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B_{12}-Cofactor Inactivation by Cobalt to Rhodium Mutation in Methylrhodibalamin: An Antivitamin B_{12} and Antibiotic

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Cobalt-corrins, such as coenzyme B_{12} (AdoCbl) and methylcobalamin (MeCbl), are indispensable enzyme-cofactors found across all kingdoms of life. Their Rh-homologues are promising coordination-chemical and structural B_{12}-mimics. Herein, the preparation of methylrhodibalamin (MeRhbl) in over 90% yield is reported, achieved through template-assisted assembly from Rh_{β}-methylrhodibyrate and the B_{12}-nucleotide. NMR and X-ray crystallography studies confirm that MeRhbl is iso-structural with the B_{12}-cofactor MeCbl. The human B_{12}-tailoring enzyme CblC binds and activates MeRhbl, but Rh-demethylation of MeRhbl is inhibited by its stable Rh—C bond, whose strength is also determined. Thus, MeRhbl meets the key criteria for a genuine antivitamin B_{12}, making it a useful tool for biomedical applications. The B_{12}-antimetabolite MeRhbl also acts as an effective growth inhibitor of the acne-causing bacterium *Cutibacterium acnes*.

1. Introduction

The corrin-bound cobalt-center[1] is central to the unique reactivity of the organometallic B_{12}-cofactors, coenzyme B_{12} (5″-deoxy-5′-adenosylcobalamin, **AdoCbl**) and methylcobalamin (**MeCbl**),[2] which are essential for a range of fascinating radical enzymes and methyl-transferases.[3] Homologous rhodium–corrins are promising B_{12}-antimetabolites that may also help answer the fundamental question, "Why is cobalt so suited to its roles in cobalamins, rather than any other metal ?".[1] Our first target in this respect, the isostructural Rh-analog of **AdoCbl**, 5′-deoxy-5′-adenosylrhodibalamin (**AdoRhbl**, **Scheme 1**), has proven to be an effective antivitamin B_{12} and inhibitor of some AdoCbl-dependent enzymes.[4] Here, we report the single step synthesis of the Rh-analog of **MeCbl**, methylrhodibalamin (**MeRhbl**), from Rh_{β}-methylrhodibyrate (**Me_{β}Rhby**)[5] and the B_{12}-nucleotide [1a,6] (**Scheme 2**). As proposed, **MeRhbl** is isostructural to **MeCbl** and exhibits the key chemical reactivity and structural features of an antivitamin B_{12}, as well as of a bacterial growth inhibitor.[4a,4c,7] The spectral data acquired here of **MeRhbl** also support its previously reported preparation.[8]

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2. Results and Discussion

The recently described Rh_{β}-methylrhodi (III)byrate (**Me_{β}Rhby**),[5] featuring an "upper" Rh-bound methyl group, served as the substrate for the condensation with the 5,6-dimethyl-benzimidazolyl-nucleotide moiety (**DMB-Nuc**) of vitamin B_{12}.[1a,6] This condensation was performed in aqueous medium at room temperature, using EDC*HCl as the activating reagent. The reaction relied on a crucial template-assistance to enable intramolecular formation of the strain-free 19-membered ring structure from the metal-pre-coordinated B_{12}-nucleotide **DMB-Nuc**, affording crystalline **MeRhbl** in 94% yield. A related strategy was pioneered by Eschenmoser and coworkers in the highly regioselective chemical self-constitution step of vitamin B_{12}.[1a] **MeRhbl** was also prepared here in 95% yield using the alternative strategy that employed fully assembled, crystalline **ClRhbl**[9] as the starting material, significantly improving on the previously reported 25% yield[8] (see Supporting Information for details).

MeRhbl exhibits a corrin-type UV/Vis-spectrum with absorption maxima of the α, β and γ-absorption bands at 507 nm,

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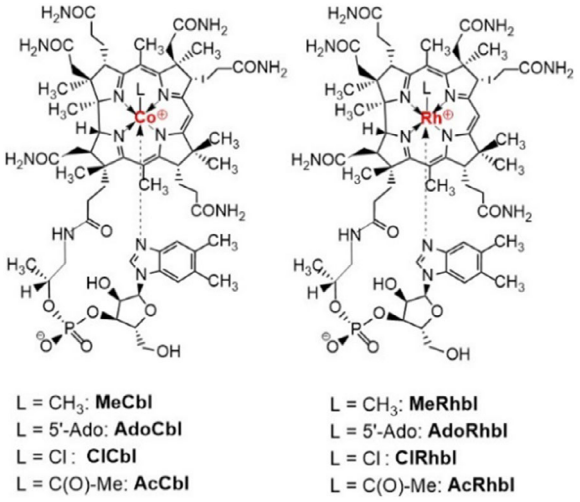
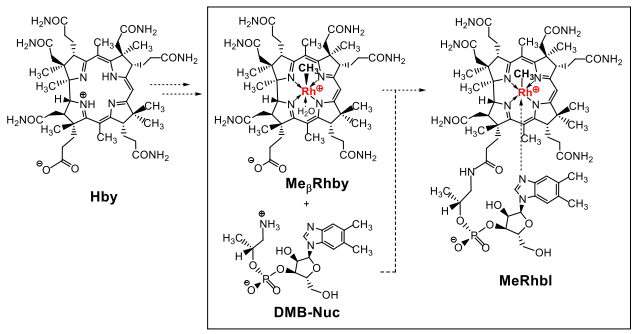


