Electronic Supporting Information (ESI)

for

Synthesis of 5,6-substituted benzimidazoles and their evaluation as potential intermediates in the anaerobic vitamin B_{12} biosynthesis pathway

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Table of contents

1. General Methods:

All chemicals and solvents were purchased from commercial sources (Sigma, BLD pharma, SRL, HiMedia, Rankem) and used as received unless stated otherwise. For synthesized compounds, purification was performed using column chromatography with Rankem silica gel (60- 120 mesh) as stationary phase. For protein purifications, Cytiva HisTrap HP (5mL) column and Bio-rad Econo-Pac 10DG desalting columns were used.

^{1}H and ^{13}C spectra were recorded on JEOL 400 MHz or Bruker 400 MHz spectrometer unless otherwise specified. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. the following abbreviations are used: m (multiplet), s (singlet), d (doublet), t (triplet), ddt (doublet of doublet of triplet) and dq (doublet of quartet).

Absorbance and fluorescence readings were obtained on a SHIMADZU UV 1900UV spectrophotometer and HORIBA Scientific Fluoromax-4 spectrofluorometer, respectively.

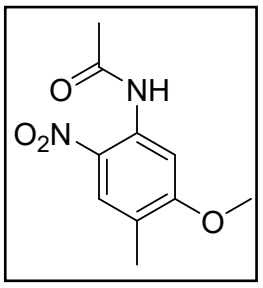
High-performance liquid chromatography (HPLC) analysis was performed using the Agilent 1260 Infinity II UHPLC system with a UV-Vis DAD detector. An Agilent Zorbax Eclipse Plus- C18 5µm 250mm x 4.60 mm column was used for analyzing CobT reactions, and an Agilent Eclipse XDB-C18, 5µm, 250 x 9.4mm column was used for collection of benzimidazolyl riboside-phosphate peaks.

High-resolution mass spectra (HR-MS) were obtained from Sciex X500R quadrupole time-of-flight(QTOF) mass spectrometer fitted with an Exion UHPLC system using a LC/MS method in the positive ion mode using high-resolution multiple reaction monitoring (MRM-HR) analysis.

Bacterial growth curve experiments were carried out in Perkin-Elmer 96 well plates (Optiplate-96) using a BMG LABTECH CLARIOstar Plus microtiter plate reader.

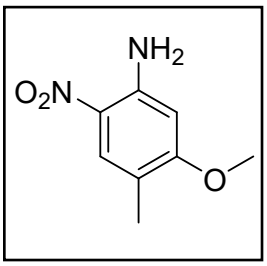
2. Synthesis and Characterization:

2.1 N-(5-methoxy-4-methyl-2-nitrophenyl) acetamide (3b')



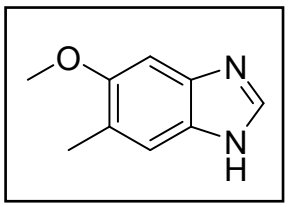
Acetic anhydride (1.14 mL, 12 mmol) was added to a solution of 3-methoxy 4-methyl aniline (**3a'**) (1.00 g, 7.3 mmol) in glacial acetic acid (3.50 mL, 61.1 mmol) and water (3.00 mL, 166.5 mmol) at room temperature in a round bottom flask. During the addition of acetic anhydride, the color of the reaction mixture changes to ink blue. After 2 hours, the round bottom flask was transferred to an ice bath to bring the temperature of the mixture to 0-5 ^{o}C. Once the reaction mixture cools down, concentrated Nitric acid (1.50 mL, 35.9 mmol) was added to the reaction mixture and the reaction mixture was stirred for 2 hours. During the addition of concentrated nitric acid, the color of the mixture changes to olive green. After 2 hours, following the consumption of the starting material by TLC, the solid product was obtained by keeping the reaction mixture at in ice bath overnight and then the obtained the solid product was filtered by vacuum filtration and was dried completely to give crude product. The crude product was further purified using column chromatography using silica gel (60-120) with 10% ethyl acetate in hexane as eluent to give 2-nitro 4-methyl 5-methoxy acetanilide (75%) as bright yellow solid. ^{1}H NMR (400 Hz, DMSO) δ 10.27 (s, 1H), 7.89 (s, 1H), 7.53 (s,1H), 3.86 (s, 3H), 2.15 (s, 3H), 2.10 (s, 3H) ppm; ^{13}C NMR (100 MHz, DMSO) δ 169.23, 162.28, 133.65, 127.21, 122.98, 105.37, 56.63, 24.58, 15.61 ppm; HRMS (ESI-TOF) for $C_{10}H_{13}N_2O_4[M+H]^+$: calcd 225.0870, found, 225.0857

2.2 2-nitro-4-methyl 5-methoxy aniline (3c')



A mixture of Claisen alkali was prepared by dissolving potassium hydroxide pellets (7.00 g, 124.8 mmol) in water (5.00 mL, 278.2 mmol) and methanol (14.00 mL, 367.2 mmol). 2-nitro 4-methyl 5-methoxy acetanilide (**3b'**) (1.27g, 5.6 mmol) was added to the solution of Claisen alkali (15 mL) in a round bottom flask and the mixture was stirred on a magnetic stirrer for 3 hours in oil bath at 70°C. After 3 hours, following the consumption of the starting material by TLC, the product was filtered using vacuum filtration and dried completely to give an orange solid product (79%). ^{1}H NMR (400 Hz, DMSO) δ 7.75 (s, 1H), 7.42 (s,1H), 6.44 (s, 1H), 3.80 (s, 3H), 2.02 (s, 3H) ppm; ^{13}C NMR (101 MHz, DMSO) δ 164.05, 148.07, 126.58, 124.45, 116.48, 98.11, 56.29, 15.52 ppm; HRMS (ESI-TOF) for $C_8H_{10}N_2O_3[M+H]^+$: calcd., 183.0764, found, 183.0777.

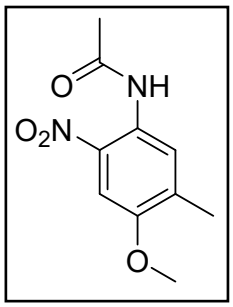
2.3 5-methoxy 6-methyl benzimidazole (3)



Ammonium chloride (1.47g, 27.4 mmol), iron powder (1.60g, 28.6 mmol) and formic acid (10.00 mL, 265.0 mmol) is added to a mixture of 2-nitro 4-methyl 5-methoxy aniline (**3c'**) (0.50 g, 2.7 mmol) and isopropanol (10.00 mL, 130.7 mmol) in a glass seal tube with a magnetic stirrer in it. The seal tube is tightly sealed and the reaction was performed at 105 °C for 4 hours. After 4 hours, following the consumption of the starting material by TLC, the reaction mixture was filtered using a celite bed with washes of isopropanol (10 mL). The filtrate is then neutralized using a solution sodium bicarbonate and the pH was checked using pH paper. The filtrate was then extracted with DCM (3*20 mL). The combined organic layer was washed with brine dried, over Na_2SO_4, filtered and

filtrate was concentrated to give a sticky mixture. The sticky mixture is then stirred with hexane (50 mL) for 12 hours. After 12 hours the hexane solution is decanted and the desired product is obtained as a white solid (52%). ^{1}H NMR (400 Hz, DMSO) δ 7.93 (s, 1H), 7.39 (s, 1H), 7.07 (s,1H), 3.86 (s, 3H), 2.32 (s, 3H) ppm; ^{13}C NMR (101 MHz, CHLOROFORM-D) δ 155.45, 139.43, 123.62, 116.11, 96.41, 55.76, 17.11 ppm; HRMS (ESI-TOF) for $C_9H_{10}N_2O$ $[M+H]^+$: calcd., 163.0866, found, 163.0854.

