# *Coα*-(1*H*-Imidazolyl)-*Coβ*-methylcob(III)amide: Model for Protein-Bound Corrinoid Cofactors

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Dedicated to Prof. *Albert Eschenmoser* on the occasion of his 75th birthday

*Coα*-(1*H*-Imidazol-1-yl)-*Coβ*-methylcob(III)amide (**4**) was synthesized by methylation with methyl iodide of (1*H*-imidazol-1-yl)cob(I)amide, obtained by electrochemical reduction of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-cyanocob(III)amide (**5**). The spectroscopic data and a single-crystal X-ray structure analysis indicated **4** to exhibit a base-on constitution in solution and in the crystal. The crucial lengths of the axial Co–N and Co–$CH_3$ bonds also emerged from the crystallographic data and were found to be smaller by 0.1 and 0.02 Å, respectively, than those in methylcob(III)alamin (**2**). The data of **4** support the view, that the 'long' axial Co–N bonds as determined by X-ray crystallography for the $B_{12}$-dependent methionine synthase, for methylmalonyl-CoA mutase, and for glutamate mutase represent stretched Co–N bonds. The thermodynamic effect (the '*trans* influence') of the 1*H*-imidazole base in **4** on the organometallic reactivity of this model for protein-bound organometallic $B_{12}$ cofactors was examined by studying Me-group-transfer equilibria in aqueous solution and using (5′,6′-dimethyl-1*H*-benzimidazol-1-yl)cobamides (cobalamins) as reaction partners (*Schemes 2–5*, *Table*). In comparison with methylcob(III)alamin (**2**), **4** was found to be destabilized for an abstraction of the Co-bound Me group by a $Co^{III}$ electrophile. In contrast, the abstraction of the Co-bound Me group by a radical(oid) $Co^{II}$ species was not significantly influenced thermodynamically by the exchange of the nucleotide base. Likewise, exploratory Me-group-transfer experiments with Me–$Co^{III}$ and nucleophilic $Co^{I}$ corrinoids at pH 6.8 provided an apparent equilibrium constant near unity. However, this finding also was consistent with partial protonation of the imidazolylcob(I)amide at pH 6.8, suggesting an interesting pH dependence of the Me-group-transfer equilibrium near neutral pH. Therefore, the replacement of the 5′,6′-dimethyl-1*H*-benzimidazole base by an 1*H*-imidazole moiety, as observed in methyl transferases and in C-skeleton mutases, does not by itself strongly alter the inherent reactivity of the $B_{12}$ cofactors in the crucial homolytic and nucleophilic-heterolytic reactions involving the organometallic bond, but may help to enhance the control of the organometallic reactivity by protonation/deprotonation of the axial base.

**1. Introduction.** – The $B_{12}$ cofactors, such as coenzyme $B_{12}$ (=(5′-deoxy-5′-adenosyl)cob(III)alamin; **1**) and methylcob(III)alamin (**2**), are highly complex, naturally occurring organometallic compounds [1][2] (see *Fig. 1*). They are coenzymes for a series of enzymes that catalyze very unusual biological reactions [2–5]. At present, coenzyme $B_{12}$ (**1**) is known to be the cofactor of eleven enzymes (of $B_{12}$-dependent ribonucleotide reductase [6] and of ten other enzymes that catalyze rearrangement reactions *via* radical intermediates [4][5]). Methylcob(III)alamin (**2**) is the cofactor in a class of enzymes that catalyze the intermolecular transfer of Me groups

[5][7–9]. In all these enzymes, critical steps of their catalytic cycles depend upon the rapid homolytic or heterolytic cleavage (and homolytic and heterolytic formation) of the organometallic bond of the protein-bound $B_{12}$ cofactors [2][4][5].

Since the early (nineteen)-sixties, isolated $B_{12}$ cofactors have been the subject of numerous structural investigations, both in the crystalline state [1][10–12] and in

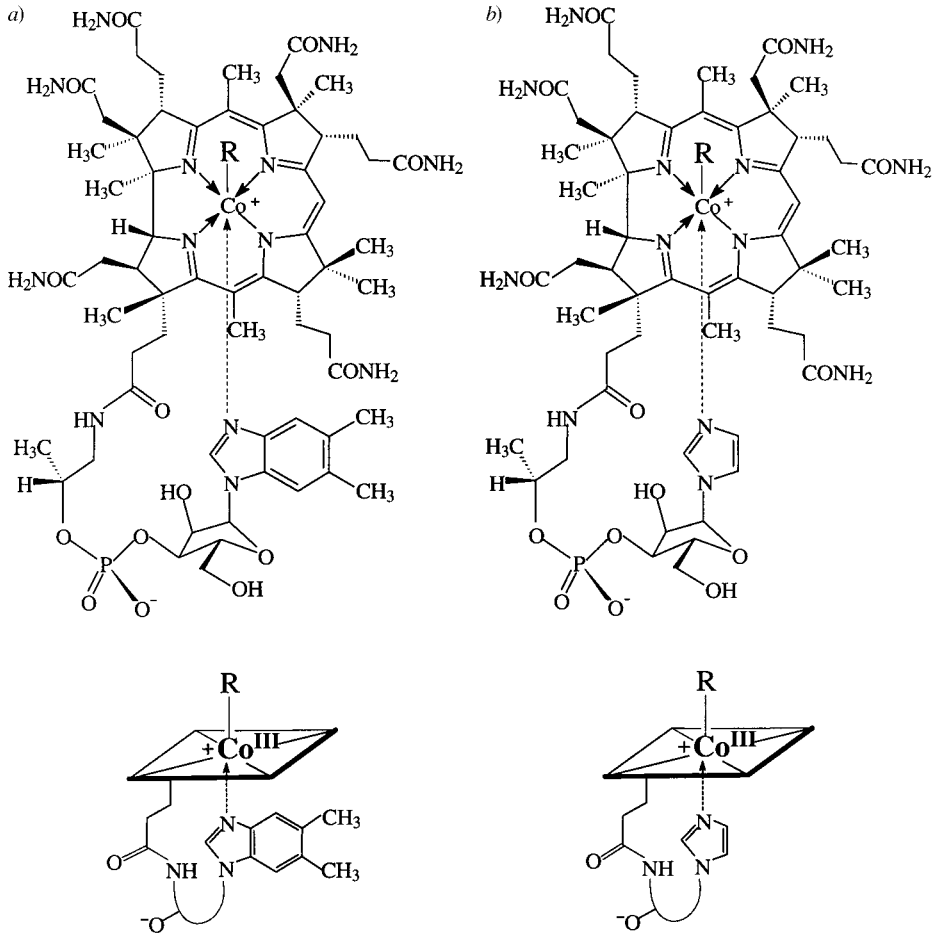



Fig. 1. *Structural formulae of vitamin-$B_{12}$ derivatives in the 'base-on' constitution:* a) *Cobalamins: (5′-deoxy-5′-adenosyl)cob(III)alamin* (=coenzyme $B_{12}$; R = 5′-deoxy-5′-adenosyl; **1**); *methylcob(III)alamin* (R = Me; **2**); *cyanocob(III)alamin* (=vitamin $B_{12}$; R = CN; **3**), *aquacob(III)alamin cation* (R = $H_2O$; $\mathbf{8}^+$); *and cob(II)alamin* (R = unoccupied; **10**). b) *Imidazolylcobamides:* Co*α-(1*H-*imidazol-1-yl)*-Co*β*-methylcob(III)amide (R = Me; **4**), Co*α-(1*H-*imidazol-1-yl)*-Co*β-cyanocob(III)amide* (R = CN; **5**), Co*α-(1*H-*imidazol-1-yl)*-Co*β-aquacob(III)amide cation* (R = $H_2O$; $\mathbf{7}^+$), *and (1*H-*imidazol-1-yl)cob(II)amide* (R = unoccupied, **9**).

solution [4][5][13][14]. Indeed, vitamin $B_{12}$ (**3**) is prominent for being the first case of a natural compound where the essential features of its structure were deduced by X-ray analysis [10–12][15]. The complex structure of the $B_{12}$ cofactors implies a correspondingly complicated biosynthesis [16–19], laboratory synthesis [20][21], and chemistry of $B_{12}$ coenzymes [2–5][14][22][23].

A well-studied feature of the isolated, intact 'complete' $B_{12}$ cofactors is the intramolecular ('base-on') cobalt coordination of their unique nucleotide function (see *Fig. 1*), which represents their most stable constitution in solution and in the solid state [1][2][4][5][10–14]. The first crystal structure of a $B_{12}$-binding protein, of the $B_{12}$-binding domain of methionine synthase (MetH) from *Escherichia coli*, revealed a surprisingly different situation for the protein-bound corrinoid cofactor methylcob(III)alamin (**2**): here, the cofactor was observed in a 'base-off/His-on' constitution, *i.e.*, instead of the dimethylbenzimidazole base, the imidazole side chain of the protein residue $His^{759}$ was found to coordinate at the corrin-bound $Co^{III}$ center of **2** (see *Fig. 2*) [24–26]. In two recent structure analyses of coenzyme-$B_{12}$-dependent mutases, methylmalonyl-CoA mutase (MCM) from *Propionibacterium shermanii* [26][27] and in glutamate mutase (Glm) from *Clostridium cochlearium* [28], the corresponding 'base-off/His-on' constitution (previously predicted from protein-sequence alignment data [29]) of the bound corrinoid was also observed. In all three protein structures, the Co-coordinating histidine and a H-bonded aspartate represent a conserved '$B_{12}$-binding' motif, and they are part of a regulatory 'ligand triad' of (typically) three amino-acid residues [25][26]. The role of the unique ($\alpha$-configurated) ribazole function of the $B_{12}$ coenzymes in their 'base-off/His-on' constitution, therefore, does not concern the control of the organometallic reactivity at the Co-atom, but primarily the anchoring of the corrinoid 'dinucleotides' coenzyme $B_{12}$ and methylcobalamin to the apoenzyme. Contrasting these crystallographic findings on the Me-group transferase MetH [24], and the two C-skeleton mutases, MCM and Glm [27][28], EPR-spectroscopic studies on the mode of binding of coenzyme $B_{12}$ to diol dehydratase (DD) and to $B_{12}$-dependent ribonucleotide reductase (RNR) indicated base-on binding of the corrinoid cofactor [30–32]. The finding on DD was confirmed by the crystal structure of the inactive vitamin-$B_{12}$ complex of this enzyme, which provided for the first time struc-

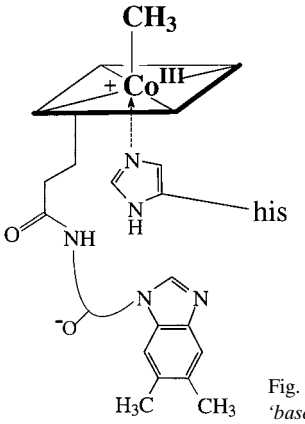



Fig. 2. *Sketch of the coordination features of methylcob(III)amin* (**2**) *bound 'base-off/His-on' in methionine synthase from* E. coli [24–26]

tural clues concerning the binding of a corrinoid cofactor in its 'conventional' base-on form [33].