Scheme 1. Structural formulae (left) of the Cbls methylcobalamin (**MeCbl**), coenzyme B_{12} (**AdoCbl**), chlorocobalamin (**ClCbl**), and acetylcobalamin (**AcCbl**) and (right) of the corresponding Rhbls methylrhodibalamin (**MeRhbl**), 5"-deoxy-5′-adenosyl-rhodibalamin (**AdoRhbl**), chlororhodibalamin (**ClRhbl**), and acetylrhodibalamin (**AcRhbl**).



Scheme 2. A template-assisted synthesis of **MeRhbl** from Rh$_\beta$-methylrhodibyric acid (**Me$_\beta$Rhby**), selectively prepared in two steps from **Hby**.[5] The condensation with **DMB-Nuc** furnished **MeRhbl** in 94% yield.

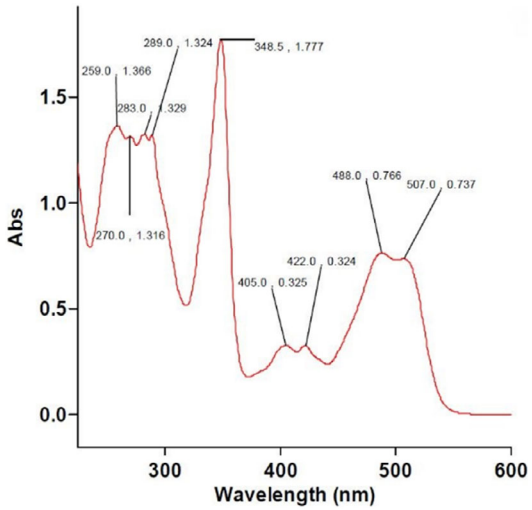


Figure 1. UV/Vis spectrum of **MeRhbl** in water ($c = 0.086$ mM).

488 and 349 nm, respectively (see **Figure 1**). The pronounced bathochromic shifts, relative to the spectrum of **Me$_\beta$Rhby**,[5] are consistent with the formation of a stable "base-on" form.[4b] High-resolution mass spectrometry of **MeRhbl** revealed a pseudo-molecular ion $[M + H]^+$ at $m/z = 1388.562$, reflecting the molecular formula $C_{63}H_{91}N_{13}O_{14}PRh$ (Figure S1,b, Supporting Information). In the 500 MHz ^{1}H-NMR spectrum of **MeRhbl** (**Figure 2**), the slightly broadened singlet of the rhodium-bound methyl group appeared at −1.80 ppm, indicating increased shielding compared to the corresponding signal in the spectrum of **Me$_\beta$Rhby.**[5] The diagnostic 2.3 Hz ^{1}H,^{103}Rh- and 26.6 Hz ^{13}C,^{103}Rh-couplings of **MeRhbl** are, respectively, seen in a 400 MHz ^{1}H-NMR and a ^{1}H-decoupled ^{13}C-NMR spectrum, recorded on a 700 MHz spectrometer (Figure S2 and S3, Supporting Information).

NMR-correlations in homo- and hetero-nuclear spectra of **MeRhbl** recorded in D_2O and H_2O enabled the assignment of 89 of its 91 H-atoms, of all 63 C-atoms and of 12 of its 13 N-atoms (Table S1/S2 and Figure S3–S6, Supporting Information). The chemical shifts of the signals of the methyl group H_3C1A at 0.60 ppm, along with those of HC4N and HC2N of the DMB-base, and key correlations observed in the ^{1}H,^{1}H-NOE-spectra confirmed the base-on nature of **MeRhbl** and the β-configuration of the Rh-bound methyl group. Coordination of the DMB base to Rh in **MeRhbl** induced a diagnostic up-field chemical shift of H_3C1A by 0.90 ppm, relative to its position in **Me$_\beta$Rhby**,[5] similar to the shift observed between adenosylrhodibyric acid (**AdoRhby**) and **AdoRhbl**.[4b] Notably, aside from an up-field shift of approximately 1.8 ppm of the signal of the Rh-bound methyl group, compared to **MeCbl**,[10] the ^{1}H-, ^{13}C-, and ^{15}N-NMR data of **MeRhbl** and **MeCbl** exhibit closely matching characteristics, supporting the high similarity of their 3D-structures in aqueoussolution.