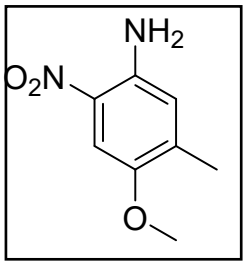
2.4 N-(4-methoxy-5-methyl-2-nitrophenyl) acetamide (3b)



Acetic anhydride (1.14 mL, 12 mmol) was added to a solution of 3-methyl-4-methoxy aniline **(3a)** (1.00 g, 7.3 mmol) in glacial acetic acid (3.50 mL, 61.1 mmol) and water (3.00 mL, 166.5 mmol) at room temperature in a round bottom flask. During the addition of acetic anhydride, the color of the reaction mixture changes to ink blue. After 2 hours, the round bottom flask was transferred to an ice bath to bring the temperature of the mixture to 0-5 ^{o}C. Once the reaction mixture cools down, concentrated Nitric acid (1.50 mL, 35.9 mmol) was added to the reaction mixture and the reaction mixture was stirred for 2 hours. During the addition of concentrated nitric acid, the color of the mixture changes to olive green. After 2 hours, following the consumption of the starting material by TLC, the solid product was obtained by keeping the reaction mixture at in ice bath overnight and then the obtained the solid product was filtered by vacuum filtration and was dried completely to give crude product. The crude product was further purified using column chromatography using silica gel (60-120) with 10% ethyl acetate in hexane as eluent to give 2-nitro 4-methoxy 5-methyl acetanilide (74%) as bright yellow solid. ^{1}H NMR (400 Hz, DMSO) δ 7.44 (s, 1H), 7.39 (s,1H), 3.86 (s, 3H), 2.20 (s, 3H), 2.02 (s, 3H) ppm; ^{13}C NMR (101 MHz,

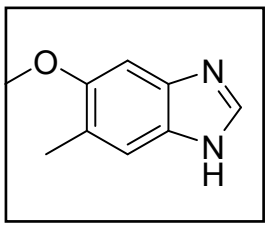
DMSO) δ 168.83, 154.50, 141.56, 133.45, 128.03, 124.69, 106.39, 56.50, 23.70, 16.50 ppm; HRMS (ESI-TOF) for $C_{10}H_{13}N_2O_4[M+H]^+$: calcd 225.0870, found, 225.1957.

2.5 2-nitro-4-methoxy 5-methyl aniline (3c)



A mixture of Claisen alkali was prepared by dissolving potassium hydroxide pellets (7.00g, 124.8 mmol) in water (5.00 mL, 278.2 mmol) and methanol (15.00 mL, 367.2 mmol). 2-nitro 4-methoxy 5-methyl acetanilide (1.27g, 5.6 mmol) was added to the solution of Claisen alkali (15.00 mL) in a round bottom flask and the mixture was stirred on a magnetic stirrer for 3 hours in oil bath at 70 ^oC. After 3 hours, following the consumption of the starting material by TLC, the product was filtered using vacuum filtration and dried completely to give an orange solid product (91%). ^1H NMR (400 Hz, DMSO) δ 7.30 (s, 1H), 7.24 (s, 1H), 6.83 (s,1H), 3.74 (s, 3H), 2.11 (s, 3H) ppm; ^{13}C NMR (101 MHz, DMSO) δ 148.47, 142.37, 138.80, 127.87, 120.71, 103.60, 55.95, 16.97 ppm; HRMS (ESI-TOF) for $C_8H_{10}N_2O_3[M+H]^+$: calcd., 183.0764, found, 183.0757

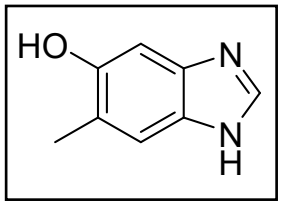
2.6 5-methoxy-6-methyl benzimidazole (3)



Ammonium chloride (1.47g, 27.4 mmol), iron powder (1.60g, 28.6mmol) and formic acid (10.00 mL, 265.0 mmol) is added to a mixture of 2-nitro 4-methoxy 5-methyl aniline **(5c)** (500mg, 2.7 mmol) and isopropanol (10 mL, 130.7 mmol) in a seal tube with a magnetic stirrer in it. The seal tube is tightly sealed and the reaction was performed at 105 ^oC for 4 hours. After 4 hours, following the consumption of the starting material by TLC, the reaction mixture was filtered using a celite bed with washes of isopropanol (10.00 mL). The

filtrate is then neutralized using a solution sodium bicarbonate and the pH was checked using pH paper. The filtrate was then extracted with DCM (3*20 mL). The combined organic layer was washed with brine dried, over Na_2SO_4, filtered and filtrate was concentrated to give a sticky mixture. The sticky mixture is then stirred with hexane (50 mL) for 12 hours. After 12 hours the hexane solution is decanted and the desired product is obtained as a white solid (50%). ^{1}H NMR (400 Hz, DMSO) δ 7.93 (s, 1H), 7.39 (s, 1H), 7.07 (s,1H), 3.86 (s, 3H), 2.32 (s, 3H) ppm; ^{13}C NMR (101 MHz, CHLOROFORM-D) δ 155.45, 139.43, 123.62, 116.11, 96.41, 55.76, 17.11 ppm; HRMS (ESI-TOF) for $C_9H_{10}N_2O$ $[M+H]^+$: calcd., 163.0866, found, 163.0854.

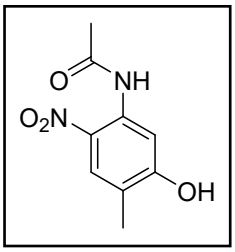
2.7 5-hydroxy-6-methyl benzimidazole (5)



Hydrobromic acid (48%) (24.29 mL, 215.99 mmol) was added to 5-Ome-6MeBza **3** (0.4 g, 2.5 mmol) in a round bottom flask and was stirred at 110 ^{o}C for 24 hours. After 24 hours, following the consumption of the starting material by TLC, the reaction mixture was neutralized with NaOH. After the reaction mixture is neutralized, the solvent present in the mixture was then evaporated by making an azeotropic mixture with ACN (10 mL). After the solvent is evaporated, a solid mixture of salt and product remains in the round bottom flask. The product was extracted from the mixture by providing multiple washes of ACN (8*15 mL), till the final product was completely extracted and was monitored by TLC. The collected ACN mixture was then evaporated to give brown colored crude product (0.4 g). The crude product was further purified using column chromatography using silica gel (60-120) with 5% MeOH in $CHCl_3$ as eluent to give 5-OH-6MeBza as brown colored solid (34 %). ^{1}H NMR (400 Hz, DMSO) δ 7.92 (s, 1H), 7.25 (s, 1H), 6.91 (s,1H), 2.19 (s, 3H) ppm; ^{13}C NMR (151 MHz, MeOD) δ 152.27, 139.47, 135.67, 131.17,

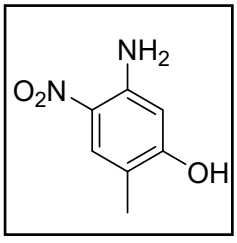
123.37, 121.99, 116.58, 115.53, 99.54, 98.46, 15.67 ppm; HRMS (ESI-TOF) for $C_8H_8N_2O$ [M+H]: calcd., 149.0709, found, 149.0704.

2.8 N-(5-hydroxy-4-methyl-2-nitrophenyl) acetamide (5b)



5-amino 2-methyl phenol **(5a)** (1.23g, 10 mmol) was dissolved in glacial acetic acid (3.00 mL, 52.4 mmol) and water (2.00 mL, 111.0 mmol) at room temperature in a round bottom flask. Acetic anhydride (2.50 mL, 26.4 mmol) was added to a solution dropwise. During the addition of acetic anhydride, the color of the reaction mixture changes to cloudy white. After 2 hours, the round bottom flask was transferred to an ice bath to bring the temperature of the mixture to 0-5 ^oC. Once the reaction mixture cools down, concentrated nitric acid (1.50 mL, 35.9 mmol) was added to the reaction mixture and the reaction mixture was stirred for 2 hours. During the addition of nitric acid, the color of the mixture changes to yellowish orange. After 2 hours, following the consumption of the starting material by TLC, the solid product was obtained by keeping the reaction mixture at in ice bath overnight and then the obtained solid product was filtered by vacuum filtration and was dried completely to give desired product (65%) as orange colored solid. ^1H NMR (400 Hz, DMSO) - δ 11.05 (s, 1H), 10.23 (s, 1H), 7.89 (s,1H), 7.62 (s, 1H), 2.12 (s, 6H) ppm; ^{13}C NMR (101 MHz, DMSO) δ 169.22, 162.17, 133.87, 131.49, 128.11, 121.56, 108.41, 24.83, 15.54 ppm; HRMS (ESI-TOF) for $C_9H_9N_2O_4$ [M]: calcd., 209.0562, found, 209.0386.