Studies concerning the axial coordination properties of corrinatocobalts are of interest, as their homolytic and heterolytic organometallic reactivity are central to their biological role [2–5][34]. Such studies have gained further relevance from the ongoing crystallographic work on $B_{12}$-dependent enzymes. In the $B_{12}$-binding domain of MetH, the Co-coordinating imidazole moiety forms a H-bond to the carboxylate of an aspartate, constituting the crucial part of the 'regulatory ligand triade' [24–26]. The latter has been suggested to undergo protonation/deprotonation as part of the catalytic cycle, which involves shuttling between hexacoordinate $Co^{III}$ and tetracoordinate $Co^{I}$ states of the corrinoid. In this way, the Co-coordinated histidine apparently helps to control the organometallic reactivity of the protein-bound corrinoid cofactor and to mediate the influence of the proteinic environment [24–26][35]. On the other hand, the bonding pattern around the Co-center in the crystallographically investigated coenzyme-$B_{12}$-dependent enzymes are remarkable, as all of them show an unusually 'long' axial Co–N bond [26–28][33]: the observed lengths of the Co–N bonds in MCM (2.53 Å), in Glm (2.27–2.35 Å), and in DD (2.50 Å) are considerably longer than those observed in the isolated corrinoid cofactors [1][10–12][36]. The crystallographically observed 'long' axial Co–N bond has been suggested to be relevant for the activation of the protein-bound corrinoid cofactor towards homolysis of its Co–C bond [26][27][33]. However, the significance of these crystallographic data must not be overestimated since their crystallographic resolution was inherently low (often $>2$ Å), and neither the oxidation state of the corrin-bound Co-centers nor the nature of their upper axial coordination partners were always clear. In the two mutase structures (MCM, Glm), presumably a mixture of corrinoid cofactors with different oxidation states was present [26]. The length of this bond was also studied by X-ray absorption spectroscopy, which yielded an indication for bond elongation for MCM [37] and a more or less 'normal' bond length for Glm [38]. However, recently we were able to show that under the conditions of a high-brilliance X-ray beam, photoreduction of the cofactor may occur, which would then suggest that at least some of the crystallographically observed bond elongation is in fact an artifact of the experimental technique [39]. However, at this stage, the 'long bond' still poses a puzzling observation. The point has been made elsewhere [2] that comparison of the lengths of the axial Co–N bond in organo-cob(III)alamins and cob(II)alamin (**10**) [10–12] would not suggest a simple stretch of the axial Co–N bond to lead to 'activation' of the protein-bound corrinoid cofactor towards homolysis.

Clearly, further chemical consequences of the axial coordination of a nitrogenous base to the Co-center of corrins are of interest [2][12][14]. Its effect on the reactivity of organometallic groups at the other axial (*trans*) position has been discussed in the context of the enigmatic mode of activation of protein-bound coenzyme $B_{12}$ [2][40–43]. Similar questions on the effect of the intramolecular coordination of the nucleotide base on the reactivity of methylcobalamin were also posed and examined experimentally [44]. So far, all such studies had to rely on a comparison between the reactivity of cobalamins and corrinoids lacking a nucleotide function all-together. With the two exceptions of the structures of the cyano-corrinoids Factor A [45] and *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-cyanocob(III)amide [46], also all high-resolution crystal structures

of 'complete' $B_{12}$ derivatives have involved cobalamins ((5′,6′-dimethyl-1*H*-benzimidazol-1-yl)cob(III)amides) [1][10–15]. Questions [24–28][35] concerning the consequences of the replacement of the 5′,6′-dimethyl-1*H*-benzimidazole (Dmb) moiety of cobalamins (such as of methylcob(III)alamin (**2**)) by an 1*H*-imidazole (Im) moiety, such as in *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcob(III)amide (**4**), on the structure and organometallic reactivity have so far not been addressed. We describe here the synthesis and spectroscopic characterization in solution of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcob(III)amide (**4**) and the crystal structure of this model for protein-bound organocorrinoid cofactors, and report on experiments concerning its organometallic reactivity.

**2. Results and Discussion.** – 2.1. *Preparation and Spectroscopic Characterization of* Coα*-(1*H*-Imidazol-1-yl)-*Coβ*-Methylcob(III)amide* (**4**). The nonnatural complete corrinoid *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-cyanocob(III)amide (**5**) was available from guided biosynthesis [46]. The electrosynthesis of the organocobamide **4** from **5** and MeI followed an established procedure [47]: Electrochemical reduction of **5** at a potential of $-1.1$ V *vs.* 0.1N calomel electrode by *ca.* 2 F/mol gave the highly $O_2$-sensitive imidazolylcob(I)amide $\mathbf{6}^-$ (see below, *Scheme 4*), characterized by the UV/VIS spectrum. Treatment of the solution containing $\mathbf{6}^-$ with an excess of MeI was accompanied by a color change (from green to red) of the reaction solution. The light-sensitive *Coα*-imidazolyl-*Coβ*-methylcobamide **4** was isolated in *ca.* 90% yield as bright red crystals. This 'complete' imidazolylcobamide **4** exhibited UV/VIS- and CD-spectroscopic data typical for 'base-on' methylcobamides (see *Fig. 3* for the UV/VIS spectrum). In the FAB-MS, the base peak at *m/z* 1266.8 and fragments at *m/z* 1251.7 indicated relatively stable pseudo-ions of intact **4** ($[M+H]^+$) and of the demethylated corrin fragment ($[M+H-CH_3]^+$). Detailed NMR studies of **4** allowed the complete assignment of the $^1$H-NMR spectrum, fully consistent with the 'base-on' constitution of **4** in neutral aqueous solution (see *Fig. 4*).

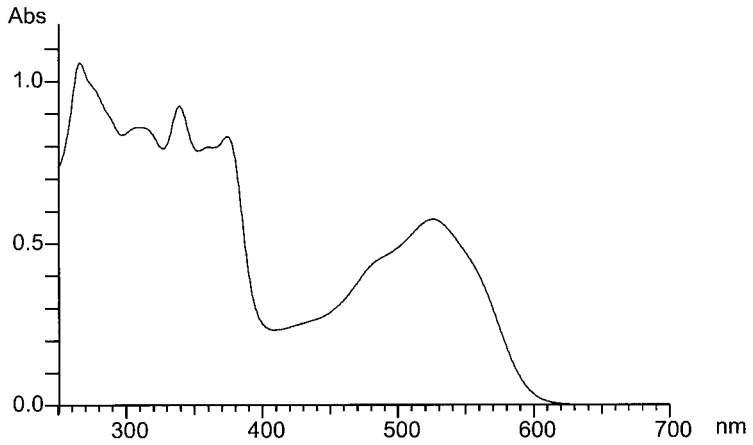



Fig. 3. *UV/VIS Spectrum of* Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcob(III)amide* (**4**) *in aqueous solution* ($c$ = 0.8 mM, 0.1M phosphate, pH 6.74)

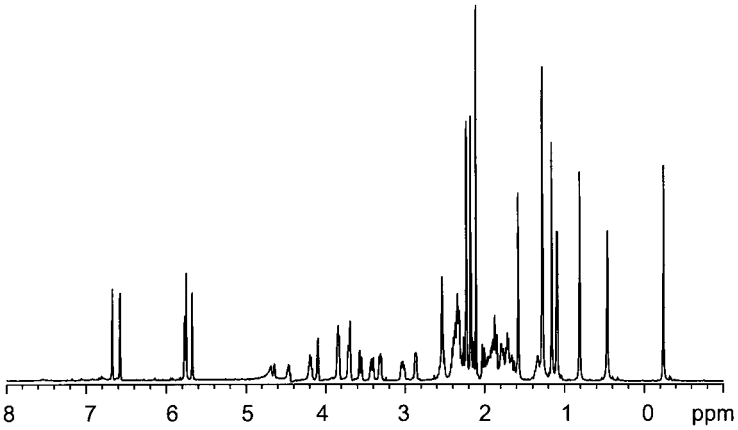



Fig. 4. *500-MHz $^1$H-NMR Spectrum of* Co*α-(1*H-*imidazol-1-yl)*-Co*β-methylcob(III)amide* (**4**) *in aqueous solution* ($D_2O$, pH 7)

The $^1$H-NMR spectrum of **4** has similar features as that of methylcob(III)alamin (**2**) [48], *i.e.* high-field *s* at $-0.21$ and 0.49 ppm for the Co-bound Me group and Me–C(1), respectively. The latter signal experiences a related upfield shift by the Co-coordinated imidazole moiety in the spectrum of **4**, as by the Co-coordinated benzimidazole moiety in the spectrum of **2**. The signal of Me–Co of **4** is shifted upfield slightly in both the $^1$H- and $^{13}$C-NMR spectra, compared to the corresponding data for **2**. All the available spectroscopic information is consistent with the expected ('conventional') base-on constitution of **4**.

2.2. *Preparation and Spectroscopic Identification of* Co*α-(1*H-*Imidazol-1-yl)*-Co*β-aquacobamide* (**7**$^+$). The *Coα*-imidazolyl-*Coβ*-methylcobamide **4** is rapidly decomposed by light, similar to other Me–Co$^{III}$ corrinoids, such as **2**. As described for the preparation of *Coβ*-aquacob(III)alamin (**8**$^+$) from **2** [49], the *Coβ*-aquacob(III)amide **7**$^+$ was prepared by photolysis of the corresponding Me–Co$^{III}$ corrinoid **4** in aerated aqueous solution. The UV/VIS spectrum of an aqueous solution of **7**$^+$ (at pH 6.3) exhibits the typical broad band with two absorbance maxima near 520 nm and a sharp band near 360 nm (see *Fig. 5*). The $^1$H-NMR spectrum of an aqueous solution of **7**$^+$ supports the assignment of its 'base-on' constitution.