Recrystallization of **MeRhbl** from an aqueous solution upon addition of acetonitrile yielded orange single crystals, orthorhombic space group $P2_12_12_1$. X-ray crystallographic analysis of **MeRhbl** revealed structural features, confirming a highly similar architecture to that of **MeCbl**[11] (**Figure 3**, **4**, S26, and S27 and Table S3–S8, Supporting Information). All six bonds at the metal-center of **MeRhbl** were slightly longer than those in **MeCbl**, consistent with the 60 mÅ larger covalent radius of Rh over Co[12] (see Figure 4). In particular, the four equatorial bonds in **MeRhbl** were elongated by an average of nearly 70 mÅ. The Rh—C and Co—C bonds in **MeRhbl** and **MeCbl** [11a] differ by about 85 mÅ. Notably, the axial Rh-N_{DMB}-bond in **MeRhbl**, measuring nearly 2.3 Å, is significantly elongated by 132 mÅ, compared to the corresponding Co-N_{DMB} bond in **MeCbl**. The pronounced structural trans-influence exerted by the axial methyl group in **MeRhbl** is comparable to that observed in **MeCbl**,[11a] albeit more extensive. In both of these organometallic methyl-corrins, the lengths of the "lower" metal-DMB bonds are consistently longer than the "upper" metal–methyl bonds, in line with similar observations for **AdoRhbl** and **AdoCbl**,[4b] as well as **AcRhbl** and **AcCbl**.[13]

MeRhbl and **MeCbl** also display very similar conformational properties of the corrin ligand, including the local conformational twists of the four pyrrolic rings (Figure 3 and S26/S27, Supporting Information). However, the corrin ligand in **MeRhbl** is slightly flatter

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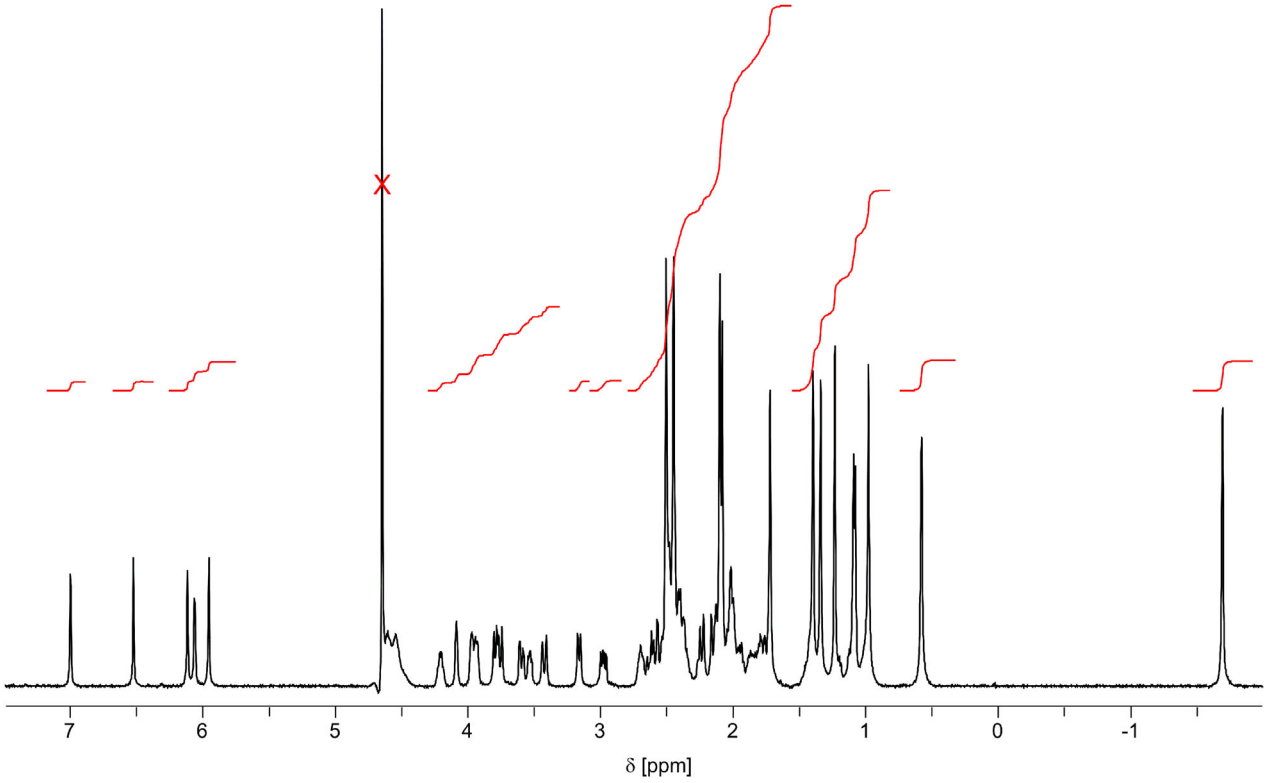

Figure 2. 500 MHz ^{1}H-NMR spectrum of **MeRhbl** (in D_2O, 298 K).

