and N(F), respectively. From the signal of H–C(8), a ^3J(N,H) value of 3 Hz can be extracted. Other long-range coupling constants only give rise to line-broadenings or are so small that they are only detected in the N,H-correlation spectrum, a phenomenon well known from homonuclear 2D-COSY spectra. We found that the following long-range coupling constants are of crucial importance for correct ^{15}N-resonance assignments: ^3J(N(A),CH$_3$–C(1)), ^3J(N(A),H–C(3)), ^3J(N(B),H–C(8)), ^3J(N(B),H–C(10)), ^3J(N(C),H–C(10)), ^3J(N(C),H–C(13)), ^2J(N(D),C(19)), ^2J(N(E),H–C(B2)), ^2J(N(F),H–C(B2)), ^3J(N(F),H–C(B7)), ^3J(N(F),H–C(R1))[2]. In *Table 1*, the δ(^{1}H) data together

[2] ^3J(N(D),H–C(18)) has never been detected neither direct nor as cross-peak, presumably because the dihedral angle is close to 90°.

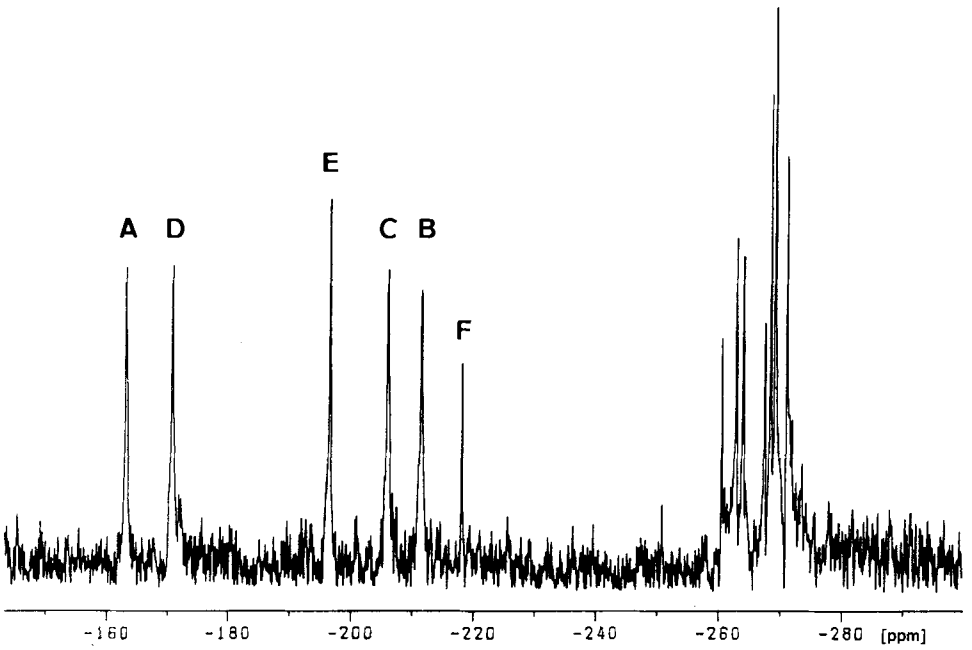


Fig. 2. *^{1}H-Decoupled 40.56-MHz ^{15}N-NMR spectrum of fully ^{15}N-labelled* **1** (4.1 mg). 23 400 Scans with an acquisition time of 0.45 s and a relaxation delay without ^{1}H decoupling of 2.6 s. A line-broadening of 2 Hz was used and the spectrum plotted in the absolute-value mode. The pulse width was 18 μs corresponding to a flip angle of 60°

Table 1. *Chemical Shifts δ(^{1}H)* [ppm] *of Selected Protons*[a])

	Base-on forms			Base-off forms	
	1	**2**	**3**	**1a**	**3a**
H–C(B2), *a*	7.084	6.605	7.047	8.370	9.2
H–C(B4), *b*	6.155	6.138	6.039	7.139	7.148
H–C(B6), *c*	6.882	6.861	6.810	6.901	7.083
H–C(B7), *d*	7.330	7.224	7.261	7.489	7.694
H–C(R1), *e*	6.325	6.218	6.266	6.353	6.565
H–C(R2), *f*	4.281	4.220	4.270	[b])	4.93[c])
H–C(R3), *g*	4.7	[b])	4.7	[b])	[b])
H–C(R4), *h*	4.040	4.007	4.084	4.580	[b])
H–C(R5), *i*	3.908	3.890	3.909	3.898	3.879
H′–C(R5), *k*	3.732	3.714	3.746	3.775	3.783
H–C(Pr1), *l*	3.597	3.615	3.564	3.309	3.312
H′–C(Pr1), *m*	2.918	2.904	3.057	3.249	3.225
H–C(Pr2), *n*	4.28	4.28	4.325	4.340	4.30
H–C(3), *o*	4.149	4.127	3.992	3.755	4.034
H–C(8), *p*	3.440	3.456	3.375	3.383	3.69
H–C(10), *q*	6.087	6.3205	6.003	5.859	6.77
H–C(13), *r*	3.320	3.489	3.158	3.134	3.42
H–C(18), *s*	2.74	2.955		2.7	2.86
H–C(19), *t*	4.067	4.240	4.008	3.674	4.418