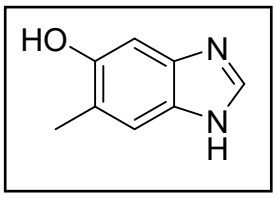
2.9 5-amino-2-methyl-4-nitrophenol (5c)



Claisen alkali was prepared by dissolving potassium hydroxide (KOH) pellets (0.27 g, 4.8 mmol) in water (3.50mL, 194.3 mmol) and methanol (6.70 mL, 165.5 mmol). N-(5-hydroxy-4-methyl-2-

nitrophenyl) acetamide (**5b**) (0.40 g, 1.9 mmol) was added to Claisen alkali solution in a round bottom flask and the mixture was stirred on a magnetic stirrer for 4 hours in oil bath at 70 °C. After 10 hours, following the consumption of the starting material monitored by TLC, the crude product was precipitated out from the reaction mixture by neutralizing the reaction mixture with 11.1N HCl. The precipitated product was filtered using vacuum filtration and dried completely to give a reddish orange crude product. The crude product was further purified using column chromatography using silica gel (60-120) with 100% $CHCl_3$ as eluent to give 5-amino-2-methyl-4-nitrophenol (66%) as dark orange solid. ^{1}H NMR (400 Hz, DMSO), δ 7.72 (s, 1H), 7.30 (s, 1H), 6.31 (s,1H), 2.00 (s, 3H) ppm; ^{13}C NMR (101 MHz, DMSO) δ 163.38, 147.92, 127.26, 124.31, 116.19, 100.87, 15.54 ppm. HRMS (ESI-TOF) for $C_7H_8N_2O_3[M+H]^+$: calcd., 169.0608, found, 169.0618.

2.10 5-hydroxy-6-methyl benzimidazole (5)



Ammonium chloride (0.37 g, 6.8 mmol), iron powder (0.39 g, 6.82 mmol) and formic acid (5.00 mL, 132.5 mmol) is added to a mixture of 5-amino-2-methyl-4-nitrophenol (**5c**) (0.12 g, 0.8mmol) and isopropanol (5.00 mL, 65.4 mmol) in a seal tube with a magnetic stirrer in it. The seal tube is tightly sealed and the reaction was monitored at 105^{o}C for 4 hours. After 4 hours, following the consumption of the starting material by TLC, the reaction mixture was filtered using a celite bed with washes of isopropanol (10.00 mL) to remove Fe powder. The filtrate is then neutralized using a solution sodium bicarbonate. The filtrate was then extracted with DCM (3*20 mL). The combined organic layer was washed with brine dried over Na_2SO_4, filtered and concentrated to give a sticky mixture. The sticky mixture is then stirred with cold hexane (50.00 mL) for 12 hours. After 12 hours the hexane solution is decanted and the desired product is obtained as a white solid (60%). ^{1}H NMR (400 Hz, DMSO) δ 7.92 (s, 1H), 7.25 (s, 1H), 6.91 (s,1H), 2.19 (s, 3H) ppm; ^{13}C NMR (150 MHz, MeOD) δ 152.27, 139.47, 135.67, 131.17, 123.37, 121.99, 116.58, 115.53, 99.54, 98.46, 15.67ppm; HRMS (ESI-TOF) for $C_8H_8N_2O$ $[M+H]^+$: calcd., 149.0709, found, 149.0717.

3. Experimental Protocols:

3.1 Heterologous expression and purification of CobT proteins using immobilized metal-affinity chromatography (IMAC):

E.coli strain BL21(DE3) containing *El*CobT expression plasmid was grown in 1L of LB medium containing 25µg of kanamycin. Protein overexpression was induced by adding an IPTG concentration of 500µM at an OD600 of 0.4-0.5. The cells were harvested by centrifugation and cell pellets were stored at -80°C. For protein purification, the cell pellets were thawed, then resuspended in 15mL binding buffer (50mM phosphate buffer, 300mM NaCl, 10mM imidazole, pH 8.0) with 7µM β-mercaptoethanol and 100µMPMSF and lysed using sonication (60% amplitude, 1 min on, 3 min off cycle). The cell lysate was clarified by centrifugation at 18000 rpm at 4°C and the clarified lysate was loaded on a Ni-NTA 5 mL column pre-equilibrated with the binding buffer. After washing with wash buffer (50 mM phosphate buffer, 300 mL NaCl, 50mM imidazole, pH 8.0), the protein was eluted using elution buffer (50 mM phosphate buffer, 300 mL NaCl, 250mM imidazole, pH 8.0). After elution, the protein was desalted using a desalting column and buffer (50mM phosphate, 150mM NaCl), the concentration was calculated to be 214µM by Bradford analysis and stored as a 12% glycerol stock at -80°C. An identical protocol was followed for the purification of *Eb*CobT, *Mt*CobT, and *Sm*CobT and their concentrations were determined to be 40µM, 27.6µM, and 120.1µM, respectively.

3.2 *In vitro* reaction with CobTs: Reactions were set with purified CobT homologs and analyzed as described earlier.[1,2] Briefly, 20µM of CobT enzyme was incubated

with 50mM Tris-HCl buffer, 2mM NMN, 1mM MgCl2, and 500μM of substrate at 25°C for 48 hours. Samples from reactions were quenched by the addition of 9% formic acid (4ul in 250ul of reaction) and the precipitated protein was removed by centrifugation before analysis using the HPLC. Ammonium acetate 10mM (solvent A), pH 6.5, and methanol (solvent B) were used as the mobile phase. 0- 2min 100% A; 2-15 min 100%A - 30%A; 15-25 min 30% - 0%A; 25-35 min 0-100% solvent A. The chromatograms were recorded at 280 nm and 300nm.

3.3 Purification of LL-riboside from the CobT reaction mixture for structural analysis:

The CobT reaction mixture was treated with 10μM shrimp alkaline phosphatase for 8 hours to get their corresponding dephosphorylated riboside derivatives. The riboside isomers were separated and collected using reverse phase HPLC on an Agilent Zorbax Eclipse Plus- C18 5μm 250mm x 4.60 mm column. The collection samples were pooled and the solvent was evaporated using centrivap and resuspended in a deuterated solvent for NMR analysis.

3.4 Analysis of Riboside products

Reactions containing CobT homologues from *E.limosum* and *S.meliloti* were used to generate 0.5−1 mg of ribosylated products as described above, purified by HPLC, and analyzed using a Bruker 400 MHz spectrometer NMR spectrometer. NMR assignments are as follows:

3.4.1 5-OMe-6MeBza-R **19**

The 1D ^{1}H spectrum shows the ribose ring protons (H1′ 6.32 ppm d, H2′ 4.52 ppm m, H3′ 4.34 ppm m, H4′ 4.34 ppm m, H5′ 3.70 ppm, H5″ 3.64 ppm), the benzimidazole ring protons (H2 8.44 ppm s, H7 7.40 ppm s, H4 7.21 ppm s), and the methoxy group protons (-OCH_3 3.84 ppm s) and methyl group proton (-CH_3 2.24 ppm s), consistent with the expected structure of a 5-Methoxy-6-methyl substituted benzimidazole riboside.

3.4.2 6-OMe-5MeBza-R**20**

The ^{1}H spectrum shows the ribose ring protons (H1′ 6.41 ppm d, H 2′ 4.61 ppm m, H3′ 4.37 ppm m, H4′ 4.38 ppm m, H5′ 3.61 ppm m), the benzimidazole ring protons (H2 8.64 ppm s, H4 7.50 ppm d, H7 7.16 ppm s), and the methyl group protons(CH_3 2.26 ppm, s), methoxy protons (-OCH_3, 3.84 ppm s), consistent with the expected structure of a 6-methoxy-5-,methyl substituted benzimidazole riboside.

3.4.3 5-OH-6-MeBza-R **21**

The 1D ^{1}H spectrum shows the ribose ring protons (H1′ 6.35 ppm d, H2′ 4.52 ppm d, H 3′ 4.35 ppm m, H4′ 4.35 ppm m, H5′ 3.76 ppm , H 5″ 3.67 ppm), the benzimidazole ring protons (H2 8.62ppm s, H7 7.42 ppm s, H4 7.10 ppm s) and the methyl group proton (-CH_3, 2.26 ppm, s), consistent with the expected structure of a 5-hydroxy-6-methyl substituted benzimidazole riboside.

3.4.4 6-OH-5-MeBza-R **22**

The 1D ^{1}H spectrum shows the ribose ring protons (H1′ 6.34 ppm d, H2′ 4.57 ppm m, H 3′ 4.44 ppm m, H4′ 4.39 ppm m, H5′ 3.92, H5″ 3.84 ppm ddd) and the benzimidazole ring protons (H2 8.69 ppm s, H7 7.08 ppm s, H4 7.49 ppm s, H 5 6.94 ppm dd), the

methyl group proton (-CH_3, 2.26 ppm, s), consistent with the expected structure of a 6-hydroxy-5-methyl substituted benzimidazole riboside.