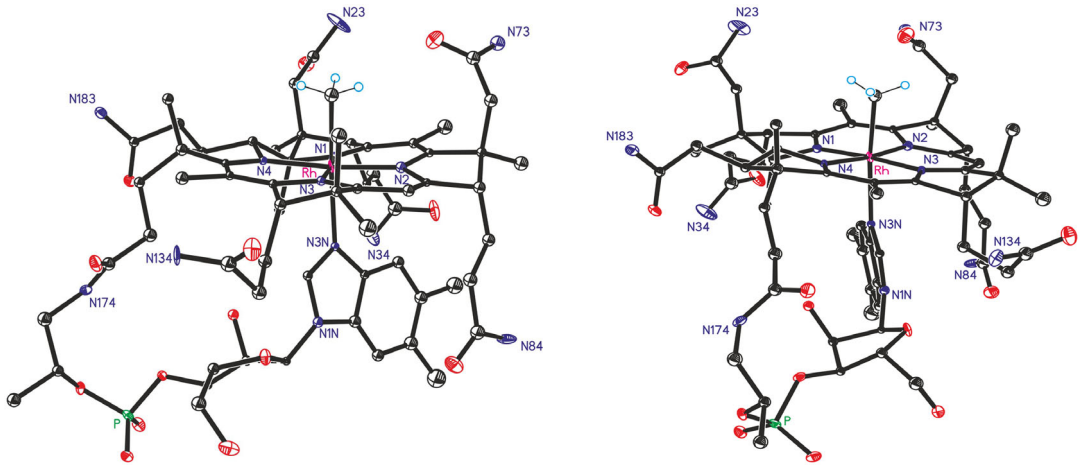

Figure 3. ORTEP-plots of the structure of **MeRhbl** in two orientations (color code: C: gray, N: blue, O: red, P: green, Rh: pink, H-atoms of Rh-bound methyl group: light blue (Figure S26 and Table S3–S8, Supporting Information)).

with a fold angle $\varphi = 10.8°$, compared to $\varphi = 15.8°$ in **MeCbl**,[11a] consistent with corresponding values for **AcRhbl** ($\varphi = 13.4°$) vs. **AcCbl** ($\varphi = 15.6°$).[13] These data suggest that the corrin macrocycle of **MeRhbl** undergoes only a minor conformational relaxation to accomodate coordination to a Rh (III)-center. In line with analogous findings of **AdoRhbl**,[4b] **ClRhbl**,[9] and **AcRhbl**,[13] the structure of **MeRhbl** suggests a slightly better geometric fit of the Rh (III)-ion, within the macrocycle. This conclusion is based on conformational criteria established by Kratky and Eschenmoser for porphyrinoids coordinating low-spin Ni (II)-ions.[14]

The UV/Vis-spectra of the Rh (III)-corrin **MeRhbl** (Figure 1) and of the related Zn (II)-corrin zincobalamin (**Znbl**)[15] display a corrin-typical absorption pattern, indicating nonspecific electronic interactions between the Rh (III)-center and the corrin ligand in **MeRhbl**. In contrast, the organometallic Co (III)-corrins **MeCbl** and **AdoCbl** have distinctive "atypical" UV/Vis-spectra reflecting an extended electronic interplay between their coordinated cobalt-ion and the corrin ligand.[1c,16] **AdoRhbl** is a light-stable Rh-analog of **AdoCbl** [4b] and functions as an anti-photoregulatory ligand by inhibiting AdoCbl-dependent bacterial CarH photoreceptors.[17]

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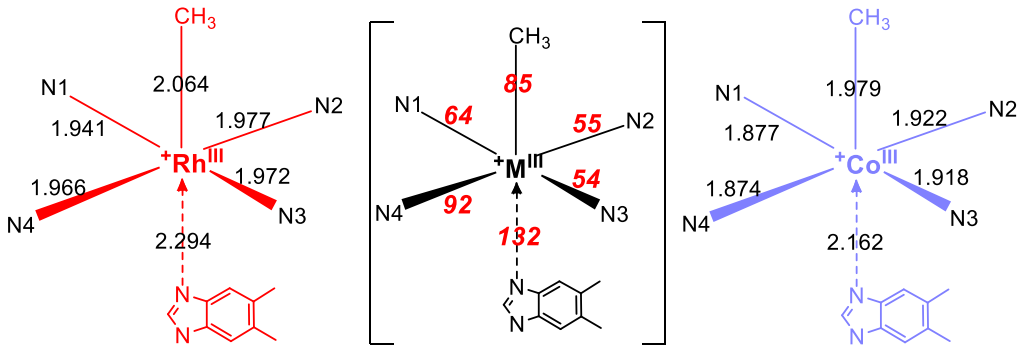

Figure 4. Lengths of bonds (Å) to the rh (III)-center of **MeRhbl** (left), to the co (III)-center in **MeCbl** (right) and differences (mÅ, center) of the bond lengths.

