The Hydrogenobyric Acid Structure Reveals the Corrin Ligand as an Entatic State Module Empowering \( \text{B}_{12} \) Cofactors for Catalysis

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Dedicated to Professor Albert Eschenmoser on the occasion of his 94th birthday

Abstract: The \( \text{B}_{12} \) cofactors instill a natural curiosity regarding the primordial selection and evolution of their corrin ligand. Surprisingly, this important natural macrocycle has evaded molecular scrutiny, and its specific role in predisposing the incarcerated cobalt ion for organometallic catalysis has remained obscure. Herein, we report the biosynthesis of the cobalt-free \( \text{B}_{12} \) corrin moiety, hydrogenobyric acid (\( \text{Hby} \)), a compound crafted through pathway redesign. Detailed insights from single-crystal X-ray and solution structures of \( \text{Hby} \) have revealed a distorted helical cavity, redefining the pattern for binding cobalt ions. Consequently, the corrin ligand coordinates cobalt ions in desymmetrized “entatic” states, thereby promoting the activation of \( \text{B}_{12} \) cofactors for their challenging chemical transitions. The availability of \( \text{Hby} \) also provides a route to the synthesis of transition metal analogues of \( \text{B}_{12} \).

The unique structural[1] and biosynthetic features[2] of coenzyme \( \text{B}_{12} \) and its biological homologues raise fundamental questions concerning the evolution and selection of the corrin ligand[3] as well as the adoption of \( \text{B}_{12} \) cofactors into key metabolic roles across the three domains of life. The combined selection of the corrin macrocycle and of cobalt as the specific transition metal center for bio-organometallic catalysis is an intriguing aspect of the \( \text{B}_{12} \) cofactors[4]. The resistance of cobalt corrins against the removal of cobalt without concomitant destruction of the corrin ligand[5] has made a study of cobalt-free natural corrins a major scientific challenge.[6] Consequently, despite the 40 years since vitamin \( \text{B}_{12} \) was prepared by total synthesis,[7] the special partnership of the ligand and the cobalt ion of the natural \( \text{B}_{12} \) cofactors remains largely unexplored.[8]

Two pathways for \( \text{B}_{12} \) biosynthesis have highlighted intriguing “ring contraction” steps[2][9] that tailor the “coordination hole” of the tetrapyrrole macrocycle to the effective size of cobalt ions.[6][8] Surprisingly, \( \text{B}_{12} \)’s own ligand, hydrogenobyric acid (\( \text{Hby} \)) (Figure 1), is not a biosynthetic intermediate in either of them.[2] However, metabolic engineering of the \( \text{B}_{12} \) biosynthetic pathway has allowed the development of strategies to access metal-free corrins by design.[9,10] We recently reported recombinant \( \text{E. coli} \) strains that generated metal-free corrins, such as hydrogenobyric acid a,c-diamide (\( \text{HBAD} \))[9,12] Normally, in the aerobic \( \text{B}_{12} \) biosynthetic pathway, \( \text{HBAD} \) is next chelated with cobalt.[2] However, when grown in the absence of cobalt, some purple sulfur bacteria produce cobalt-free corrins,[11] including a compound tentatively identified as \( \text{Hby} \)[11,14] providing hope for the biological synthesis of \( \text{Hby} \).[20] Herein, we describe an engineered \( \text{B}_{12} \) biosynthesis pathway variant containing the enzyme \( \text{CobQ} \) for the effective preparation of \( \text{Hby} \), and present a thorough analysis of the structure of this metal-free corrin, which is critical for binding cobalt ions and for bestowing \( \text{B}_{12} \) biocatalysts with their exceptional reactivity.[4,12]

A pathway variant was explored for the biosynthesis of \( \text{Hby} \) by integrating \( \text{cobQ} \) from a purple sulfur bacterium[11a] into the existing repertoire of \( \text{HBAD} \) biosynthetic genes to generate a \( \text{Hby} \)-operon in an \( \text{E. coli} \) strain called ED661.