3.5 *Growth curve using ΔmetE and supplementation:*

E.coli MG1655 *ΔmetE* cells were grown in LB-Kan media at 37° C, 180 rpm for 4-5 hours. The cultures were centrifuged at 6500 rpm for 1 minute and the pellets were washed thrice with 1X M9 salts by re-suspending them using a vortex for each wash. These cells were used to start P1 cultures (first subcultures in M9 medium) in [M9 + NH_4Cl + Glucose)] medium, at a starting O.D. 600 of 0.1, and were incubated aerobically at 37°C, 180 rpm, for 12-14 hours. P1 cells were centrifuged at 6500 rpm for 1 minute and the pellets were washed thrice with 1X M9 salts by re-suspending them using a vortex for each wash. This step was used to make sure that the cells do not carry over any residual nutrients from the P1 stage. Cells washed after P1 were used to start their growth curves in P2 cultures (second subcultures in M9 medium) at a starting O.D. 600 of 0.01, in a 96-well plate with lid, with 200µL medium in each well. These cultures were incubated aerobically at 37°C, 180 rpm orbital shaking in the (M9 +NH_4Cl+Glucose) media with supplemented B_{12} or dicyancobinamide (Cbi) or dicyancobinamide (Cbi) + lower ligands alongside a control with nothing added.

4. Supplementary Tables

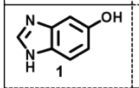
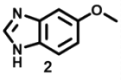
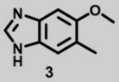
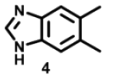
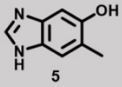
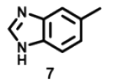
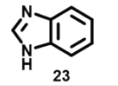
Bza	Cobamide	Source	Primary reference
 1	(5-OHBza)-Cba	*Methanobacillus omelianskii*	Lezius, Barker, 1965
 2	(5-OMeBza)-Cba	*Clostridium thermoaceticum*	Irion, Ljunngdahl, Wood,1965
 3	(5-OMe-6-MeBza)-Cba	*Clostridium fermitoaceticum*	Stupperich, Eisinger, Krauetler, 1988
 4	(DMB)Cba (Cobalamin)	*Streptomyces griseus, Ox liver*	Smith, Parker, 1948
 5	(5-OH-6-MeBza)-Cba	Predicted biosynthesis intermediate	Renz et al., 1993
 7	(5-MeBza)-Cba	*Methanothrix soehngenii*	Freidrich, Bernhauer, 1958,Kohler 1988
 23	(Bza)-Cba	Anaerobic sewage sludge	Freidrich, Bernhauer 1958

Table S1: The structures of the benzimidazolyl lower ligands of the cobamides and the organism/ environment from which these cobamides were first isolated.[3–9]

5. Supplementary Figures

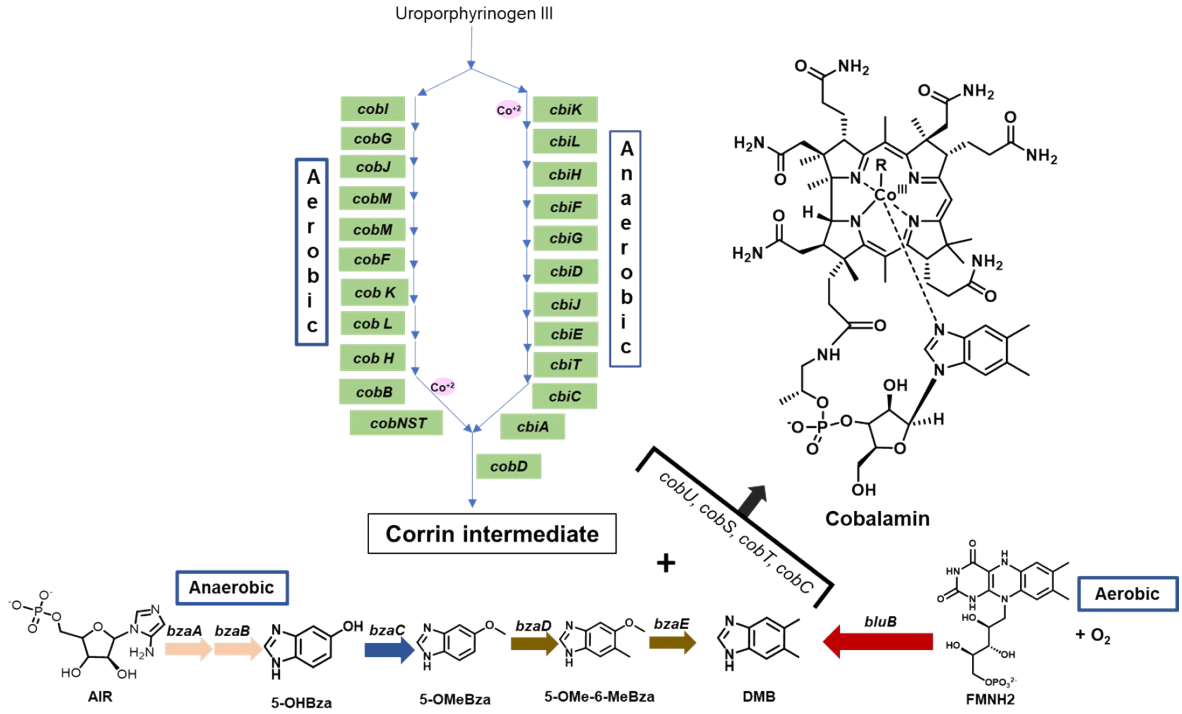


Figure S1: Complete biosynthesis pathway for cobalamin with biosynthesis of lower ligand with enzymes involved in aerobic and anaerobic routes. Incorporation of the lower ligand into corrin ring structure is carried out with the help of enzymes CobU, CobS, CobT, CobC where both aerobic and anaerobic routes converge. Action of these enzymes on the intermediates of the anaerobic DMB biosynthesis give rise to different cobamides. [10–12]

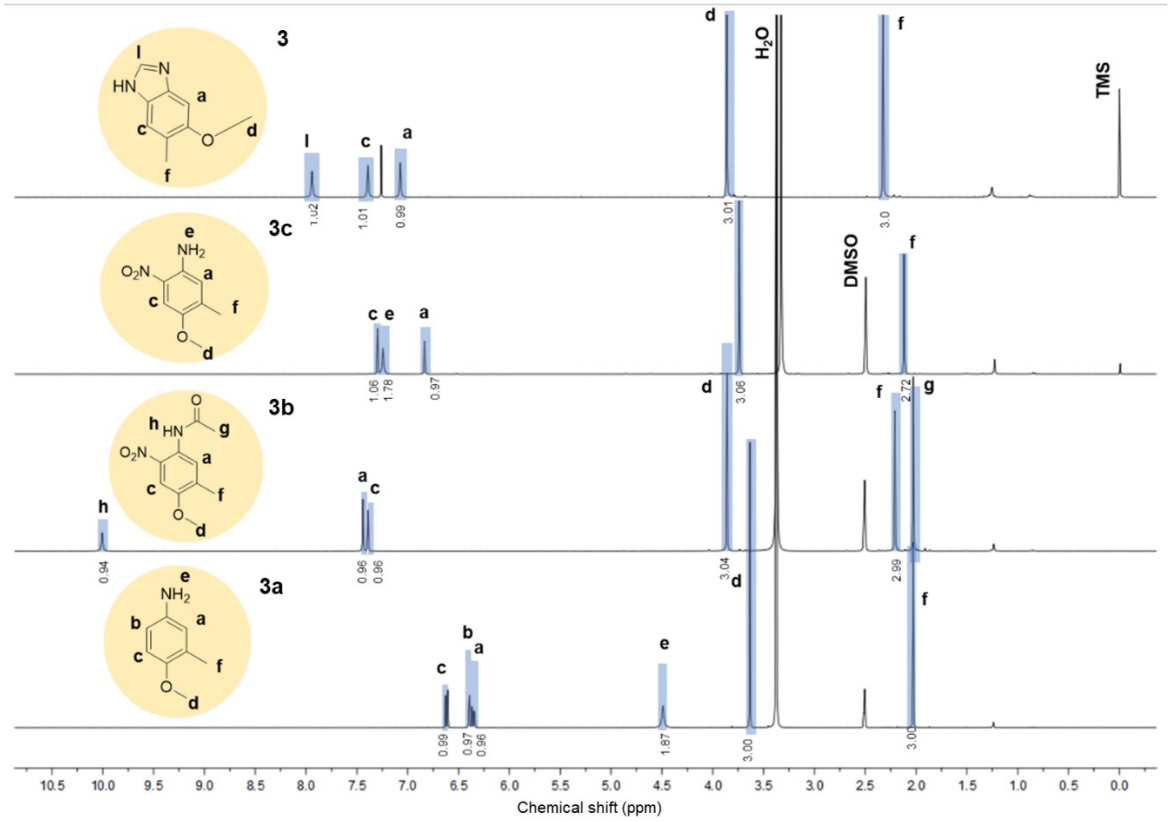


Figure S2: ^{1}H-NMR for the starting material and intermediates for the synthesis of 5-methoxy-6-methyl benzimidazole **(3)**: From bottom to top, starting material 4-methoxy-3-methyl aniline **(3a),** N-(4-methoxy-5-methyl-2-nitrophenyl) acetamide **(3b),** 2-nitro-4-methoxy 5-methyl aniline **(3c)**, and product 5-methoxy-6-methyl benzimidazole **(3)**