Similarly, unlike the light-sensitive organometallic Cbls,[1c] **MeRhbl** underwent only very slow photo-decomposition under daylight in aerated aqueous solution. Compared to **MeCbl**, its photoreaction was about 250 times less efficient. Notably, this reaction did not involve the typical photolytic loss of the organometallic methyl group, as in **MeCbl**, but instead resulted in the formal addition of dioxygen, as shown by mass spectrometry (Figure S8–S16, Supporting Information). The UV/Vis spectrum of the main photoproduct from **MeRhbl** suggested oxidative cleavage of the corrin ring, presumably involving $^1O_2$ from photosensitization from a metal-corrin triplet state, as previously observed in a synthetic $(CN)_2$Rh (III)-corrin[18] and in certain $(CN)_2$Co (III)-corrins derived from vitamin B_{12}.[19]

We also investigated the acid-induced de-coordination of the DMB base of **MeRhbl**, to evaluate its ability to mimic the biologically relevant "base-on" to "base-off" transition of the cobamides. In strongly acidic aqueous solution, characteristic blue shifts of the dominant absorption bands were observed, most notably for the α-band from 508 to 465 nm (Figure S17–S23, Supporting Information). Analysis of the pH-dependent spectra yielded a pKa $= 0.7 \pm 0.1$ for the DMB de-coordinated species **MeRhbl-H$^+$**. As with its organometallic analog **AdoRhbl**,[4d] **MeRhbl** is over 100 times more resistant to protonation than its natural cobalt-counterpart **MeCbl**. The low propensity of the DMB moiety to undergo protonation and de-coordination in aqueous solution indicates strong stabilization in **MeRhbl** by the bond of the DMB-base to the Rh (III)-center. Remarkably, while **MeRhbl** is protonated at the DMB-base under highly acidic conditions, it largely resists the proteolytic loss of its organometallic methyl group.

To evaluate the proposed classification of **MeRhbl** as an anti-vitamin B_{12},[4c,20] we investigated its resistance to methyl group abstraction by the key human enzyme MMACHC (also named CblC),[7b,21] which rapidly removes the "upper" ligand of **MeCbl**[22] as prerequisite for further cellular processing of the Cbl-moiety. CblC bound **MeRhbl** with high affinity and triggered its conversion to the activated nucleotide-decoordinated "base-off" form, as indicated by a blue shift in the **MeRhbl** spectrum in the presence of CblC (**Figure 5**). The protein's affinity for **MeRhbl** was enhance to a $K_D < 0.2$ µM by the tightly binding and protein-restructuring natural co-substrate glutathione (GSH),[23] causing a

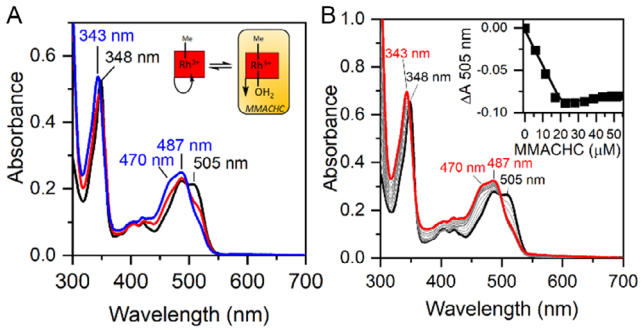

Figure 5. MeRhbl binding and activity of MMACHC (also named CblC[7b]). A) Comparison of the spectra of free **MeRhbl** (20 µM, black) and in the presence of MMACHC (40 µM) and in the absence (red) or presence (blue) of 5 mM glutathione (GSH). B) Binding of **MeRhbl** to MMACHC in the presence GSH at 25 °C with increasing amounts of MMACHC added, as monitored by UV/Vis spectroscopy. *Inset:* The change in absorbance at 505 nm versus CblC concentration yielded $K_D < 0.2$ µM (N = 2) (see Supporting Information for details).

further hypsochromic shift in the absorption maxima (Figure 5). However, an HPLC analysis of the incubated mixture (Figure S24, Supporting Information) showed that **MeRhbl** remained intact, revealing that MMACHC (or CblC) was unable to abstract the methyl group from **MeRhbl** under transfer to GSH.

To assess the efficacy of **MeRhbl** as a potentially useful microbial B_{12}-antimetabolite, we examined the effect of **MeRhbl** on growth of *Cutibacterium acnes* DSM1897. This is a common commensal opportunistic pathogen, which can influence the skin microbiome and contributes to the development of some forms of acne. It features a functional B_{12} biosynthesis-pathway and known susceptibility to elevated B_{12} levels. In skin environments, *C. acnes* primarily uses lactate as a carbon source and grows well on a defined lactate medium. We observed markedly inhibited growth of *C. acnes* in the presence of **MeRhbl** (**Figure 6**), an effect that was reversed upon addition of vitamin B_{12} (CNCbl). Additionally, **MeRhbl** also inhibited a strain of *Salmonella enterica* that relies on exogenous B_{12} for growth (Figure S25, Supporting Information).