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With the Hby-operon integrated in the genome under the control of a T7 promoter, Hby was found to be excreted into the culture medium. A 4 L fermentation of this strain furnished 11.8 mg (12.8 μmol) of crystalline Hby (Figure 1 and Supporting Information, SI), providing an unprecedented opportunity to study a metal-free natural corrin. When buffered to pH 5–7, and kept in the dark, aqueous solutions of Hby were found to be relatively stable at room temperature (at higher pH Hby was converted into “yellow corrinoids”).[11a,b]

In aqueous solution, Hby exhibited UV/Vis absorption[11b] with maxima at 270 nm, 330 nm, 499 nm and 524 nm, and emitted fluorescence with maxima at 552 nm and 609 nm (Figure 2), comparable to a natural “metal-free red corrin”.[13] The absorption and emission maxima (at 524 and 552 nm, respectively) position the lowest singlet excited state of Hby at 223 kJ mol⁻¹ (for additional data see SI, Figure S2). NMR- and mass spectra (Figure 2 and see SI) established the structure of Hby. The signals of all H, C and Na atoms of Hby were assigned via (¹H, ¹H)-homonuclear and (¹H, ¹³C)- and (¹H, ¹⁵N)-heteronuclear single and multiple bond correlations. Two lowfield signals gave evidence for two “inner” H-atoms at N2 and N4, specifying the structure of the cationic corrin ligand core in metal-free Hby. Other NH tautomers, such as ¹⁵⁷Hby with “inner” H-atoms at N1 and N3, were not detected (Figure 2). However, the HN2 and HN4 protons undergo unsymmetrical transannular H-bonding with N1 and N3, detected with ¹⁵N-labelled Hby, clarifying the question[13] of the location and H-bonding pattern of the “inner” H-atoms in a natural metal-free corrin. The H atoms H(N2) and H(N4) of Hby were also observed to interact mutually by NOE correlations and by an additional nonbonding through-space interaction, diagnosed through substitution of either one of these H(N)s by D (see SI, Figure S4).

Both of the two “inner” H atoms are tightly bound by the corrin ligand, despite their fast exchange with water with rates of 21.9 s⁻¹ (HN2) and 6.3 s⁻¹ (HN4) at 308 K (SI, Figure S5). Indeed, the corrin moiety of Hby, a weak acid with pKₐ (Hby) = 11.2,[11b] is deprotonated at the corrin periphery, presumably at C8 (Figure 2), as was first deduced by Eschenmoser and Fischli for the model corrin HCor⁺ (formula and crystal structure in SI, Figure S6).[6,14,15] Poignantly, a monoprotonated “neutral” corrin ligand[15] remains elusive. These features of Hby are supported by DFT analyses, which are consistent with the experimentally found stable zwitterionic form of Hby with two unsymmetrical H-bonds N1–HN2 and N3–HN4, support peripheral C8 as the most acidic position of Hby and indicate protoners of Hby with a single “inner” H atom, either at N4 or at N2, to be significantly less stable (see SI, Figures S7 and S8; Table S5).

Hby generated single crystals from H₂O/MeCN at 5°C, with space group P2₁. X-ray analysis revealed a pseudo-C₂-symmetric helical arrangement of the core part of Hby, with similar structural features observed in the crystal as in solution (Figure 3 and SI, Figures S6 and S9). Electron density for two “inner” H atoms was located at N2 and N4, which were at a distance of only 2.27 Å from each other. The two H atoms are also close to N1 and N3 with distances of 1.91 Å and 2.06 Å, respectively, consistent with the NMR-derived unsymmetrical H-bonding. The distance between N2 and N4 of Hby is 3.97 Å, that is, about 0.3 Å longer than that between N1 and N3 (3.67 Å). By contrast, in HCor⁺, the “inner” H atoms are located at N1 and N3.[6,14,15] However, H(N1) of HCor⁺ undergoes H-bonding interactions to an EtOH molecule, giving the C4–C5 bond of HCor⁺ a 24.8° twist.[6,15] In both, Hby and HCor⁺, the “inner” H atoms break...
the inherent $C_2$ symmetry of the corrin core, contrasting with the situation in the more regularly structured cobalt corrins and in the “expanded”, symmetrical porphyrins.[14]