2.3. *Acid/Base Properties of* Co*α-(1*H-*Imidazol-1-yl)*-Co*β-methylcob(III)amide* (**4**) *in Solution*. The UV/VIS absorbance spectra of aqueous solutions of **4** are strongly pH-dependent, indicating protonation of **4** at low pH with concominant decoordination of the nucleotide base (see *Fig. 6* and *Scheme 1*). A spectrophotometric analysis of buffered aqueous solutions of **4** in the pH range between pH 0.3 and 7.8 allowed the determination of the p$K_a$ of protonated ('base-off') **4**·**H**$^+$ as $4.3 \pm 0.1$ at room temperature. The value of this p$K_a$ is higher by *ca.* 1.4 units than that of **2**·**H**$^+$, the corresponding p$K_a$ of the protonated, 'base-off' form **2**·**H**$^+$ of methylcob(III)alamin (**2**) [50]. Comparison of the p$K_a$ of **2**·**H**$^+$ (=2.9) with that of protonated 5,6-dimethyl-1-(α-D-ribofuranosyl)-1*H*-benzimidazole (=α-ribazole; 5.56 at 25°) indicated the equilibrium constant for the formation of 'base-on' methylcobalamin **2** to amount to *ca.* 460 (at 25°) [50]. The corresponding p$K_a$ of protonated 1-(α-D-ribofuranosyl)-1*H*-imidazole is not available at this stage, but may be estimated to be close to 7, based on the values for Me-group-transfer equilibria, determined here (see below).

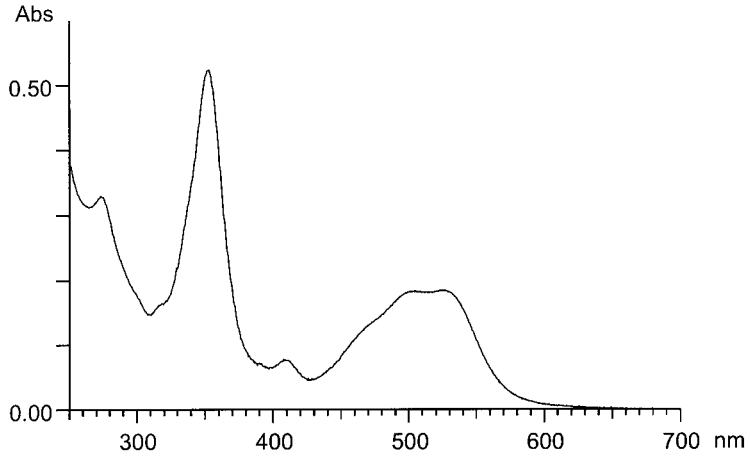



Fig. 5. *UV/VIS Spectrum of* Co*α-(1*H*-imidazol-1-yl)*-Co*β-aquacob(III)amide* (**7**$^+$) *in aqueous solution* (0.02M phosphate, pH 6.3)

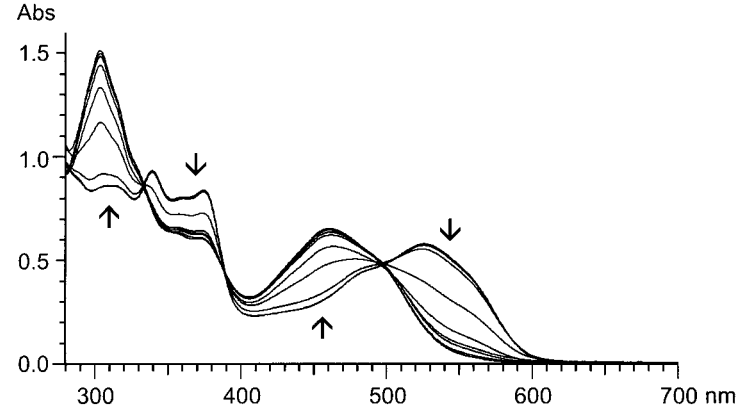
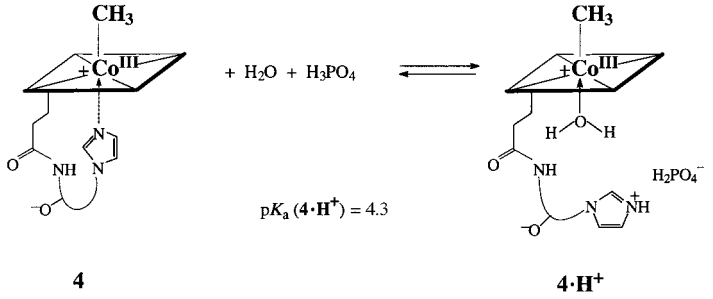



Fig. 6. *pH Dependence of UV/VIS Spectra of* Co*α-(1*H*-imidazol-1-yl)*-Co*β-methylcob(III)amide* (**4**) *in buffered aqueous solutions* (see *Exper. Part* for conditions)

*Scheme 1*

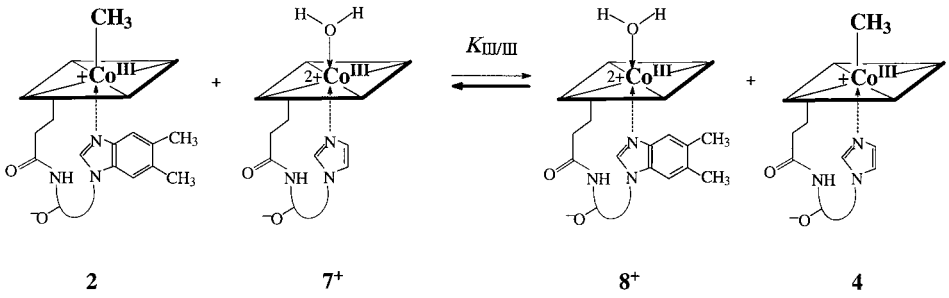


2.4. *Methyl-Group-Transfer Equilibria in Solution Characterizing the Organometallic Reactivity of* Co*α-(1*H-*Imidazol-1-yl)*-Co*β-methylcob(III)amide* (**4**). 2.4.1. *Preamble.* Early observations have suggested the occurrence of Me-group transfer reactions between various Co-complexes [23]. Methyl-group transfer from methylcob(III)alamin (**2**) to diaquacob(III)inamide was reported by *Hogenkamp* and coworkers [51]. Systematic studies on the equilibria in aqueous solution between Me–$Co^{III}$, $Co^{II}$, and $Co^{I}$ forms involving the cob(III)alamin **2**, cob(II)alamin (**10**), and cob(I)alamin (**11**$^-$; see below, *Scheme 4*), as well as the corresponding cobinamides (methylcob(III)-, cob(II)-, and cob(I)inamide), were carried out and informed on the (thermodynamic) effect of the Dmb base on the three Co–C bond dissociation energies in methylcorrinoids [2][44][52]. They indicated a small effect of the coordinated Dmb base on the homolytic bond strengths of the investigated methylcorrinoids, as well as on the related adenosylcorrinoids [44]. In contrast, these experiments revealed significant stabilization of ('base-on') methylcob(III)alamin (**2**) by the intramolecular coordination of the base against abstraction of the Co-bound Me group by a nucleophile [44]. Exploratory experiments comparing cobalamins and (5′-hydroxy-1*H*-benzimidazol-1-yl)cobamides (found in some methanogens [53]) also indicated small effects of the differing bases on the heterolytic mode of nucleophilic cleavage of the Co–C bond, but negligible effects on the homolytic mode of cleavage [52]. The experiments described here were carried out to compare the thermodynamic (*trans*) effects of Im and of Dmb as nucleotide bases on the three elementary modes of Co–C bond cleavage of the methylcob(III)amides **2** and **4**.

2.4.2. *Equilibration Experiments with Methylcob(III)amides and Aquacob(III)amides.* The following experiments were carried out to investigate the Me-group-transfer equilibrium indicated in *Scheme 2*, which allowed the determination of $K_{III/III}$ of *Eqn. 1*.

$$K_{III/III} = ([\mathbf{8}^+] \cdot [\mathbf{4}])/([\mathbf{2}] \cdot [\mathbf{7}^+]) \quad (1)$$

*Scheme 2*




**2** **7**$^+$ **8**$^+$ **4**

A buffered aqueous solution of about equimolar amounts of methylcob(III)alamin (**2**) and of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-aquacob(III)amide (**7**$^+$) was stored in an NMR tube at room temperature for 30 days with protection from light (reaction A). In a parallel experiment (reaction B), a corresponding buffered solution of *Coα*-(1*H*-

imidazol-1-yl)-*Coβ*-methylcob(III)amide (**4**) and of aquacob(III)alamin chloride (**8**·**Cl**) was also stored in an NMR tube at room temperature for the same period of time with protection from light. The changes in the two solutions (A and B) were observed $^1$H-NMR-spectroscopically. The reaction mixture A only underwent minor, $^1$H-NMR-spectroscopically manifest changes, but small signals due to traces of **4**, of aquocobalamin cation **8**$^+$, and of an intermediate **12** (see below) appeared over the course of the measurements. In contrast, the observed changes in reaction mixture B were more apparent. Within the first 2 h, the signals of the intermediate **12** (no formula shown) had grown to almost their maximal intensity, while those of **2** and **7**$^+$ appeared more slowly, to reach by the end of the experiment (30 days) essentially the same solution composition as in reaction A. The position of two signals of the intermediate **12**, a *singlet* at $-0.36$ ppm (a high-field position typical of signals due to Co-bound Me groups) and a *singlet* at 0.95 ppm, would make it rather likely that **12** is a *Coα*-methylcorrinoid in a 'base-off' form [54]. Further experiments are planned to reveal the identity of **12**.