Cellular uptake and transport of **MeRhbl** are expected to proceed with similar efficiency as those to the isostructural B_{12}-cofactor **MeCbl** and the previously studied **AdoRhbl**.[17]

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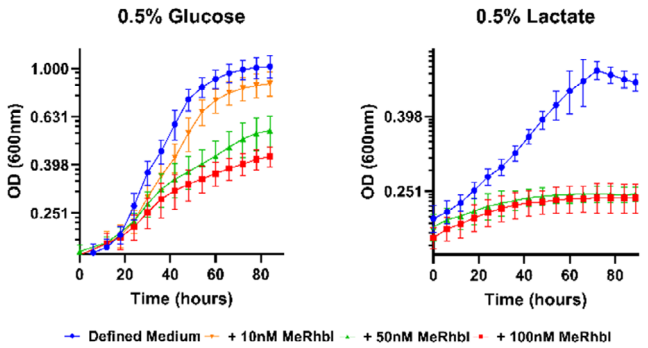

Figure 6. Growth of the opportunistic skin pathogen *Cutibacterium acnes* is inhibited by MeRhbI. *C. acnes* subsp. acnes (DSM1897) was grown on a defined medium containing glucose (left) or lactate (right) as carbon and energy source.[24] Added **MeRhbI** inhibited the growth in a concentration-dependent manner (see Supporting Information for details), monitored as optical density (error bars indicate the standard deviation).

Bacterial uptake of **MeRhbI** is further supported by the growth inhibition studies reported here with *C. acnes* and *S. enterica* (see Supporting Information), as well as by early exploratory studies involving *Escherichia coli* and *Lactobacillus leichmanii*.[8] However, as verified in this work, **MeRhbI** resists modification by the human cobamide deligase CblC and, thus, qualifies as a genuine antivitamin B_{12}.[4a,25] **MeRhbI** resistance to enzymatic cleavage of its Rh—C bond mirrors the behavior observed for **AdoRhbI**.[4d] This can be rationalized by the comparatively greater strength of the Rh—C bond. Data on methyl-Rh (III)-porphyrins[26] suggest that the Rh—C bond in **MeRhbI** is considerably stronger than the Co—C bond in methyl-Co (III)-corrins.[27] Notably, the ^{13}C,^{103}Rh-coupling observed in the NMR-spectra of **MeRhbI** (see Figure S2d, Supporting Information) closely matches that reported for a Rh-porphyrin, indicating similarly strong Rh-C bonds with BDEs around 40 kcal/mol.[28]

Returning to the question, "Why is cobalt so suited to its roles in the B_{12}-cofactors?", one is naturally led to ask specifically "Why not rhodium?" Indeed, when it comes to mimicking the cobalt chemistry no other metal could rival rhodium, though a major biological role for rhodium is unlikely, given its natural scarcity on Earth.[29] In fact, the Rh (III)-ion experiences a slightly better coordinative fit within the corrin ligand, than Co (III).[4b,9,13] However, the Rh (III)-analogs of B_{12}-cofactors are characterized by critically reduced reactivity in organometallic B_{12}-dependent enzymes,[4b,4d] a limitation that can be rationalized by the stronger Rh-C BDEs, deduced in this study. The unique cobalt-centered organometallic reactivity of the B_{12} cofactors, including their photo- and redox-chemistry,[2f,3c] is a direct consequence of the specific electronic interactions between the cobalt ions and the corrin ligand. In contrast, the interactions between the Rh (III)-ion and corrin ligand are rather nonspecific,[4b,4d,9,13] rendering typical B_{12}-like organometallic redox-reactivity inaccessible.

The growth inhibition of *C. acnes*, an opportunistic pathogen implicated in common acne,[30] and *Salmonella enterica*, by **MeRhbI** highlights its potential as a B_{12}-based antibiotic.[20] Direct enzyme-inhibition by **MeRhbI** may not fully explain its antibacterial effect, the gene-regulatory activity of structural B_{12}-mimics acting as ligands for bacterial B_{12}-riboswitches,[31] should be considered, as well. The high structural similarity between **MeRhbI** and **MeCbl** suggests that **MeRhbI** could bind effectively to class-II B_{12}-riboswitches that are selective for **MeCbl**.[4c,31c,32] In addition, **MeRhbI** is expected to serve as a highly effective and specific inhibitory surrogate for **MeCbl** in the wide-spread B_{12}-dependent methyl transferases, which are essential in microorganisms, particularly in fundamental processes such as methanogenesis,[33] and in CO_2-fixation in acetogens.[34] We look forward to studies investigating **MeRhbI** as a specific inhibitor for representative B_{12}-dependent methyl transferases, such as methionine synthase[35] and radical SAM-dependent methyl transferases.[36] In summary, beyond inducing "functional B_{12}-deficiency" in humans and animals[4a] as a true antivitamin B_{12},[20] **MeRhbI** holds promise as a multifunctional growth-inhibitor of bacteria and other B_{12}-dependent microorganisms.[4c,20,33a]