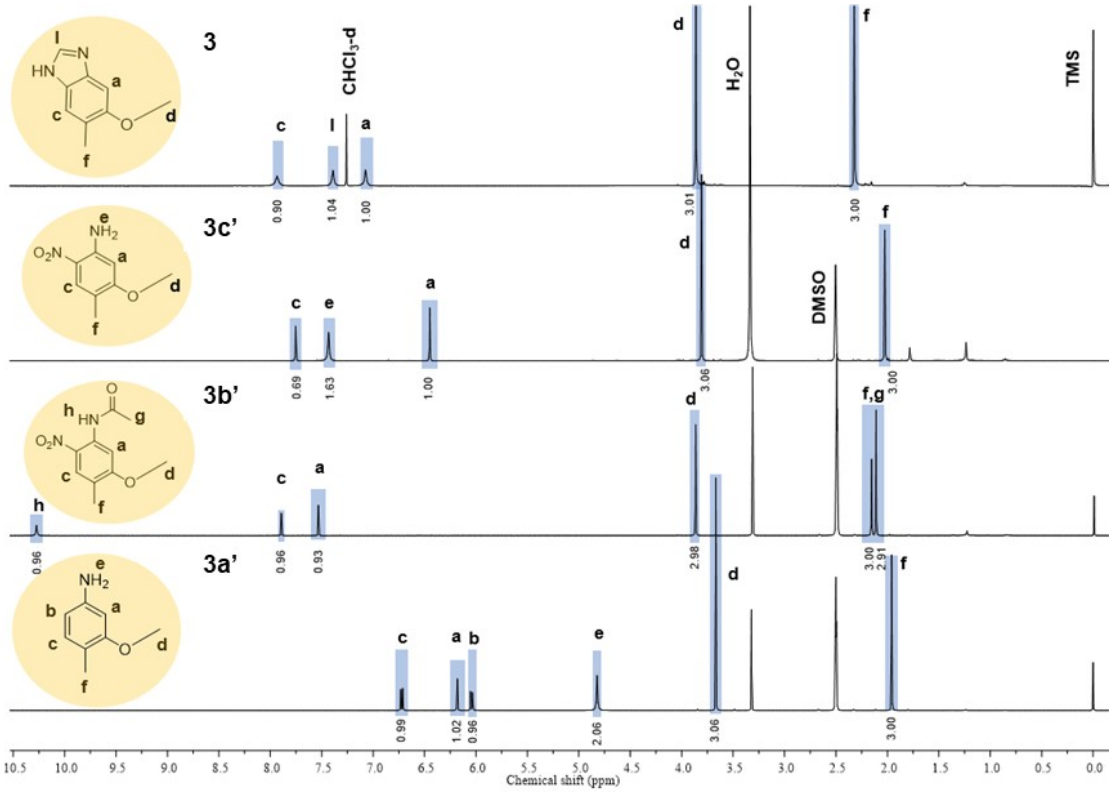


Figure S3: ^{1}H-NMR for the starting material and intermediates for the synthesis of 5-methoxy-6-methyl benzimidazole **(3)**: From bottom to top, starting material 3-methoxy-4-methyl aniline **(3a'),** N-(5-methoxy-4-methyl-2-nitrophenyl) acetamide **(3b')** and 2-nitro-4-methyl 5-methoxy aniline **(3c')** and product 5-methoxy-6-methyl benzimidazole **(3)**

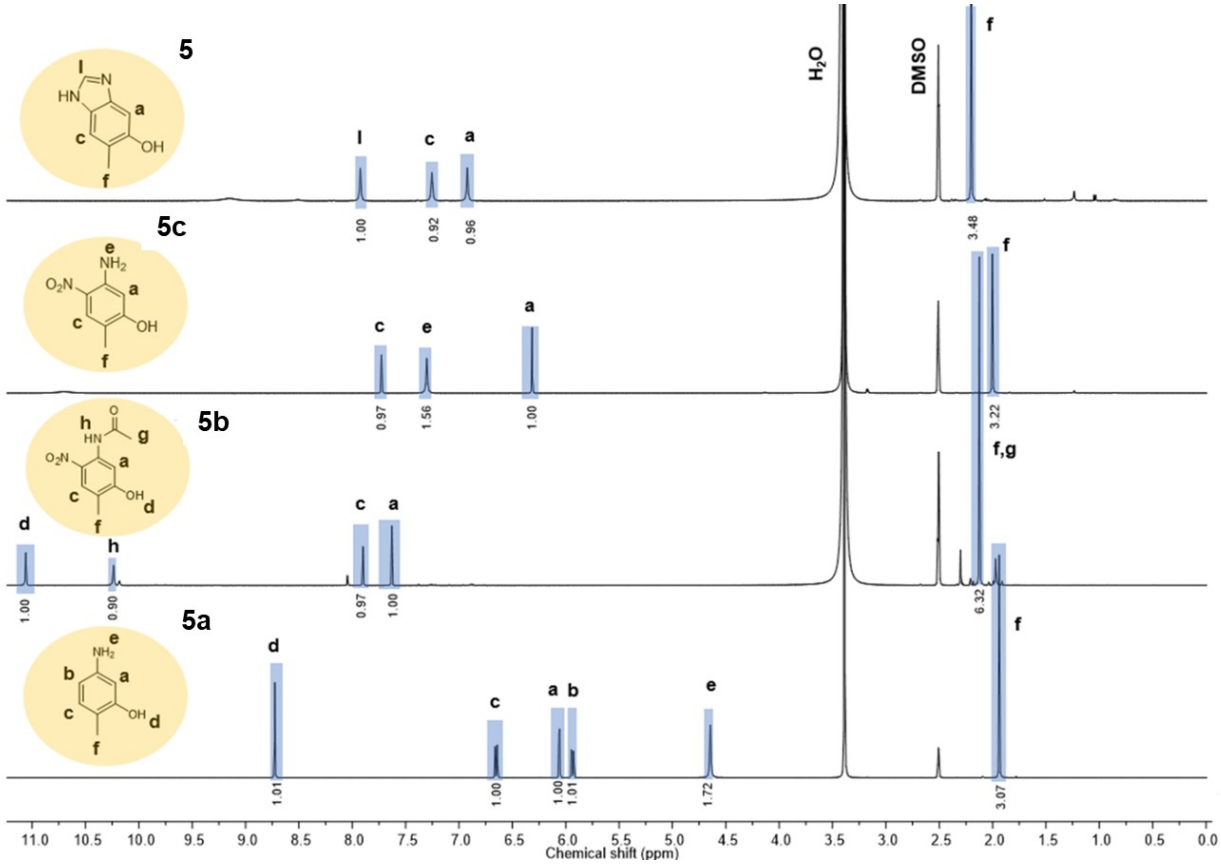


Figure S4: ^{1}H-NMR for the starting material and intermediates for the synthesis of 5-Hydroxy-6-methyl benzimidazole **(5):** From bottom to top, starting material 5-amino-2-methyl-phenol **(5a),** N-(5-hydroxy-4-methyl-2-nitrophenyl) acetamide **(5b),** 5-amino-2-methyl-4-nitrophenol **(5c),** and product 5-Hydroxy-6-methyl benzimidazole **(5)**