For further analysis of both experiments, the two reaction mixtures were treated with a slight excess of KCN in $H_2O$ (with protection from light) and were analyzed once more by $^1$H-NMR spectroscopy. Accordingly, the cyanide treatment of both reaction mixtures converted the aquacorrinoids in their cyano analogues and induced the disappearance of the small remaining signals of **12**. In the reaction mixture from experiment A, the corrinoids **2**–**5** were present in a ratio of $4.8:1.2:0.8:2.5$, corresponding to a product ratio of $0.08 \pm 0.04$ (see *Table*). Likewise, in the reaction mixture from experiment B, the corrinoids **2**–**5** were present in a ratio of $1.8:1.1:0.3:1.4$, corresponding to an equilibrium value of $0.12 \pm 0.02$. Excluding significant equilibration after the cyanide quenching, the contents of the equilibrating reaction mixtures (**2**, **8**$^+$, **4**, and **7**$^+$) would be reflected by the ratios of the four corrinoids **2**, **3**, **4**, and **5**, respectively, and the equilibrium constant for the equation given in *Scheme 2* (see *Eqn. 1*) could be estimated as $K_{III/III} = 0.10 \pm 0.02$ (see *Table*). These experiments indicate that the thermodynamic enhancement of the electrophilic demethylation of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcob(III)amide **4** by the coordinated Im base is significantly larger than that by the Dmb base in methylcob(III)alamin (**2**). This suggests a particularly strong stabilization of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-aquacob(III)amide (**7**$^+$) by the intramolecular coordination of the Im base. In view of

Table. *Concentration Ratios of Cyanocorrinoids* **3** *and* **5** *and of Methylcorrinoids* **2** *and* **4**, *from Methyl-Group-Transfer Experiments at Room Temperature and pH 6.8 and Calculated* (apparent) *Equilibrium Constants for Methyl-Group-Transfer Equilibria*

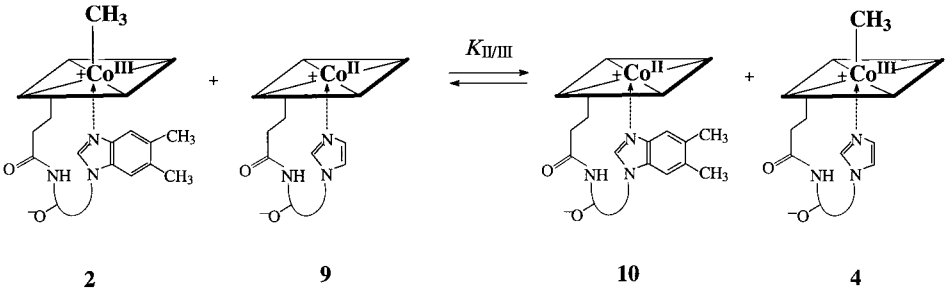
| Starting materials | Products (rel. amount, ±10%) | | | | Calc. ratio (**3**·**4**)/(**5**·**2**) | Calc. (apparent) equilibrium constants |
|---|---|---|---|---|---|---|
| | **2** | **3** | **4** | **5** | | |
| **2**, **7**$^+$ | 4.77 | 1.24 | 0.76 | 2.52 | 0.08 | $K_{III/III} = 0.1 \pm 0.02$ |
| **4**, **8**$^+$ | 1.83 | 1.09 | 0.30 | 1.43 | 0.12 | |
| **2**, **9** | 0.61 | 1.01 | 0.90 | 0.72 | 2.06 | $K_{II/III} = 1.7 \pm 0.4$ |
| **4**, **10** | 1.80 | 1.04 | 3.62 | 1.55 | 1.34 | |
| **2**, **6**$^-$ | 1.22 | 1.04 | 1.01 | 1.08 | 0.80 | $K_{I/III}$(app) $= 0.9 \pm 0.2$ |
| **4**, **11**$^-$ | 1.31 | 1.05 | 1.70 | 1.30 | 1.05. | |

the observed strong upwards folding in aquacobalamin, ascribed to steric strain imposed by the strongly coordinating, bulky Dmb base [55], an increased stabilization in $\mathbf{7}^+$ by the intramolecular coordination of the less bulky Im base is in agreement with basic expectations on the coordination behavior of the corrin-bound $Co^{III}$-center and on the higher nucleophilicity of Im (compared to Dmb).

2.4.3. *Equilibration Experiments with Methylcob(III)amides and Cob(II)amides.* The following two experiments were carried out to investigate the Me-group-transfer equilibrium given in *Scheme 3*, which led to the determination of $K_{II/III}$ of *Eqn. 2*.

$$K_{II/III} = ([\mathbf{10}] \cdot [\mathbf{4}])/([\mathbf{2}] \cdot [\mathbf{9}]) \quad (2)$$

*Scheme 3*




A buffered aqueous solution of (1*H*-imidazol-1-yl)cob(II)amide (**9**) was prepared by electrochemical reduction of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-cyanocob(III)amide **5**. The extent of the reduction was monitored coulometrically and UV/VIS-spectroscopically (see below) [56]. The solution of **9** was then mixed (with protection from light and air) with a buffered aqueous solution of an equimolar amount of methylcob(III)alamin (**2**). The mixture was left to stand at room temperature (with protection from light and air). After an equilibration time of 2 h, the mixture was taken out of the glove box, and $O_2$-saturated 1% HCN in MeOH was injected. The reaction mixture contained significant amounts of the four corrinoids **2**–**5** (according to TLC) and was further analyzed by recording a 500-MHz $^1$H-NMR spectrum (see *Table*). Accordingly, the corrinoids **2**–**5** were present in a ratio of 0.61 : 1.0 : 0.90 : 0.72 (see *Fig. 7*), corresponding to a ratio of products of $2.06 \pm 0.4$.

A buffered aqueous solution of cob(II)alamin (**10**) was prepared likewise by electrochemical one-electron reduction of vitamin $B_{12}$ (**3**) (see below) [54]. An aliquot of the solution of **10** was then mixed (with protection from light and air) with a buffered aqueous solution of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcob(III)amide (**4**) and left to stand at room temperature. After workup as described above, the $^1$H-NMR analysis (500 MHz) revealed the presence of the corrinoids **2**–**5** in a ratio of 1.8 : 1.04 : 3.6 : 1.55, corresponding to a ratio of products of $1.34 \pm 0.4$ (see *Table*). Assuming insignificant equilibration after the oxidative quenching, justified by the experiences with aquacorrinoids [44], the contents of the equilibrating reaction mixtures (**2**, **10**, **4**, and **9**) would be reflected by the ratios of the four corrinoids **2**, **3**, **4**, and **5**, respectively, and the equilibrium constant for the equation given in *Scheme 3* (see *Eqn. 2*) could be

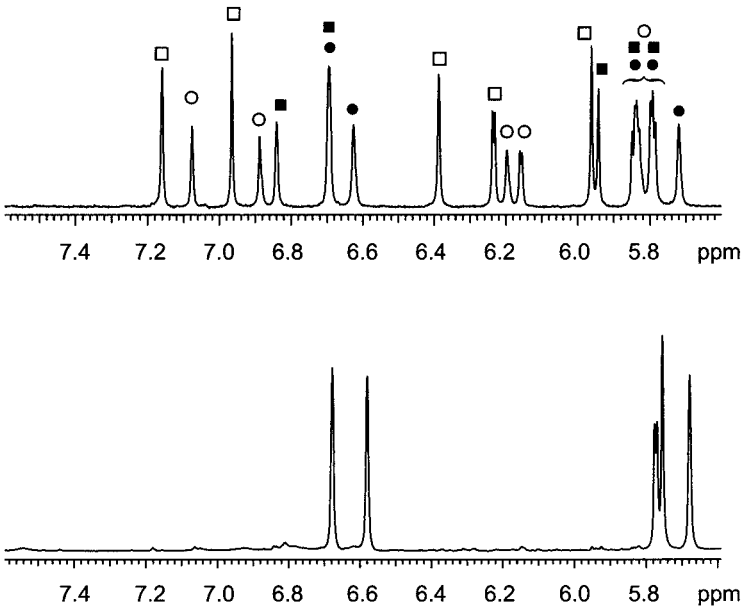



Fig. 7. *Comparison of the low-field section of the* $^1$*H-NMR spectra of* Co*α-(1*H*-imidazol-1-yl)-*Co*β-methylcob(III)amide* (**4**) *and of the reaction mixture from methyl-group transfer starting with methylcob(III)alamin* (**2**) *and (1*H*-imidazol-1-yl)cob(II)amide* (**9**). ○: signals of **2**, □: signals of **3**, ●: signals of **4**, and ■: signals of **5**.

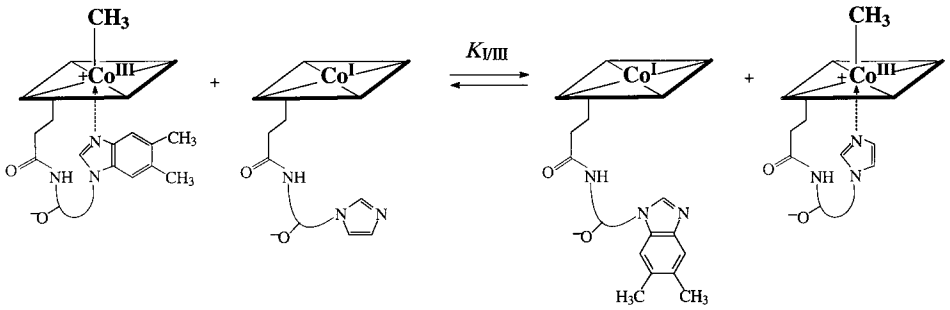
estimated as $K_{II/III} = 1.7 \pm 0.4$. This value indicates a slight stabilization of the imidazolyl(methyl)cobamide **4** upon homolytic methylation, compared to methylcobalamin **2**. The (thermodynamic) effect of the coordination of the Im base of **4** on the homolytic Co–C bond strength thus is small, when compared to that of the Dmb base of **2**, but tends to stabilize the imidazolyl(methyl)cob(III)amide **4**. A similar result is likely to characterize also the related homolytic Co–C bond strengths of adenosylcobamides, such as coenzyme $B_{12}$ (**1**) and its imidazolyl analogue. Accordingly, the substitution of the Dmb base by a histidine residue, as observed in the adenosylcobamide-dependent mutases MCM and Glm, is not likely to result in an inherently increased reactivity towards homolysis of the Co–C bond.