[a]) Small letters (in italics) refer to the assignment of ^{15}N-coupled resonances in *Figs. 3* and *4*.
[b]) Hidden under the HDO resonance.
[c]) Identified through its correlation with N(F).

with other easily assignable resonances are given for all the compounds studied. The differentiation between H–C(19) and H–C(3) is sometimes prevented because of overlap of these signals with H–C(R4) or H–C(Pr2). In these cases, decoupling experiments between H–C(18) and H–C(19) are very useful. The relative chemical shifts of H–C(8) and H–C(13) are best confirmed with a NOE experiment: irradiation (saturation) of H–C(10) leads to a NOE for H–C(8) only.

In the light of conflicting ^{1}H-assignments for vitamin B_{12} and derivatives in the literature, it cannot be emphasized enough that assignments done in analogy to derivatives or under different experimental conditions, as for instance different solvents, must be corroborated by suitable NMR experiments as spin-decoupling, COSY, or NOE (NOESY, ROESY).

With the known ^{1}H-NMR assignments the ^{15}N assignments of factor III follow in a straightforward manner from the ^{15}N,^{1}H-correlation spectrum, as examplified in *Fig. 3*.

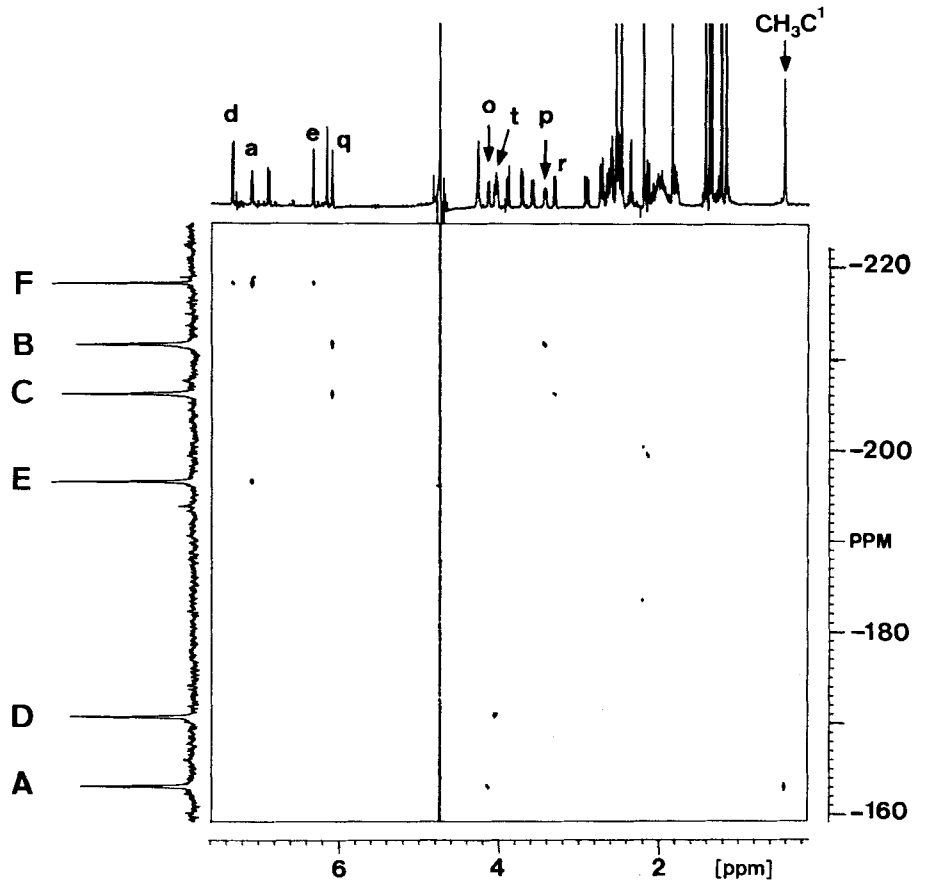


Fig. 3. *Two-dimensional ^{1}H-detected ^{15}N,^{1}H shift-correlation spectrum of* **1** *at 600.138 and 60.834 MHz, respectively.* The *F*2 axis shows the conventional ^{1}H spectrum, the *F*1 axis the conventional ^{15}N spectrum. The data matrix was 2 × 512 × 2 K with 24 scans plus 4 dummy scans per t_1 value and a delay of 3 s between scans giving a measuring time of 12 h. For the evolution of the heteronuclear coupling, a delay of 90 ms was used, corresponding to J(N,H) = 5.6 Hz. A shifted sine-bell filter was used in t_1 and t_2, and an absolute-value representation is shown.