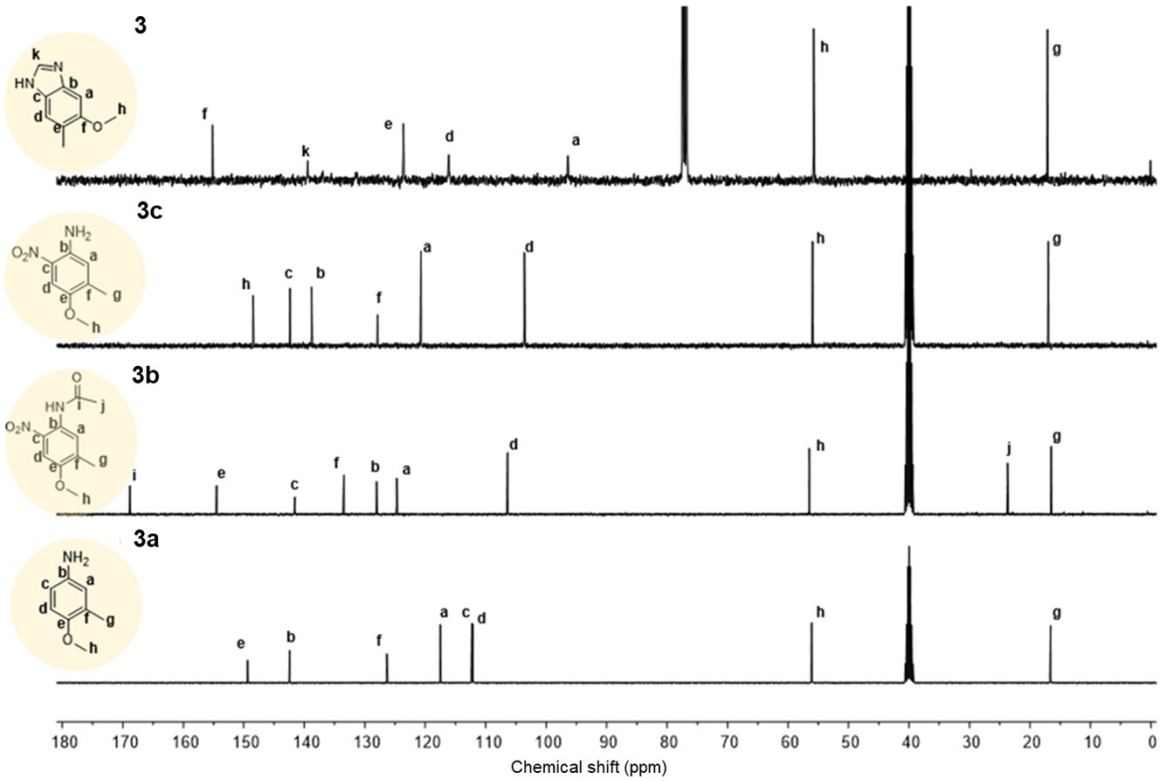


Figure S5: ^{13}C-NMR for starting material and intermediates for the synthesis of 5-Methoxy-6-methyl benzimidazole **(3):** From bottom to top, starting material 4-methoxy-3-methyl aniline **(3a),** N-(4-methoxy-5-methyl-2-nitrophenyl) acetamide **(3b)**, 2-nitro-4-methoxy 5-methyl aniline **(3c)**, and product 5-methoxy-6-methyl benzimidazole **(3)**

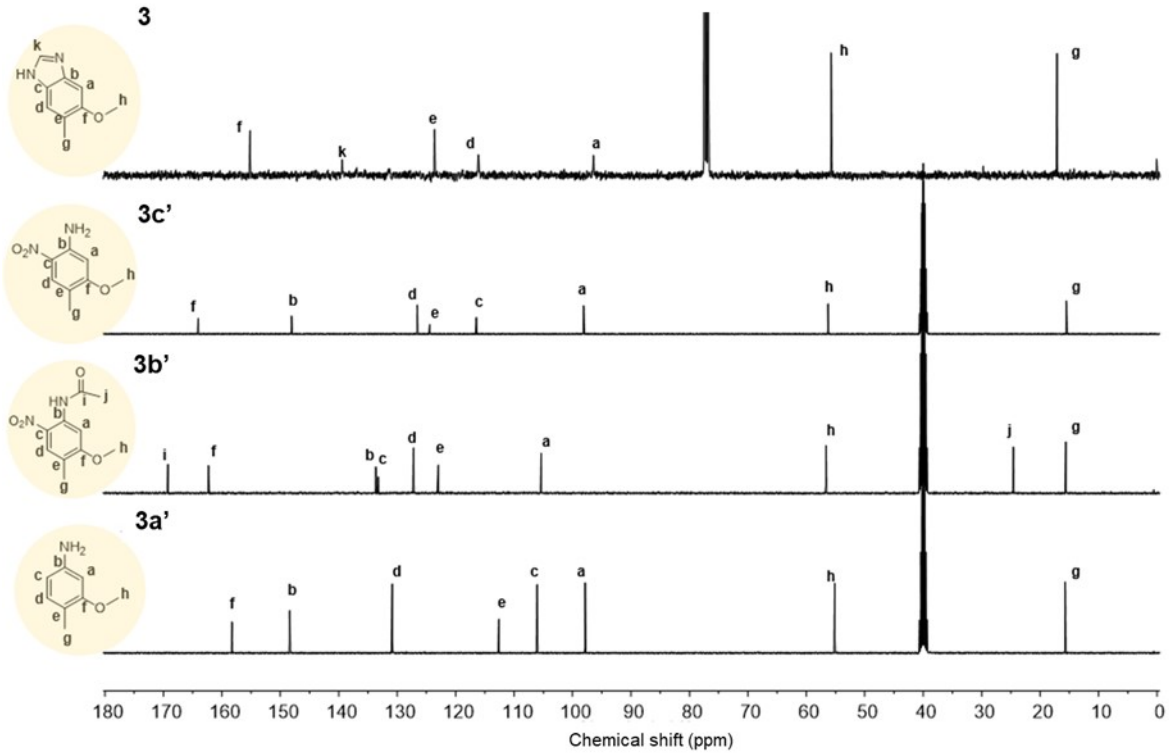


Figure S6: ^{13}C-NMR for starting material and intermediates for the synthesis of 5-Methoxy-6-methyl benzimidazole **(3):** From bottom to top, starting material 3-methoxy-4-methyl aniline **(3a'),** N-(5-methoxy-4-methyl-2-nitrophenyl) acetamide **(3b')**, 2-nitro-4-methyl 5-methoxy aniline **(3c')**, and product 5-methoxy-6-methyl benzimidazole **(3)**