2.4.4. *Exploratory Equilibration Experiments with Methylcob(III)amides and Cob(I)amides.* With the intention of investigating the Me-group transfer equilibrium given in *Scheme 4* and of determining the equilibration constant $K_{I/III}$ of *Eqn. 3*, the following two exploratory experiments were carried out.

$$K_{I/III} = ([\mathbf{11}^-] \cdot [\mathbf{4}])/([\mathbf{2}] \cdot [\mathbf{6}^-]) \quad (3)$$

In one experiment, crystalline methylcob(III)alamin (**2**) was dissolved in a buffered aqueous solution (pH 6.8) containing a nearly equimolar amount of (1*H*-imidazol-1-yl)cob(I)amide (**6**$^-$), prepared electrochemically and identified by a UV/VIS spectrum of the electrolysis product (see *Fig. 12* in the *Exper. Part*). The mixture was left to stand at room temperature for 15 min (with protection from light and air), after which time a

*Scheme 4*




**2** **6**$^-$ **11**$^-$ **4**

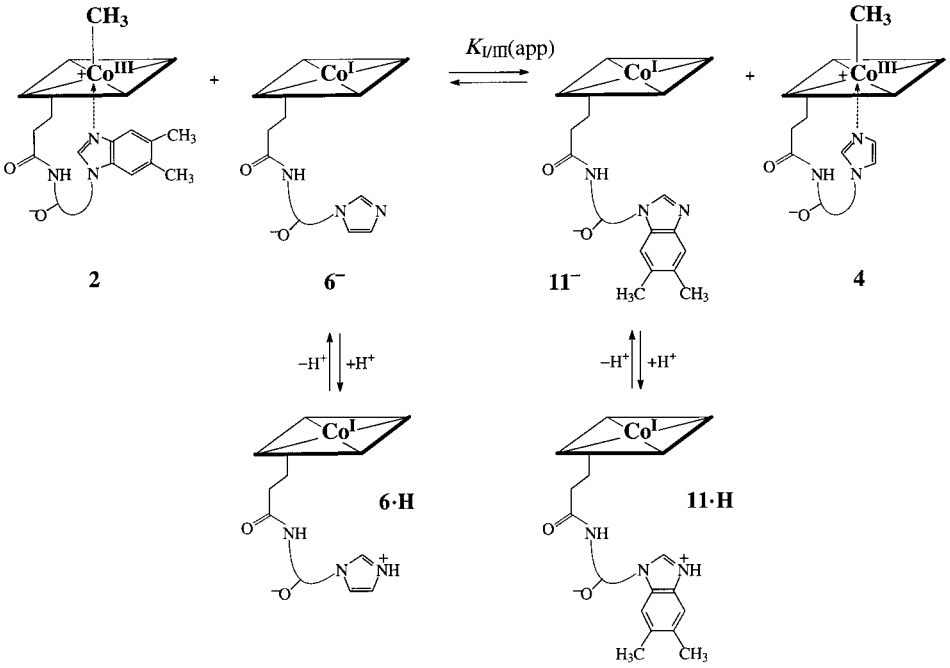
sample was analyzed by UV/VIS spectroscopy. The UV/VIS spectrum showed absorbance characteristics of a mixture of cob(I)amides and of alkylcob(III)amides. Thus, the flask containing the reaction mixture was tightly stoppered and taken out of the glove box. Injection of 0.5 ml of $O_2$-saturated 1% HCN in MeOH into the flask rapidly oxidized the mixture, which then consisted of significant amounts of all of the four corrinoids **2**–**5** (according to TLC). The $^1$H-NMR analysis (500 MHz) established the presence of the corrinoids **2**–**5** in a ratio of 1.2 : 1.04 : 1.0 : 1.1, corresponding to a ratio of products of $0.80 \pm 0.2$ (see *Table*).

Likewise, a buffered aqueous solution of cob(I)alamin (**11**$^-$), prepared electrochemically, was added to a buffered aqueous solution (pH 6.8) of an equimolar amount of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcob(III)amide (**4**). The mixture was left to stand at room temperature for 30 min (with protection from light and air), and then analyzed and worked up as described in the preceding paragraph. The reaction mixture again contained significant amounts of all of the four corrinoids **2**–**5** (according to TLC), and the $^1$H-NMR spectrum (500 MHz) indicated the corrinoids **2**–**5** to be present in a ratio of 1.3 : 1.05 : 1.7 : 1.3, corresponding to a ratio of the products of $1.05 \pm 0.2$ (see *Table*). Based on earlier experiences with the analysis of Me-transfer mixtures [44], significant equilibration during the oxidative quenching process can be assumed to be unlikely. Accordingly, the contents of the equilibrating Me–Co$^{III}$ and Co$^{I}$ corrinoids would be reflected by the ratios of the four cobamides **2**–**5**, and the apparent equilibrium constant for the equation given in *Scheme 4* could be estimated as $K_{I/III}(app) = 0.9 \pm 0.1$. These results appear to suggest an unexpectedly similar stabilization of methylcob(III)alamin (**2**) and of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcob(III)amide (**4**) by the intramolecular coordination of their nucleotide bases Dmb or Im, respectively.

However, from an earlier comparison of the p$K_a$ of **2**·**H**$^+$ (=2.9) with the p$K_a$ of protonated $\alpha$-ribazole (5.56 at 25°), the equilibrium constant for the formation of 'base-on' methylcob(III)alamin (**2**) was deduced to amount to *ca.* 460 (at 25°) [50]. Indeed, the size of $K_{I/III}(app)$ similarly suggests the equilibrium constant for the formation of base-on *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcobamide **4** to be roughly 500 too (at room temperature). Accordingly, the p$K_a$ of protonated 1-($\alpha$-D-ribofuranosyl)-1*H*-imidazole would be indicated to exceed the p$K_a$ of **4**·**H**$^+$ similarly and to amount to *ca.*

7.0, at least. Indeed, such acid/base characteristics of 1-(α-D-ribofuranosyl)-1*H*-imidazole or of the imidazolylcob(I)amide **6**$^-$ would be consistent with the higher acidity of protonated Dmb ($pK_a = 6.0$ [57]) than of protonated 1*H*-imidazole ($pK_a = 7.0$ [58]). However, assuming the acidity of the protonated Im base of the base-off imidazolyl cobamides to be similar to that of the protonated 1-(α-D-ribofuranosyl)-1*H*-imidazole, a significant fraction of the imidazolylcob(I)amide **6**$^-$ would be expected to be protonated at the solution pH of 6.8, and the protonated form of the imidazolylcob(I)amide, *i.e.*, **6**·**H**, would also have to be taken into consideration (see *Scheme 5*).

*Scheme 5*




Indeed, the product ratios of the four cobamides **2**–**5**, therefore, provide the apparent equilibrium constant $K_{I/III}(app)$, as given in *Eqn. 4*.

The product ratios represent, in fact, a measure of the extent of Me-group equilibration between (all of) the cobalamins and the imidazolylcobamides present in solution at a given pH (near neutral). Accordingly, near neutral pH $K_{I/III}(app)$ can be correlated with $K_{I/III}$ by taking into account the pH-dependent presence of **6**·**H** (and of **11**·**H**). With $pK_a$ (**11**·**H**) < $pK_a$ (**6**·**H**) ($pK_a$ (**11**·**H**) *ca.* 5.6 [47]), an approximate correlation between $K_{I/III}(app)$ and $K_{I/III}$ for the case of equilibria at neutral pH or slightly basic solutions (where [**11**·**H**] is neglected) is given by *Eqn. 5*.

$$K_{I/III}(app) = (\{[\mathbf{11}^-] + [\mathbf{11}\cdot\mathbf{H}]\}[\mathbf{4}])/([\mathbf{2}]\{[\mathbf{6}^-] + [\mathbf{6}\cdot\mathbf{H}]\}) \quad (4)$$

$$K_{I/III} = K_{I/III}(app)\cdot\{1 + 10^{[pK_a(\mathbf{6}\cdot\mathbf{H}) - pH]}\} \quad (5)$$

*Eqn. 5* shows that $K_{I/III}$ exceeds $K_{I/III}$(app), but $K_{I/III} \lesssim 2\ K_{I/III}$(app) at pH values close or slightly above the p$K_a$ of **6**·**H**. However, *Eqn. 5* also indicates $K_{I/III}$ to exceed $K_{I/III}$(app) more significantly at pH values in the range between p$K_a$ (**11**·**H**) and p$K_a$ (**6**·**H**). Accordingly, protonation of the axial base of the cob(I)amide **6**$^-$ near neutral pH is due to assisting (thermodynamically) the demethylation of the *Co*$\alpha$-imidazolyl-*Co*$\beta$-methylcob(III)amide **4** by nucleophiles. To explore the effects from (partial) protonation of the imidazolylcob(I)amide **6**$^-$ under the conditions of the Me-group-transfer equilibria, further experiments at various (higher) solution pH values are, therefore, needed and are currently in progress.

Our Me-group-transfer experiments indicated the Co-bound Me group to be similarly deactivated against nucleophilic abstraction by the intramolecular coordination of the Dmb base of **2**, as by the Im base of **4**. Accordingly, the substitution of the Dmb base by a histidine residue, as observed in MetH and as indicated in other Me-group transferases [24–26][59], is not likely to result in an inherently altered reactivity towards nucleophilic heterolysis of the Co–C-bond. However, the estimated p$K_a$ value for protonated 1-($\alpha$-D-ribofuranosyl)-1*H*-imidazole is close to 7 and is significantly more positive than those of protonated $\alpha$-ribazole. The higher p$K_a$ values of protonated imidazole bases, when compared to Dmb and other natural nucleotide bases, are likely to be very significant from the enzymological point of view: protonation/deprotonation steps involving the His-Asp pair of the regulatory 'ligand triad' were observed to accompany both the enzymatic heterolytic demethylation/methylation reactions of MetH near neutral pH, as well as the one-electron redox reactions involving protein-bound cob(II)alamin (**10**) and cob(I)alamin (**11**$^-$) [26]. Protonation/deprotonation steps of protein-bound methylcorrinoids in their 'base-off/His-on' constitution thus control and tune the thermodynamics of the Me- and electron-transfer reactions of MetH and of other methyl transferases. Clearly, in methyl transferases, the substitution of the Dmb base (and of other nucleotide bases, such as adenines) of the naturally occurring complete corrinoids by the more basic imidazole base (of His in the 'ligand triad') thus contributes significantly to the accessibility of the crucial protonation/deprotonation steps at physiological pH. On the other hand, it is far from being understood, to what extent the more basic character of Im (compared to Dmb) and the associated protonation/deprotonation steps would contribute to the function of the protein-bound adenosyl-cobamides in the coenzyme-$B_{12}$-dependent mutases.

2.5. *Crystal-Structure Analysis of* Co$\alpha$-*(1*H-*Imidazol-1-yl)*-Co$\beta$-*methylcob(III)amide* (**4**). Crystals of **4** were grown from an aqueous acetone solution at 5°, and X-ray diffraction data were collected at cryotemperature (100 K) at one of the EMBL beamlines at the DESY synchrotron in Hamburg, Germany. Structure refinement against diffraction data extending to a crystallographic resolution of 1.0 Å converged at a residual of $R = 0.0871$ für all 9011 symmetry-independent reflections. Projections of the $B_{12}$ moiety of the crystal structure of **4** are shown in *Fig. 8*.