The corrin $\text{Hby}$ features a coordination hole with an average diameter of 3.83 Å, indicating an effective ring contraction of roughly 0.3 Å, compared to octaethylporphyrin (HOEP).[15] Hence, the effective coordination radius in $\text{Hby}$ (1.916 Å) is close to the average equatorial (Co–N) bond in $\text{AdoCbl}$ (1.897 Å).[17] $\text{MeCbl}$ (1.898 Å)[18] and in $\text{Cbl}^{\text{II}}$ (1.88 Å).[19] At first sight, the corrin ligand appears to be well adapted for coordination of $\text{Co}^{\text{II}}$ and $\text{Co}^{\text{III}}$ ions.17–19 Consequently, the four chelating N atoms of the corrin macrocycle of $\text{Hby}$ represent a screw-like coordination hole, leading to a coordinative misfit for cobalt ions that is particularly strong for $\text{Co}^{\text{II}}$.

The mutual conformational adaptation of the corrin ligand and the coordinated cobalt ions was evaluated by two structure parameters: i) The corrin helicity $h$ of the innermost coordination space of the corrin ligand provided by the four corrin nitrogen atoms, defined by the dihedral angle $\text{N1-N2-N3-N4}$ (see Figure 4). In the metal-free corrin $\text{Hby}$ it amounts to $h(\text{Hby}) = 12.9^\circ$. $\text{Co}^{\text{II}}$ corrins feature strongly reduced $h$ values, e.g., $h(\text{AdoCbl}) = 3.5^\circ$ and $h(\text{MeCbl}) = 4.6^\circ$. Hence, the ligand is strongly flattened by $\text{Co}^{\text{III}}$ binding in $\text{AdoCbl}$ and $\text{MeCbl}$. On the other hand, the four-coordinate $\text{Co}^{\text{III}}$ center ($\text{Cbl}^{\text{III}}\text{ACA}$) of the human adenosyltransferase ACA fits the corrin ligand better, displaying $h(\text{Cbl}^{\text{III}}\text{ACA}) = 8^\circ$.20 Five-coordinate $\text{Co}^{\text{II}}$ corrins display lower intermediate levels (see Figure 4), ii) The interplanar angle $\phi$, which concerns the equatorial coordination sphere at the cobalt center, indicating coordinative strain in cobalt corrins when deviating from $0^\circ$ (see Figure 4 and SI for details). The reference value of $\text{Hby}$ is $\phi = 13.5^\circ$. In $\text{Cbl}^{\text{III}}\text{ACA}$ $\phi = 17^\circ$, in the two $\text{Co}^{\text{II}}$ corrins, $\text{Cbl}^{\text{II}}$ and $\text{Cbin}^{\text{II}}$ $\phi$ is $12.5^\circ$, respectively.7,6 In $\text{Co}^{\text{III}}$ corrins, like $\text{AdoCbl}$ and $\text{MeCbl}$, $\phi$ is only $4–5^\circ$. Hence, $h$ and $\phi$ decrease in a roughly correlated fashion from $\text{Hby}$ to $\text{Co}^{\text{II}}$ and to $\text{Co}^{\text{III}}$ corrins, indicating significant directional coordinative misfit in $\text{Co}^{\text{III}}$ corrins.

The structural analysis of the helical corrin ligand $\text{Hby}$ of $\text{B}_{12}$ derivatives has revealed key elements helping to “demy?tify vitamin $\text{B}_{12}$.”1,4 It has confirmed the postulated “fit”1,4,4 of the “ring-contracted” corrin ligand $\text{Hby}$ to the size of $\text{Co}^{\text{III}}$ and $\text{Co}^{\text{II}}$ ions (in $\text{AdoCbl}$ and $\text{Cbl}^{\text{II}}$). However, the corrin ligand $\text{Hby}$ is distinctly helical, dissatisfying the octahedral coordination preference of $\text{Co}^{\text{III}}$ centers, while better meeting the requirements of $\text{Co}^{\text{III}}$ and $\text{Co}^{\text{II}}$ ions (Figures 4 and 5). The inferior accommodation of $\text{Co}^{\text{III}}$ over $\text{Co}^{\text{II}}$ centers implies a previously overlooked coordinative strain for $\text{Co}^{\text{III}}$ corrins that promotes homolytic (Co–C) bond cleavage. This effect is crucial for the homolysis of $\text{AdoCbl}$ to $\text{Cbl}^{\text{II}}$ in the $\text{B}_{12}$-dependent radical isomerization reactions.16,21 The same type of strain also activates the cobalt-bound methyl group of
enzyme catalysis.[20] Herein, we infer that cobalt corrinoids have been selected[44] since they represent “entatic state” complexes in which ligand-imposed strain activates CoIII centers for catalysis. A related situation exists in coenzyme F430—a Ni corphinoid, in which radial strain results from a misfit between the size of the coordinated Ni ions and the porphyrinoid macrocycle.[46,29]