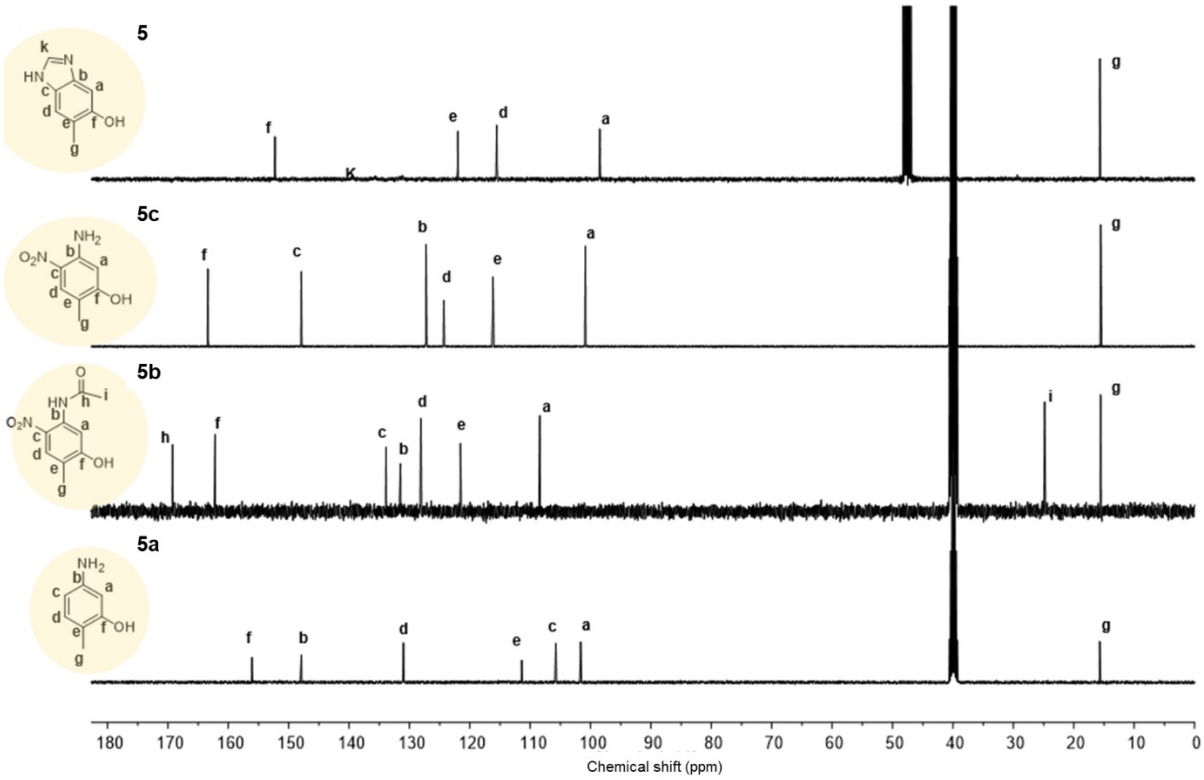
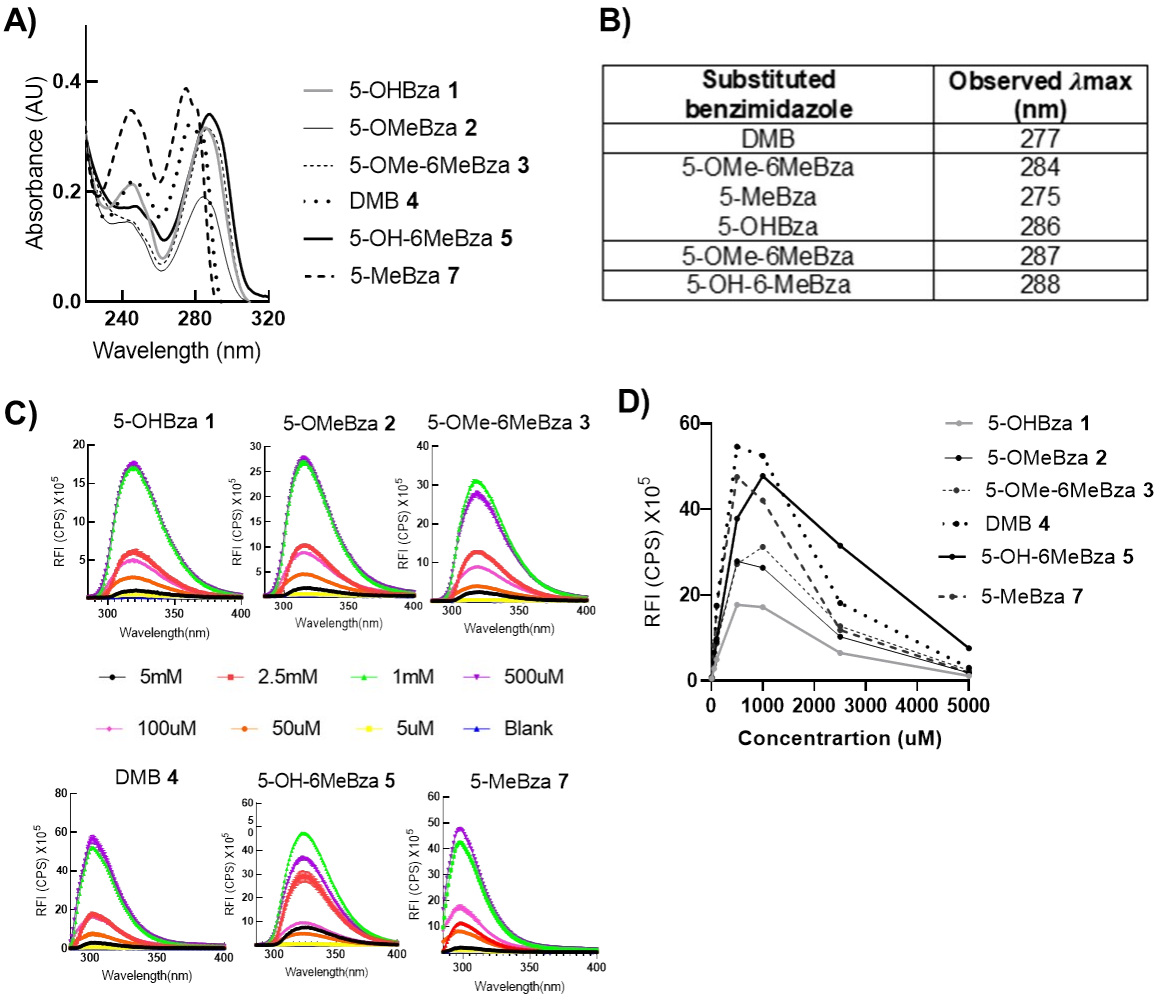


Figure S7: ^{13}C-NMR for starting material and intermediates for the synthesis of 5-hydroxy-6-methyl benzimidazole **(5):** From bottom to top, starting material 5-amino-2-methyl-phenol **(5a),** N-(5-hydroxy-4-methyl-2-nitrophenyl) acetamide **(5b),** 5-amino-2-methyl-4-nitrophenol **(5c)**, and product 5-hydroxy-6-methyl benzimidazole **(5)**



Substituted benzimidazole	Observed λmax (nm)
DMB	277
5-OMe-6MeBza	284
5-MeBza	275
5-OHBza	286
5-OMe-6MeBza	287
5-OH-6-MeBza	288

Figure S8: UV-Vis and Fluorescence analysis of benzimidazolyl intermediates **A)** UV-visible absorbance profiles of six benzimidazole intermediates (5-OHBza**1**, 5-OMeBza **2**, 5-OMe-6MeBza **3**, DMB **4**, 5-OH-6MeBza **5,** 5-MeBza **7**) involved in the study. **B)** All of them show a characteristic benzimidazole smaller peak around 243, followed by a different maximum. The observed λmax of the set is noted in the table. **C)** Emission profiles of benzimidazoles at different concentrations ranging from 5μM to 5mM while excitation at a common wavelength (243 nm). **D)** Maximum fluorescent intensity at each concentration was plotted against increasing concentration. Interestingly, fluorescent intensity increases from 5μM to 500μM, after which it remains steady at 1mM followed by a sharp decline from 1mM to 5mM. This can be attributed to fluorescence quenching at higher concentrations, as molecules starts stacking up on each other owing to its planar structural scaffold and π-π interactions.[13]