A superposition of the crystal structure of **4** with the $B_{12}$ cofactors observed in the 'base-off/His-on' proteins [24][27][28] reveals the imidazole ring of **4** to occur in a very similar conformation ('north-south' orientation of the plane of the imidazole ring) with respect to the corrin ring as the protein-derived imidazole of the protein-bound $B_{12}$ cofactors (*Figs. 9* and *10*). The distance between the Co-center and the axially coordinated 1*H*-imidazole N-atom of **4** is 2.09 Å, which is 0.10 Å shorter than the

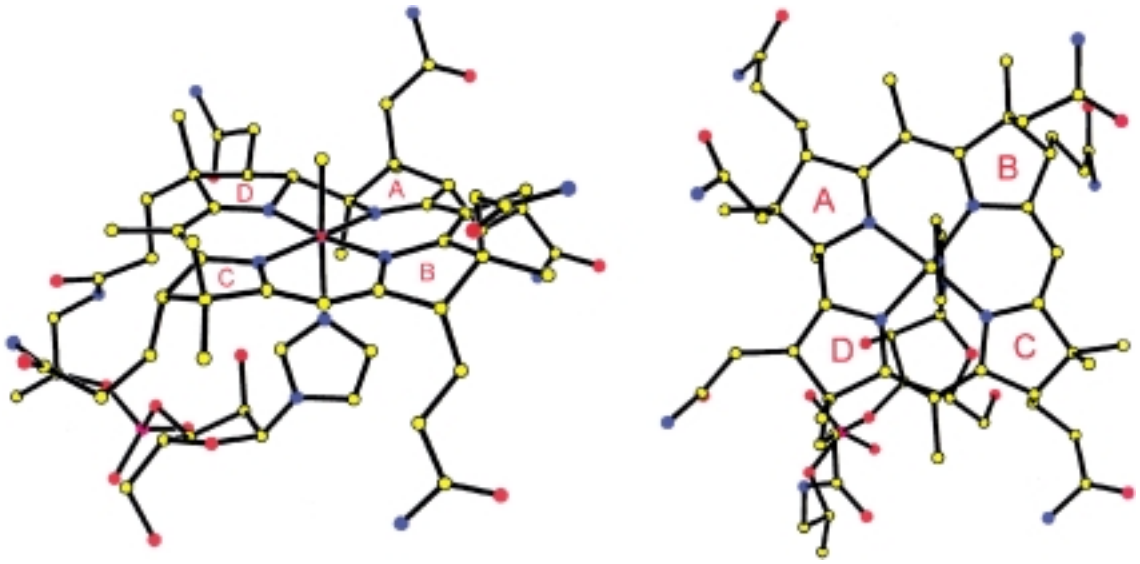



Fig. 8. *Two projections of the crystal structure of* Co*α-(1*H*-imidazol-1-yl)*-Co*β-methylcob(III)amide* (**4**)

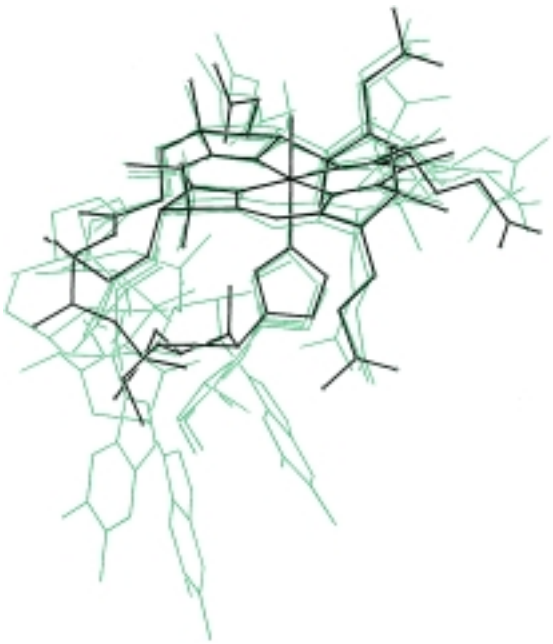
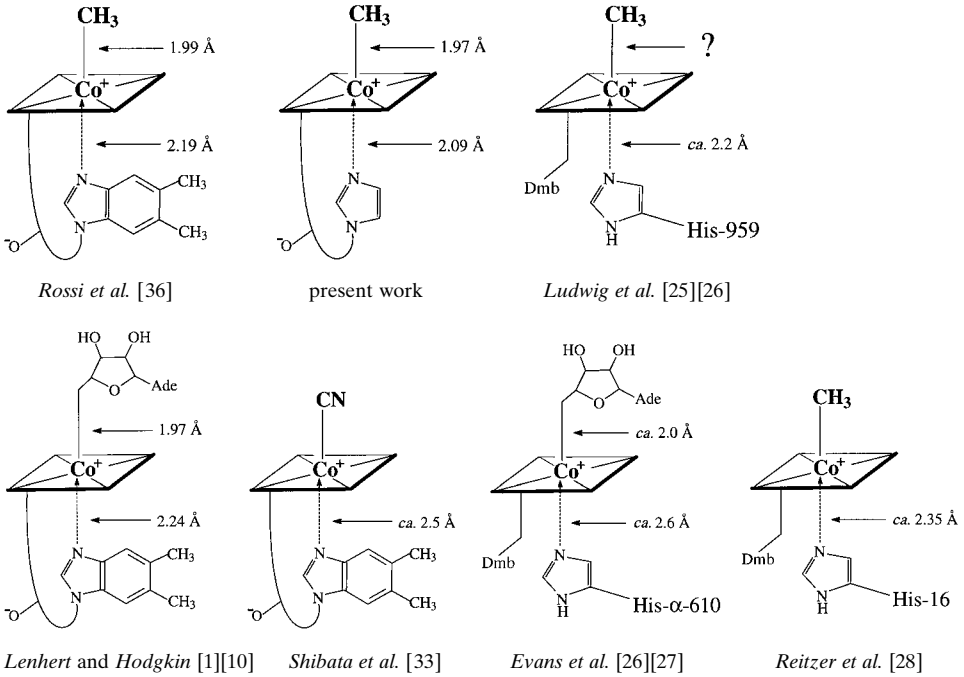


Fig. 9. *Structure superposition of the* $B_{12}$ *cofactors obtained from the crystal structures of methylmalonyl-CoA mutase* (MCM), *glutamate mutase* (Glm), *and of the* $B_{12}$*-binding domain of methionine synthase* (MetH; green), *with that of* Co*α-(1*H*-imidazol-1-yl)*-Co*β-methylcob(III)amide* (**4**; black)

Fig. 10. *Structure superposition of the corrin ligands of* Co*α-(1*H*-imidazol-1-yl)*-Co*β-methylcob(III)amide* (**4**; black) *with those of the $B_{12}$ cofactors obtained from the crystal structures of methylmalonyl-CoA mutase* (MCM), *glutamate mutase* (Glm), *and of the $B_{12}$-binding domain of methionine synthase* (MetH; green)

corresponding distance to the Dmb N-atom in the crystal structure of methylcob(III)alamin (**2**) [36]. This 'shortening' of the axial Co–N bond as a consequence of the replacement of Dmb by Im underpins the similar observation in the crystal structure of **5** [46]. The shorter bond in **4** (and in **5**) correlates with the smaller size and the higher nucleophilicity of 1*H*-imidazole as compared to Dmb ($pK_a(Im \cdot H^+) = 7.0$ [57], $pK_a(Dmb \cdot H^+) = 6.0$ [58]), and $pK_a(\alpha\text{-ribazole} \cdot H^+) = 5.6$ [60]).




*Rossi et al.* [36] present work *Ludwig et al.* [25][26]

*Lenhert* and *Hodgkin* [1][10] *Shibata et al.* [33] *Evans et al.* [26][27] *Reitzer et al.* [28]

Fig. 11. a) *Comparison of axial bond lengths of crystalline methyl-corrinoids and of methylcob(III)alamin bound 'base-off/His-on' in methionine synthase from* E. coli [24–26]. b) *Comparison of axial bond lengths of crystalline coenzyme $B_{12}$ and of corrinoid cofactors bound in methylmalonyl-CoA mutase from* P. shermanii [26][27], *in glutamate mutase from* C. cochlearium [28], *and in diol dehydratase* [33].

The length of the Co–$N_{ax}$ bond in the crystal structure of **4** (2.09 Å) is thus shorter than the corresponding bond to the protein-derived 1*H*-imidazole moiety in proteins with a methylcob(III)alamin (**2**) [24] or (5′-deoxy-5′-adenosyl)cob(III)alamin (**1**) cofactor (see *Fig. 11*) [27][28][37]. These data appear to suggest that protein-bound methylcob(III)alamin (**2**) in the 'base-off/His-on' constitution of methionine synthase has a strained Co–$N_{ax}$ bond, since the crystallographically observed bond length was reported to be 2.2 Å. However, the experimental observation has to be taken with caution since the crystal structure of MetH is of relatively low resolution (2.4–8.0 Å) [24][26], and it is conceivable that, during the crystallographic refinement, the bond length was constrained to the value (2.2 Å) observed in the structure of methylcob(III)alamin (**2**). Nevertheless, the presence of such a stressed Co–$N_{ax}$ bond would favor the cleavage of both axial bonds to the $Co^{III}$ center. This may be necessary during the catalytic cycle of this enzyme (and of other methyl transferases), which involves heterolytic release of the *Coβ*-methyl group to be transferred to a nucleophile. Such a heterolytic Co–C bond cleavage results in a (tetracoordinated) $Co^{I}$ metal center.

An important and frequently cited structural parameter concerns the conformation of the corrin ligand, whose most pronounced deviation from planarity involves a 'folding' of the corrin ring about an axis running 'east-to-west', *i.e.*, through the *meso*-atom C(10) and intersecting the C(1)–C(19) bond [61]. This deformation was termed 'upward folding', and it was suggested to be involved in enzyme-assisted Co–C homolysis (for a discussion, see, *e.g.*, [55]). The deformation is measured as the dihedral angle between best planes through rings A + B and C + D, respectively. Observation of a negative correlation of this parameter with the length of the Co–$N_{ax}$ bond in crystal structures of cobalamins led to the conclusion that large upward folding is primarily the result of steric repulsion between the Dmb base and the lower face of the corrin ring [11]. This view was initiated by the observation of a small fold angle (10.8°) in the crystal structure of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-cyanocob(III)amide (**5**) [46]. In the crystal structure of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcob(III)amide (**4**), the observed upward folding is 12.5°, *ca.* 2° smaller than in the structure of methylcob(III)alamin (**2**) (14.6°) [36]. Lack of resolution of presently available crystallographic evidence of proteins with a $B_{12}$ cofactor [24][27][28][33] prevents a detailed appraisal of the relevance of the cofactor's 'upward folding' for the catalysis of the corresponding enzymes.