The two N-atoms which show a correlation (cross-peak) with H–C(10) must be N(B) and N(C). They can be assigned individually through the cross-peaks with H–C(8) and H–C(13), respectively. N(A) correlates with H–C(3) and the CH_3 group at $\delta(H) \approx 0.5$ ppm, which had been assigned to CH_3–C(1) in base-on corrinoids. Finally, N(D) shows a cross-peak with H–C(19). The two N-atoms which correlate with H–C(B2) belong to the 5-hydroxybenzimidazole and are differentiated by the ^3J(N,H) to H–C(B7) and H–C(R1), which unequivocally assign N(F). The $\delta(^{15}N)$ values obtained from the correlation experiment were confirmed by a conventional (direct) ^{15}N-NMR spectrum shown parallel to the δ(N) axis of *Fig. 3*.

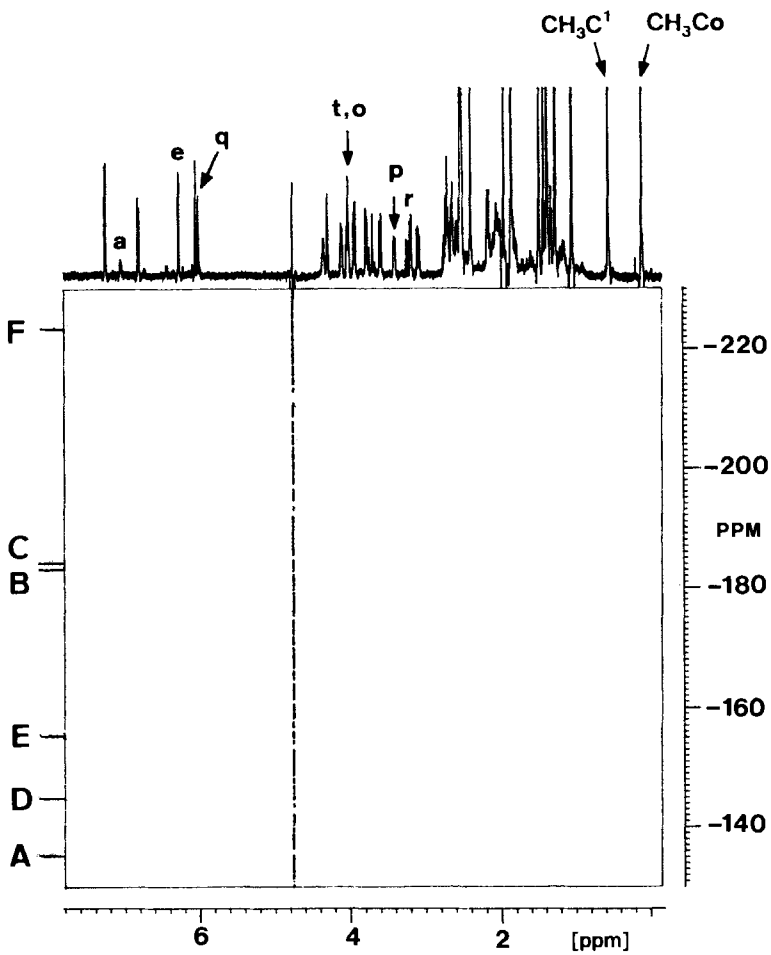


Fig. 4. *Two-dimensional ^1H-detected $^{15}N,^1H$ shift correlation spectrum of* **3** (1 mg) *at 600.138 and 60.834 MHz, respectively.* The *F*2 axis shows the conventional ^{1}H spectrum. The data matrix was 2 × 512 × 2 K with 48 scans plus 4 dummy scans per t_1 value and a delay of 2 s between scans giving a measuring time of 16 h. For the evolution of the heteronuclear coupling, a delay of 120 ms was used corresponding to J(N,H) = 4.2 Hz. A shifted sine-bell filter in t_1 and t_2 and an absolute-value representation were used.