3. Conclusion

The remarkable biocatalytic potency of the cobalt–corrin pair in organometallic B_{12}-biocatalysis stems from the specific electronic interactions between the metal center and its ligand.[37] This finely tuned reactivity remains unmatched by any other transition metal–ligand interactions. While Rhbls serve as excellent structural mimics of Cbls, they lack the exceptional reactivity of B_{12}-cofactors.[38] As a result, Rhbls offer utility as inhibitors of Cbl-dependent enzymes[4b,4d] and as deceptive ligands in bacterial gene-regulation via riboswitches.[39,40] These unique properties make Rhbls especially valuable tools in biochemical and structural studies of B_{12}-related metabolic processes[4d] and position them as promising candidates for the multifunctional growth inhibition of B_{12}-dependent drug-resistant microorganisms.

4. Experimental Section

General

RV, rotatory evaporator; RT, room temperature (23 °C); 10 mM aqueous potassium phosphate buffer pH 6 (KP6-buffer); 10 mM aqueous potassium phosphate buffer pH 7 (KP7-buffer); HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance (spectroscopy).

Solvents

Acetic acid (HOAc, Sigma–Aldrich, glacial, ACS reagent, ≥99.7%, distilled under Ar); water ddH_2O (Millipore MiliQ Academic, HPLC gradient grade); methanol (MeOH; Fisher, ≥99.8%, HPLC grade); t-butanol (t-BuOH, Fluka, puriss. p.a.); acetone (Fluka, puriss. p.a.); acetonitrile (ACN, Fluka, puriss. p.a.); D_2O (Euriso-top, 99.96%D).

Chemicals and Materials

Rh_{β}-methylrhodibyrate (**Me_{β}Rhby**)[5] was obtained by chemical synthesis from hydrogenobyric acid (**Hby**);[1b] the B_{12}-nucleotide [1a,6] and human CblC (also named MMACHC) (lacking the C-terminal disordered residues 244–296)[41] were isolated and prepared as published. Glutathione (GSH, purity > 98%, Sigma–Aldrich) (see Supporting Information for further details).

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Spectroscopy

UV-Vis spectra: Agilent Technologies Cary 60; λ_{max} (log ε) in nm. NMR: 500 MHz Agilent DD2 and 700 MHz Bruker Avance Neo spectrometers. ^{1}H-NMR spectra: coupling constants J given in Hz, referenced against TMS (calculated frequency) or internal water (at 4.70 ppm at 298 K).[42] Hetero-nuclear NMR-spectra were acquired using the listed 500 and 700 MHz spectrometers. ^{1}H- and ^{13}C-NMR chemical shift values (the latter referenced against the calculated frequency for TMS) were from ^{1}H-decoupled ^{13}C-spectra, as well as ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-ROESY, ^{1}H,^{13}C-HSQC, and ^{1}H,^{13}C-HMBC spectra; ^{15}N-NMR chemical shift values and signal assignments were derived from ^{1}H,^{15}N-HSQC and ^{1}H,^{15}N-HMBC spectra (referenced against the calculated frequency for liquid NH_3 at 298 K). ESI-MS. Finigan LCQ classic, HR-ESI-MS: Finnigan/Thermo-Fischer LTQ Orbitrap spectrometer; positive ion mode: m/z (relative intensity in % in reference to basis signal); spray solutions of samples in H_2O : methanol.

HPLC

Hitachi Pump, type L-2130; Diode Array Detector L2450 L-P19596-1/07 (detection at $\lambda = 480$ nm); injection: manual loading of the injection loop (20–100 µl); stationary phase: Phenomenex HyperClone ODS (C18); column: 250 × 4.60 (5 µm); solvent composition: 10 mM KP7-buffer / MeOH: 0–34.5 min (20–95% MeOH), 34.5–38.5 min (95% MeOH), 38.5–39.5 min (95–20% MeOH), 39.5-44.5 min (20% MeOH).

X-Ray Crystallography

A sample of about 3 mg of **MeRhbl**, first dissolved in about 200 µL of water, started to crystallize spontaneously upon dropwise addition of ACN until turbidity was observable. The sample was stored overnight at room temperature in order to crystallize to practical completion. A single crystal of **MeRhbl** was selected for the X-ray diffraction experiments. These were carried out with a Bruker D8 Quest diffractometer at 173 K, Photon 100 Detector. The structure was solved by direct methods and refined against F^2-values using the program SHELXL-97.[43]

Preparation of Methylrhodibalamin (MeRhbl) from Rh$_\beta$-Methylrhodibyrate (Me$_\beta$Rhby)