The observed elongation of the Co–$N_{ax}$ bond (*ca.* 2.5 Å) of the protein-bound coenzyme $B_{12}$ (**1**) in the crystal structure of methylmalonyl-CoA mutase, on the other hand, is experimentally highly significant. Therefore, this bond was considered as strained [27]. Likewise, the value observed for the length of this bond in the crystal structure of glutamate mutase (2.27–2.35 Å) is significantly longer than the value observed for crystalline **5**. In view of recent XAS evidence [38][62], it remains to be shown whether and how such a strained Co–$N_{ax}$ bond can be linked to activation towards Co–C bond homolysis in this class of coenzyme-$B_{12}$-dependent mutases.

**3. Conclusions.** – In the present work, we studied some main patterns of the organometallic reactivity of (1*H*-imidazol-1-yl)cobamides and the details of the crystal structure of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-methylcobamide (**4**), considered to be a model for protein-bound (organometallic) corrin(III)cofactors. This work was

intended to provide insights into relevant effects of the substitution of the Dmb base of the cobalamins by an imidazole moiety, as observed (in the 'ligand triad') in several $B_{12}$-dependent proteins. First of all, these studies supported the view that the observed 'long' axial Co–N bonds of the Co-coordinated histidine in MetH [26], in the C-skeleton mutases MCM [26][27] and Glm [28] would represent stretched Co–N bonds, provided that these observations do not represent experimental artifacts [39]. The long axial Co–N bond has been suggested to contribute in a crucial way to the activation of the corrin-bound Co-center of the $B_{12}$ cofactors, relevant for the initiation of the radical reactions [26][27]. One of us has raised the alternative that the 'stretched Co–N bond', if crystallographically significant, may not mainly play a role in the activation of the bound (5′-deoxy-5′-adenosyl)cobamides towards homolysis of their Co–C bond, but may be relevant for a later stage of the enzymatic radical rearrangement reactions [2]. The studies presented here also provide further experimental insights into the importance of the inherent basicity of the imidazole moieties of the His-Asp part of the 'ligand triad', of particular relevance in enzymatic Me- and electron-transfer reactions that are accompanied by the coordination/decoordination of a N-ligand at the corrin-bound Co-center.

## Experimental Part

1. *General*. Materials. Methylcob(III)alamin (**2**), prepared as described in [48]. Crystalline cyanocobalamin **3**, supplied by *Hoffmann-La Roche & Co. Ltd.*; aquacobalamin chloride **8**·**Cl**, *Roussel Uclaf*, water (purified using *Epure*, *Barnstead Co.*); acetone, Hg, $(Bu_4N)PF_6$, MeI, AcOH, KCl, KCN, $KH_2PO_4$, $K_2HPO_4$, NaOAc, aq. HCl soln., all *Fluka puriss. p.a.,* or *Fluka MicroSelect*; $Me_3CN$, $ClCH_2COOH$, HCOOH, benzoic acid, *Fluka purum*; $H_3PO_4$ crystallized *puriss.*; MeOH, $CH_2Cl_2$ redistilled; 1% HCN in MeOH [63]. $O_2$-Sensitive manipulations were done in a glove box (*Mecaplex GB-80*, $< 15$ ppm $O_2$). The electrochemical syntheses were carried out in an electrolysis cell with two compartments, separated by a medium porosity glass frit; Hg-pool working electrode, Pt-wire counter electrode, 0.1N calomel electrode (0.1N CE) as reference electrode; potentiostat *Amel 550*. pH values were determined with a *WTW SenTix 41* electrode connected to a *WTW inoLab digital pH meter*. Reaction mixtures were desalted with *Waters Sep-Pak Classic RP-18 cartridges*. TLC: *RP-18 F254s* TLC plates 0.25 mm (*Merck* No. 115389). UV/VIS Spectra: *Hitachi-U3000;* $\lambda_{max}$ (log $\varepsilon$) in nm. CD Spectra: *Jasco J715;* $\lambda_{max}$ or $\lambda_{min}$ ($\Delta\varepsilon$) in nm. $^1$H-NMR Spectra: *Varian Unity 500plus;* $\delta$(H) in ppm referenced to HOD (=4.66). FAB-MS: *Finnigan MAT 95S*, positive-ion mode; 3-nitrobenzyl alcohol matrix; Cs gun.

2. *Syntheses*. Co*α*-(1*H-Imidazol-1-yl)*-Co*β-methylcob(III)amide* (**4**). In a glove box ($<10$ ppm $O_2$), 18 mg (14.1 μmol) of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-cyanocob(III)amide (**5**) and 2.2 mg (18.3 μmol) of benzoic acid were dissolved in 4 ml of 0.1M $Bu_4N(PF_6)$/MeOH. The red soln. was placed into the cathode chamber of a two-compartment electrolysis cell and was stirred magnetically. It was reduced at a Hg electrode at a potential of $-1.1$ V *vs.* a 0.1N CE reference (at r.t.) for 4.5 h, when 2.44 Coulombs were consumed. To the resulting green soln. of the (1*H*-imidazol-1-yl)cob(I)amide, 6.9 μl (140.9 μmol) of MeI were added under protection from light, while the cathode was kept at the same potential. After 140 min, the mixture was transferred into a separatory funnel (with protection from light), that contained 5 ml of $H_2O$. The dark red mixture was extracted with $CH_2Cl_2$ ($3 \times 5$ ml), the aq. phase evaporated, and the dried residue (18.8 mg) taken up in 0.1 ml of $H_2O$. Acetone was added dropwise, until a slight turbidity appeared. After storage in a refrigerator overnight, 16 mg (89.7%) of crystalline **4** was obtained. TLC: uniform: UV/VIS ($c = 8.69 \cdot 10^{-4}$M, $H_2O$): 268(4.16), 314(4.07), 341(4.11), 376(4.07), 528(3.91); *Fig. 3*. CD ($c = 8.69 \cdot 10^{-4}$M, $H_2O$): 219 (37143), 258 (−45322), 284 (4314), 314 (−27490), 337 (−2511), 358 (−18218), 398 (13483), 417 (6401), 477 (37432), 564 (−11228). $^1$H-NMR (pH 5, $H_2O$ (600 μl)/$D_2O$ (100 μl), 500 MHz; *Fig. 4*): −0.21 (*s*, *β*-Me–Co); 0.49 (*s*, Me–C(1)); 0.82 (*s*, 3 H); 1.06 (*d*, $J = 6.6$, 3 H); 1.17 (*s*, 3 H); 1.28 (*s*, 3 H); 1.29 (*s*, 3 H); 1.36 (*m*, 1 H); 1.59 (*s*, 3 H); 1.64 (*m*, 1 H); 1.71 (*m*, 2 H); 1.79 (*m*, 1 H); 1.89 (*m*, 2 H); 1.93 (*m*, 2 H); 1.97 (*m*, 1 H); 2.04 (*m*, 1 H); 2.16 (*m*, 1 H); 2.18 (*s*, 3 H); 2.24 (*s*, 3 H); 2.30 (*m*, 1 H); 2.33 (*m*, 2 H); 2.35, 2.37 (*m*, 4 H); 2.55 (*m*, 2 H); 2.91 (*t*, $J = 5.9$, 1 H); 3.04 (*dt*, $J = 14.4$, 7.3, 1 H); 3.31 (*dd*, $J = 10.3$, 4.6, 1 H); 3.40 (*dd*, $J = 14.4$, 1.9, 1 H); 3.56 (*dd*, $J = 12.7$, 4.2, 1 H); 3.71 (*dd*, $J = 12.7$, 2.5, 1 H); 3.74 (*d*,

$J = 9.4$, 1 H); 3.81 (*d*, $J = 8.8$, 1 H); 3.88 (*dt*, $J = 8.1, 2.9$, 1 H); 4.12 (*dd*, $J = 4.0$, 1 H); 4.19 (*dq*, $J = 6.8, 1.9$, 1 H); 4.47 (*dt*, $J = 7.9, 4.6$, 1 H); 5.76 (*s*, 1 H); 7.78 (*d*, $J = 3.55$, 1 H); 5.80 (*s*, 1 H); 6.61 (br. *s*, 1 H); 6.68 (*s*, 1 H); 6.71 (*s*, 1 H); 6.76 (br. *s*, 2 H); 6.79 (br. *s*, 1 H); 6.92 (br. *s*, 1 H); 6.93 (br. *s*, 1 H); 7.06 (br. *s*, 1 H); 7.46 (br. *s*, 1 H); 7.49 (br. *s*, 1 H); 7.52 (br. *s*, 1 H); 7.62 (br. *s*, 1 H); 7.76 (br. *s*, 1 H); 8.03 (*t*, $J = 6.1$, 1 H). FAB-MS: 1268.8 (32), 1267.8 (61), 1266.8 (100, $[M+H]^+$); 1253.7 (26), 1252.7 (55), 1251.7 (82, $[M+H-CH_3]^+$).

Co*α*-*(1*H-*Imidazol-1-yl)*-Co*β*-*aquacob(III)amide* (**7**$^+$). A soln of **4** (6.0 mg, 4.5 μmol) in $H_2O$ (4 ml) was saturated with $O_2$ and irradiated with light (500 W) for 50 min. The $H_2O$ was evaporated, the residue taken up in a minimal amount of $H_2O$, and the product precipitated by diffusion of acetone vapor into the aq. soln. at r.t. The precipitate was washed with acetone (3×): 5.1 mg (85%) of pure *Coα*-(1*H*-imidazole-1-yl) *Coβ*-hydroxocob-(III)amide. TLC (*RP18*, MeCN/$H_2O$ 3 :7): $R_f$ 0.05). UV/VIS (0.02M phosphate buffer pH 6.3; rel. *ε*; *Fig. 5*): 273 (0.61), 355 (1.00), 410 (0.16), 532 (0.35). $^1$H-NMR (9 mM aq. phosphate, pH 6.8): 0.66 (*s*, Me–C(1)); 1.12 (*s*, 3 H); 1.13 (*s*, 3 H); 1.20 (*s*, 3 H); 1.26 (*s*, 3 H); 1.42 (*s*, 6 H); 1.71 (*s*, 3 H); 2.37 (*s*, 3 H) superimposed by 1.1–3.0 (various *m*, in total *ca.* 86 H), 3.2–4.2 (*m*, *ca.* 13 H); 4.43 (*m*, H–C(3′)(Rib)); 5.44 (*s*, 1 H); 5.71 (*s*, 1 H); 6.16 (2*s*, 2 H); 6.69 (*s*, H–C(5) (Im)).