As a second example, the ^{15}N,^{1}H-correlated spectrum of methyl-factor III **3** is shown in *Fig. 4*. The ^{1}H-NMR spectrum parallel to the δ(H) axis shows a CH_3 resonance at *ca.* 0.0 ppm, which belongs to the Co–CH_3 group. It shows cross-peaks with all five ^{15}N-atoms coordinated to Co. The ^{3}J(HCCoN) coupling constants must be very small (< 0.2 Hz), and cross-peaks due to these couplings were unexpected, since the correlation experiment was optimized for a coupling constant of 4.2 Hz. All cross-peaks observed and discussed in the case of **1** are found again. In *Table 2*, the results are summarized.

Table 2. *Chemical Shifts* $\delta(^{15}N)$ [ppm] *of the Corrin and Benzimidazole N-Atoms*

	N(A)	N(B)	N(C)	N(D)	N(E)	N(F)
1	−163.1	−211.7	−206.2	−170.8	−196.6	−218.3
2	−160.8	−208.3	−200.2	−166.8	−214.9	−214.7
3	−135.0	−183.5	−184.3	−144.9	−156.0	−223.9
1a	−167.3	−216.4	−220.4	−179.7	−151.6	−221.1
3a	−131.1	−169.1	−179.2	−142.0	[a])	−210.1

[a]) Under the acidic conditions, H–C(B2) exchanges so fast with ^{2}H that no cross-peaks with N(E) and N(F) could be detected. For an analogous observation, see [8].

3. Discussion. – The ^{15}N-NMR data are best visualized in *Fig. 5*. The four ^{15}N resonances of the macrocyclic ring are arranged into two groups with N(A) and N(D) at higher frequencies. This seems to imply that the electron distribution within the conju-

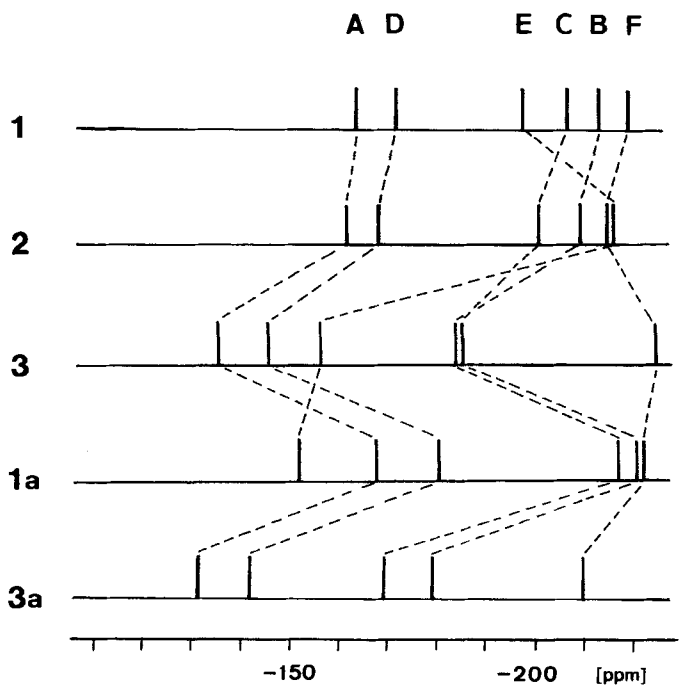


Fig. 5. *Stick plot representation of the* ^{15}N*-NMR spectra*

gated system is as suggested by the structural formula of *Fig. 1*, *i.e.* N(A) and N(D) of imine-types and N(B) and N(C) as a tautomeric mixture of imine and enamine leading to a plane of symmetry perpendicular to the N_4-plane and going through the Co-atom and C(10). The bond-length variations within the N(A) to N(D) chromophore observed in the crystal, on the other hand, cannot be described by a regular alternation of single and double bonds [17].

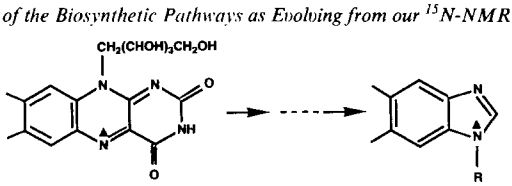
Changing the β-face ligand from CN to OH has moderate deshielding effects on all *cis*-ligands ranging from 2 ppm (N(A)) to 6 ppm (N(C)), whereas the *trans*-ligand N(E) is shielded by −18 ppm. It seems that this ligand change leads to some electron flow from the corrin ring to N(E).