The availability of the metal-free Hby has also opened the door to the direct preparation of transition metal analogues of the cobalamins, the “metbalamins” (Metbls), a “Holy Grail” of bioinorganic chemistry.[6,9,11b,c,30] Hence, Hby has served as an effective starting material for the synthesis of transition metal B12 analogues, to be reported shortly. As described with AdoRbl, the RhIII analogue of AdoCbl[9] suitably structured Metbls hold a significant potential as “antivitamins B12”, in biological imaging or as novel antibiotics.[31] The exciting prospect of investigations with transition metal complexes of the skewed corrins will interest experimental scientists and theoretical chemists alike.

**Experimental Section**

CCDC 1881269 (Hby, see SI) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre.

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**Conflict of interest**

The authors declare no conflict of interest.

**Keywords:** cobalamins · cobalt · synthetic biology · vitamins · X-ray structures

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**Figure 5.** The helical corrin ligand binds cobalt centers in a strained state, promoting the cleavage of axial bonds and formation of reduced corrinoids. This is symbolized at the top for the CoVI corrin AdoCbl (before and after Co–C bond cleavage), for four-coordinate CoII and CoIII cobalamins, and for Hby. Middle and bottom: The corrin ligand is flattened and interplanar angle φ decreased most strongly at CoIII centers, less at CoII and CoIV ions. Both parameters indicate an increasing misfit and strain in the series CoII/CoIII and CoIV/CoV coronins; numerical data for h and φ are collected in Figure 4.

**MeCbl for abstraction by radicals[24] in B12-dependent radical SAM enzymes.[25] A similar strain decrease may also accompany the heterolytic abstraction of the cobalt-bound methyl of MeCbl by nucleophiles in B12-dependent enzymatic methyl group transfer, producing CoIV cobalamin.[20]** In the critical adenosyl-transferase ACA, an unstable four-coordinate form of ChI (ChI ACA)[28] undergoes the reduction to the four-coordinate CoII species. Such essential four-coordinate CoII and CoIV forms, which are hard to generate metabolically,[25b,27] appear to be well accommodated by the helical coordination hole of the corrin ligand. Since CoIII coronins are not structurally characterized, model DFT calculations were used. They indicate a reduction of coordinative strain, by about 7 kJ mol−1, for the transition from six-coordinate CoIII to four-coordinate CoII ions, when bound by four N atoms in a nonplanar arrangement, as in Hby. The analogous CoIII-to-CoII transition experiences a strain decrease of about 10 kJ mol−1 (SI, Figure S10). Hence, the inherently helical corrin ligand acts as a “Procrustean bed” that destabilizes CoIII centers towards loss of axial ligands and formation of CoII or CoIV forms, enhancing catalysis by the B12 cofactors.

The previously unrecognized role of the flexible helical corrin ligand in activating organometallic CoIII coronins for catalysis classifies the B12 cofactors AdoCbl and MeCbl as “entatic state” molecules. The term “entatic” state was initially applied to proteins with metal centers bound in a strained coordination sphere to lower activation barriers for enzyme catalysis.[20] Herein, we infer that cobalt coronins have been selected[44] since they represent “entatic state” complexes in which ligand-imposed strain activates CoIII centers for catalysis. A related situation exists in coenzyme F430—a Ni corphinoid, in which radial strain results from a misfit between the size of the coordinated Ni ions and the porphyrinoid macrocycle.[46,29]

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