3. *Determination of the p*$K_a$ **4**·**H**$^+$. Aq. solns. of **4** ($8 \cdot 10^{-4}$M), 0.1M buffer (HCl, chloroacetate, acetate, formate, or phosphate) and KCl (total ionic strength 1.0M) were prepared (with pH values 7.76, 6.50, 5.65, 4.48, 3.84, 3.24, 2.93, 2.10, and 0.30). Absorbance spectra of these solns. obtained at r.t. showed isosbestic points at 498, 388, and 334 nm (see *Fig. 6*). The spectral changes at 538, 455, and 304 nm were analyzed according to *Eqn. 6* by the method of least squares, where $A_x$ is the absorbance at a given wavelength of the solution at $pH_x$, and $A_{AH}$ and $A_{A^-}$ are the corresponding absorbances of **4**·**H**$^+$ and **4**, determined (in duplicate) at the titration end points. The intercepts resulting from these fits of the data determined at 538, 455, and 304 nm, resp., were 4.22, 4.33, and 4.35, yielding a value of $4.3 \pm 0.1$ for $pK_a$ (**4**·**H**$^+$).

$$pH_x = pK_a + \log(|A_x - A_{AH}| / |A_{A^-} - A_x|) \quad (6)$$

4. *Methyl-Transfer Experiments.* 4.1 *Equilibration Experiments with Methylcob(III)amides and Cob(III)-amides (Scheme 2)*. The reactions were carried out in stoppered NMR tubes in 9 mM aq. phosphate buffers (10% $D_2O$), pH 6.8, and monitored directly by $^1$H-NMR. Reaction mixture A was produced by dissolving *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-hydroxocob(III)amide (4.5 mg, 3.55 μmol) and methylcob(III)alamin (4.4 mg, 3.27 μmol; **2**) in aq. phosphate (0.7 ml). Reaction mixture B was prepared likewise by dissolving **4** (3.5 mg, 2.77 μmol) and aquacob(III)alamin chloride (5.3 mg, 3.83 μmol; **8**·**Cl**). Two hours after the preparation, the 500-MHz $^1$H-NMR revealed significant changes in mixture B and only small changes in mixture A. Further NMR spectra were recorded after 2, 8, 22, and 30 days. Before the final analysis, the equilibrated mixtures were treated each with aq. KCN (0.5 mg, *ca.* 8 μmol) soln. yielding mixtures of *Coβ*-methyl- and *Coβ*-cyanocob(III)amides. The solvent was evaporated at r.t., the residue of both mixtures dissolved in $D_2O$, and the $^1$H-NMR recorded (see *Table*).

4.2. *Equilibration Experiments with Methylcob(III)amides and Cob(II)amides* (*Scheme 3*). By electrochemical one-electron reduction of *Coα*-(1*H*-imidazol-1-yl)-*Coβ*-cyanocob(III)amide (3.6 mg, 2.8 μmol; **5**) in 6 ml of a buffered $O_2$-free aq. soln. (0.01M phosphate buffer, pH 6.8; 0.1M KCl) at $-1.0$ V *vs.* 0.1N CE, a buffered aq. soln. of 2.8 μmol of (1*H*-imidazol-1-yl)cob(II)amide (**9**) was prepared (UV/VIS (**9** in $H_2O$; rel. *ε*): 264 (0.75), 312 (1.00), 473 (0.38)). The extent of the reduction was monitored coulometrically, and the UV/VIS of the reduced reaction mixture exhibited the absorbance characteristics of a base-on cob(II)amide [58]. The soln. of **9** was then mixed (with protection from light and air) with 0.5 ml of an aq. soln. of **2** (3.7 mg, 2.8 μmol), buffered similarly (0.01M phosphate, pH 6.8; 0.1M KCl), and left to stand at r.t. (with protection from light and air). After an equilibration time of 2 h, the stoppered reaction flask was taken out of the glove box and $O_2$-sat. 1% HCN/MeOH (0.5 ml) was injected (with protection from light). For further analysis, the mixture was first desalted by filtration through an *RP-18* cartridge and then examined by TLC (4 spots of **2**–**5**) and by $^1$H-NMR (500 MHz) (see *Table*).

Likewise, by electrochemical one-electron reduction of vitamin $B_{12}$ (**3**; 14 mg, 10.1 μmol) in 4 ml of a buffered $O_2$-free aq. soln. (0.01M phosphate buffer, pH 6.8; 0.1M KCl), a buffered aq. soln. of cob(II)alamin (**10**) was prepared (coulometrical monitoring; UV/VIS base-on cob(II)alamin [58]; see above). An aliquot of 1 ml of the soln. (containing 2.8 μmol) of **10** was then mixed (with protection from light and air) with 0.5 ml of an aq. soln. of **4** (3.5 mg, 2.8 μmol), buffered similarly (0.01M phosphate, pH 6.8; 0.1M KCl). The mixture was left to stand for 2 h at r.t. (with protection from light and air). The stoppered flask was taken out of the glove box, treated with $O_2$-sat. 1% HCN/MeOH, desalted, and analyzed by TLC (4 spots of **2**–**5**) and $^1$H-NMR as described above (see *Table*).

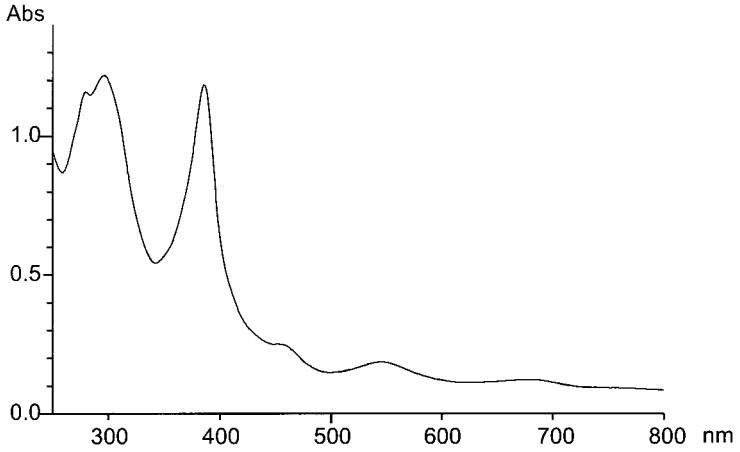



Fig. 12. *UV/VIS Spectrum of electrochemically produced (1*H*-imidazol-1-yl)cob(I)amide* (**6**$^-$) *in aqueous solution*

4.3. *Equilibration Experiments with Methylcob(III)amides and Cob(I)amides* (*Scheme 4*). By electrochemical reduction at $-1.1$ V *vs.* 0.1N CE of **5** (3.5 mg, 2.7 μmol) in 5 ml of a buffered $O_2$-free aq. soln., a buffered aq. soln. of 2.7 μmol of (1*H*-imidazol-1-yl)cob(I)amide (**6**$^-$) was prepared. (UV/VIS (**6**$^-$ in $H_2O$, rel. $\varepsilon$): 280 (0.95), 296 (1.00), 386 (0.97), 453 (sh, 0.21), 546 (0.16); see *Fig. 12*). The extent of the reduction was monitored amperometrically and UV/VIS-spectroscopically [50]. To the soln. of **6**$^-$, crystalline **2** (3.4 mg, 2.5 μmol) was added (glove box, protection from light). The mixture was left to stand at r.t. for 15 min (with protection from light and air). UV/VIS Analysis indicated the presence of a mixture of cob(I)amides and alkylcob(III)amides. The mixture was then taken out of the glove box, treated with $O_2$-sat. 1% HCN/MeOH, desalted, and analyzed by TLC (significant amounts of all **2**–**5** and $^1$H-NMR as described in *Exper. 4.2* (see *Table*).

Likewise, by electrochemical reduction at $-1.1$ V *vs.* 0.1N CE of vitamin $B_{12}$ (14 mg, 10.3 μmol; **3**) in 4 ml of a buffered $O_2$-free aq. soln., a buffered aq. soln. of 10.3 μmol of cob(I)alamin (**11**$^-$) was prepared. The extent of the reduction was monitored amperometrically and UV/VIS-spectroscopically [56]. One fourth of the soln. of **11**$^-$ (ca. 2.7 μmol) were added (glove box, protection from light) to a soln. of crystalline **4** (3.5 mg, 2.8 μmol) in 0.5 ml of 10 mM phosphate buffer (pH 6.8). The mixture was left to stand at r.t. for 30 min (with protection from light and air). UV/VIS Analysis indicated the presence of a mixture of cob(I)amides and alkylcob(III)amides. The mixture was then taken out of the glove box, treated with $O_2$-sat. 1% HCN/MeOH, desalted, and analyzed by TLC (significant amounts of all **2**–**5**) and $^1$H-NMR (see *Table*).

5. *Crystal-Structure Analysis of* Co*α*-(*1*H-*Imidazol-1-yl)*-Co*β*-*methylcob(III)amide* (**4**). Crystals were grown from $H_2O$/acetone under exclusion of light. A crystal of approximate dimensions $0.2 \times 0.1 \times 0.1$ mm was removed from its mother liquor and immersed in a drop of hydrocarbon oil. After removing adhering mother liquor from the surface of the crystal with a strip of filter paper, the crystal was picked up with a glass fiber and shock-cooled by dumping into liq. $N_2$ [46]. Low-temp. (92 K) intensity data ($\lambda$ 0.9076 Å) were collected at the EMBL beamline X11 at DESY in Hamburg, Germany, using a MAR345 image-plate goniometer. The images were integrated with the program DENZO [64], subsequent scaling steps were performed with the CCP4 [65] program suite, yielding 17820 reflections ($2\theta_{max} = 54°$, resolution limit 1.0 Å), of which 9011 symmetry-independent ($R_{int} = 0.0307$). Space group $P2_12_12_1$, $a = 15.95(1)$, $b = 22.10(1)$, $c = 25.92(1)$ Å. The structure was solved with direct methods and refined against $F^2$ quantities by full-matrix least squares. $C_{57}H_{83}N_{13}O_{14}$ plus 16 partly disordered solvent molecules; $\mu = 0.28$ mm$^{-1}$; $d_x = 1.111$ g cm$^{-3}$; $R = 0.0857$ for 8713 $F_o > 4\sigma(F_o)$ and 0.0871 for all 9011 reflexions (155 restraints); $wR_2 = 0.2542$. The following computer programs were used: SHELXTL Version 5 [66]; SHELXL-93 [67]. Crystallographic data (excluding structure factors) have been deposited with the *Cambridge Crystallographic Data Centre* as supplementary publication no. CCDC 144874.

Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033, e-mail: deposit@ccdc.cam.ac.uk).

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