In a 25 ml round bottom flask and with reduced indirect day light, a powder sample of 5.3 mg (5.3 µmol) of Rh$_\beta$-methylrhodibyrate (**Me$_\beta$Rhby**) was dissolved in 6 ml of water. Then, 6.7 mg of crystalline B_{12}-nucleotide (**DMB-Nuc**,16 µmol) were added to the yellow-orange solution, which changed its color immediately to red-orange. Subsequently 4.2 mg HOBt (32 µmol) were added to the reaction solution. The flask was cooled with ice externally to about 0 °C and a cold solution of 21 mg EDC*HCl (100 µmol, in 250 µL of water) was added. After a reaction time of 1 h roughly 95% conversion was indicated by HPLC analysis. The reaction mixture was kept at about 0 °C for additional 30 min, warmed up to RT and stirred for 1.5 h. An HPLC-analysis indicated complete conversion to **MeRhbl**. The reaction solution was applied to a conditioned RP-18 cartridge, which then was washed with 30 ml of water. The orange red fraction of **MeRhbl** was eluted by roughly 12 ml each of water:MeOH (3:1) and of water:MeOH (2:1). The eluate containing about 5.2 µmol orange **MeRhbl** (UV/Vis) was concentrated under light protection to about 7 ml using an RV at T < 40 °C und was stored in the freezer overnight. On the next day it was dried under light protection using an RV at T < 40 °C. The residue was dissolved in MeOH and applied to a SiO_2 column (length 6 cm, $\varphi = 2.5$ cm). With about 15 ml of MeOH the remaining B_{12}-nucleotide was washed out and the orange **MeRhbl** fraction was eluted with about 25 ml of MeOH:H_2O (3:1), identified by UV/Vis analysis. The solvents of the eluate were evaporated off with protection from light; the sample was dried (HV, RT, 20 min) and taken up in roughly 2 ml of MeOH. After the solvents were removed again the sample was dried (HV/RT, 5 h) and weighed (7.1 mg, 5.1 µmol, 97% yield). For crystallization, the purified **MeRhbl** was dissolved in about 300 µL of water and acetone was added slowly until turbidity was observed. Orange red crystals started to form and the batch was stored in the refrigerator overnight. The crystals were separated from the mother liquor and washed with copious amounts of acetone and then dried (HV, RT), furnishing 6.9 mg (5.0 µmol, 94% yield) of orange red crystalline **MeRhbl**. It was identified by HPLC, HR-MS, UV/Vis, and ^{1}H-NMR spectra; 500 MHz spectrum in D_2O (Figure 2) and spectra at 700 MHz in H_2O /5% D_2O (SI). For the here improved alternative procedure for preparing **MeRhbl**[8] from crystalline chlororhodibalamin (**ClRhbl**),[9] see Supporting Information.

UV/Vis

(H_2O, $c = 0.086$ mM): λ_{may} (log ε)= 507 (3.93), 488 (3.95), 422 (3.58), 405 (3.58), 349 (4.32), 289 (4.19), 283 (4.19), 270 (4.18), 259 (4.20) (Figure 1).

HR-MS

(ESI, positive ions, LTQ-Orbitrap, MeOH/H_2O (9:1)): m/z (%) = 1428.52 (6), 1427.52 (13), 1426.51 (17, $[M + K]^+$); 1412.55 (29), 1411.54 (81), 1410.54 (100, $[M + Na]^+$); 1390.57 (8), 1389.56 (22), 1388.56 (29, $[M + H]^+$), m/z_{calc} ($C_{63}H_{92}N_{13}O_{14}PRh$) = 1388.57 (Figure S1a,b, Supporting Information).

MeRhbl Demethylation Activity of MMACHC (also Named CblC)

MMACHC (or CblC) [7b, 41] (40 µM final concentration) was added to a solution of **MeRhbl** (20 µM) in 100 mM HEPES,[41] 150 mM KCl 10% glycerol pH 7.4, and incubated for 20 min at 20 °C. Then, GSH (5 mM) was added and incubated for 60 min. UV/vis spectra were recorded every 1 min. For HPLC analysis (see Figure S24, Supporting Information), the reaction was terminated by precipitating protein with trifluoracetic acid (1% v/v), centrifuged for 3 min at 13,600 x *g* and immediately analyzed (by HPLC). The supernatant (100 µl) was injected onto a C18 column (Phenomenex Luna C-18−2, 4.6 × 250 mm). The column was eluted using the following system: Buffer B: 10 mM potassium phosphate pH 7.0 and Buffer C: acetonitrile, and the following gradient at a flow rate of 1 ml min^{-1} (time, % Buffer C): 0–2 min, 2%; 2—12 min, 15%; 12–25 min, 18%; 25–39 min, 40%; 39–43 min, 60%; 43–44 min, 2%; 44–50 min, 2%.

MeRhbl Inhibiton of Growth of Cutibacterium Acnes DSM1897

Cutibacterium acnes subsp. *acnes* (DSM1897) was grown on a defined medium with glucose or lactate added as a carbon and energy source as described by Holland et al.[24] **MeRhbl** was added to the medium at a final concentration of 10, 50, or 100 nM. Growth was monitored as a function of optical density at 600 nm at 37 °C in five independent biological replicates (see Figure 6).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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