The effect of an axial CH_3 group as in **3** is even more pronounced. All N-atoms coordinated (bound) to Co are deshielded, the values observed go from 28 ppm for N(A) to 41 ppm for N(E). Interestingly enough, the Co-atom is shielded by almost 500 ppm as a consequence of this β-face ligand change [10]. These results could be interpreted that, by the axial electron donor, the bonds between Co and its ligands are weakened (less back-bonding). This bond weakening has been observed as a change in bond length between Co and the β-face N-atom (our N(E)), which is 1.97 to 2.06 Å in cyanocobalamin and 2.24 Å in adenosylcobalamin (coenzym B_{12}) [18]. Since metal coordination of sp^2-N-atoms, as well as protonation or H-bonding, usually leads to an N-shielding, the reverse should occur on partial de-coordination, as is indeed observed.

In the base-off derivative **1a**, where the α-face ligand is replaced by CN, all corrin N-atoms are shielded moderately by this ligand replacement in accord with a stronger coordination of these atoms induced by the more electron-withdrawing β-CN group. It should also be mentioned that dicyanocobalamin has a ^{59}Co resonance shielded by 770 ppm compared with cyanocobalamin [10], an effect assigned to the special shielding property of the cyanide ion. As the 5-hydroxybenzimidazole becomes free, the observed δ(N) values resemble those of 1-methylbenzimidazole [19], *i.e.* −218.0 ppm (N(1)) and −119.5 ppm (N(3)) in DMSO. In **3a**, the α-face ligand is OH or OH_2, possibly a mixture of both species. The δ(N) values of the corrin N-atoms are not much different from those of **3**, and all are deshielded.

The correct assignment of N(E) and N(F) has implications on possible biosynthetic pathways [20] [21]. When *Kurumaya et al.* [11] fed 5-[^{15}N]riboflavin to B_{12}-producing bacteria, they isolated ^{15}N-enriched B_{12} with one N-atom enriched at a chemical-shift position that corresponds to our N(F). The authors' assumption that the Co-coordinated N-atom was enriched (our N(E)) is not consistent with our data. Concerning the biochemical pathway [22], this means that N(5) of riboflavin corresponds to N(1), our N(E), of the benzimidazole residue (*Scheme*).

Scheme. *Part of the Biosynthetic Pathways as Evolving from our ^{15}N-NMR Assignments*

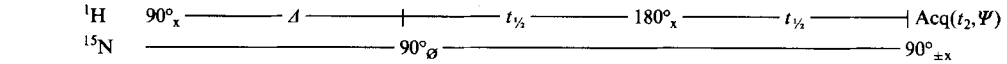


This work has been supported by the *Deutsche Forschungsgemeinschaft* and the Dr. *Helmut Legerlotz-Stiftung*. We gratefully acknowledge the cooperation of *W. von Philipsborn* and *B. Kräutler*.

Experimental Part

Material and Methods. Methanobacterium thermoautotrophicum was grown in 10-l mineral medium, which contained 20 mM (^{15}N)NH_4Cl (98%; *MSD Isotopes*, Munich, Germany) as the nitrogen source. The cobamide was extracted from wet cells by HCN in AcOH at pH 5.5 and purified by *XAD-2*, neutral aluminum-oxide column chromatography and *RP-C_{18}* HPLC as described previously [23]. The HO derivative was obtained from the CN form on reduction with about 0.5 mM titanium(III) citrate at pH 6.5. The light-sensitive methyl-cobamide **3** was synthesized in darkness from the HO derivative **2** by adding a 10-fold excess of CH_3I after the reduction by titanium citrate. Titanium citrate and minor impurities were removed from the cobamide samples by *XAD-2* and *RP-C_{18}* HPLC. The base-off compound **1a** was obtained by adding *ca.* 10 µl of NaCN soln. containing *ca.* 2 mol-equiv. of $CN^{\ominus}$. Under our experimental conditions, the ligand-exchange is slow on the NMR time-scale so that the signals of *ca.* 5% of the base-on form are also visible. The base-off compound **3a** was obtained by the addition of DCl.

Spectra. The spectra were recorded in 5-mm tubes on *Bruker AMX-600* or *AM-400* spectrometers at a temp. of 300 K. The compounds were dissolved in 99.95% D_2O and pD 7.4 by phosphate buffer to give pD 7.4. ^1H Spectra are referenced to 3-(trimethylsilyl)propionate (TSP). The HDO signal appeared at 4.75 ppm. The ^{15}N spectra are referenced to a capillary of nitromethane. [^{15}N]-Long-range correlations were performed in the inverse mode, utilizing the ^1H-sensitivity [24] and the following pulse sequence:



$\Delta = 1/(2J(N,H))$

The phases $\emptyset$ and Ψ are cycled along the 4 axes leading to a 8-pulse cycle.

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