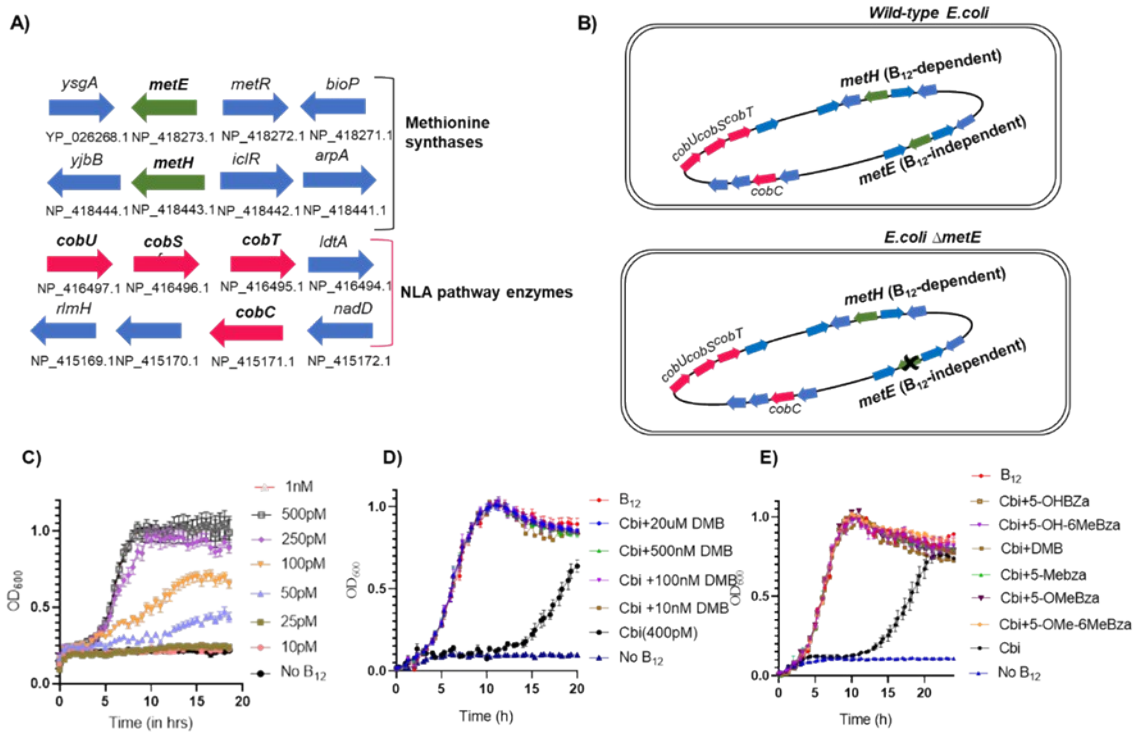


Figure S9: Supplementation studies with a cobamide auxotroph strain *E. coli* Δ*metE* to determine conditions for testing the role of synthesized benzimidazoles as potential cobamide lower ligands **A)** Genomic context of the two methionine synthase enzymes (*metE and metH*) and nucleotide loop assembly pathway (NLA) enzymes **(***cobU, cobS, CobT, cobC*) in *E. coli* **B)** Wild type *E. coli* contains B_{12}-independent methionine synthase MetE and B_{12}-dependent methionine synthase MetH in its genome. When the *metE* gene is deleted, the *E. coli* Δ*metE* strain is a B_{12} / methionine auxotroph. The *E. coli* Δ*metE* strain is the same ones used in Hazra et al., 2015 **C)** Different concentration of B_{12} provided to the cultures of B_{12} auxotrophic strain of *E. coli* Δ*metE* to understand the saturating and minimum concentration required to support the growth in M9 minimal media. **D)** Increasing concentrations of DMB with 400pM Cbi to determine saturating concentration of DMB, with no B_{12} and B_{12} supplemented controls **E)** Growth curves of *E. coli* Δ*metE* supplemented with different lower ligands (20μM each) with 400pM Cbi. We observe that only Cbi control showed delayed growth whereas all other lower ligand and B_{12} supplementation shows a similar growth trend indicating saturating concentrations of benzimidazole supplementation.

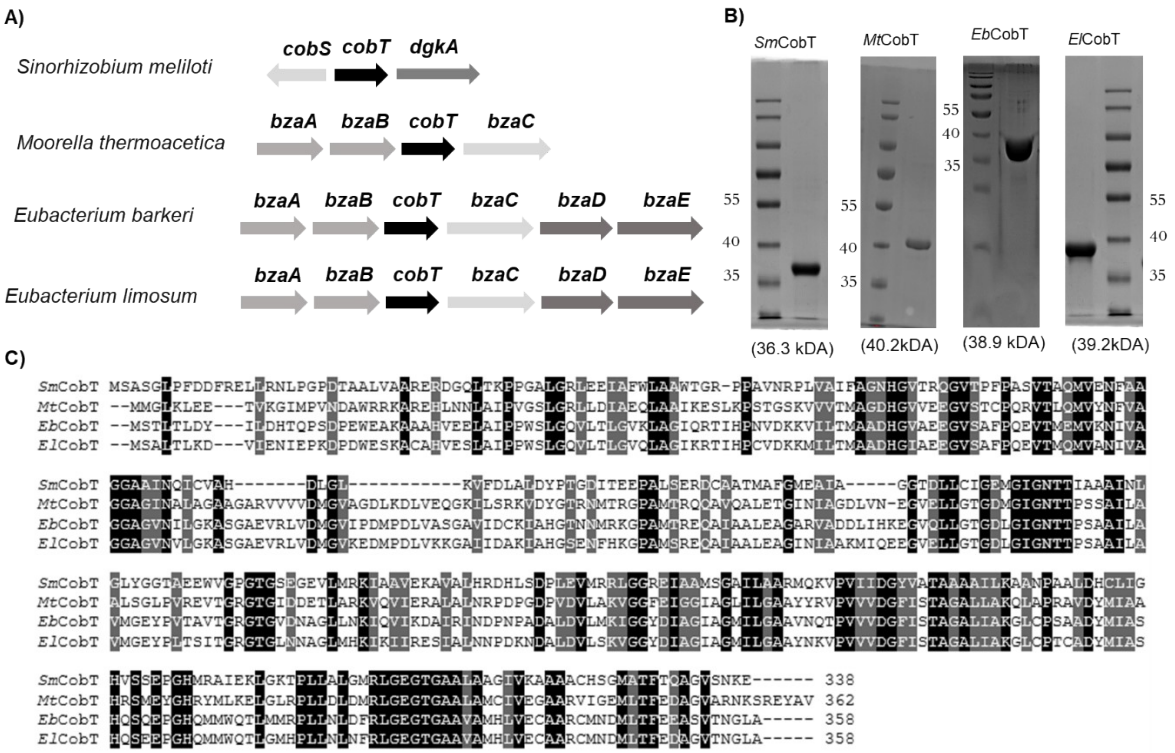


Figure S10: **A)** Gene neighborhood of the CobT homologs from *Eubacterium barkeri*, *Eubacterium limosum*, *Moorella thermoacetica*, and *Sinorhizobium meliloti.* The CobT enzyme in *E. barkeri*, *E. limosum* and *M. thermoacetica* (all strict cobamide-producing anerobic bacteria) lie within the *bza* operon, which is responsible for anerobic benzimidazole biosynthesis. The CobT from *S. meliloti* (aerobic bacterium) lies next to CobS, another enzyme involved in the assembly of the nucleotide loop of the cobamide B**)** SDS-PAGE gel of the purified CobT proteins indicating their molecular weights. **(C)** Sequence alignment of the four CobTs studied.

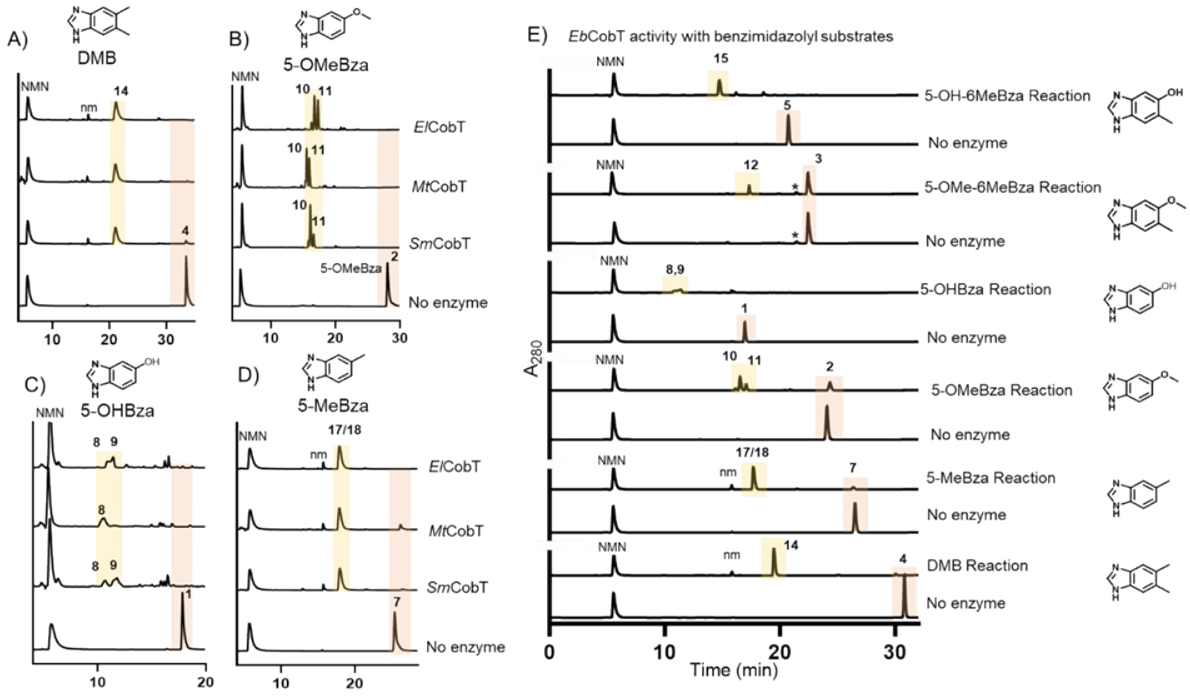


Figure S11: **A)** HPLC traces for reactions of CobT homologs (*Sm*CobT, *Mt*CobT and *El*CobT) with DMB **4 B)** with 5-OMeBza **2**, **C)** with 5-OHBza **4** and **D)** with 5-MeBza **7**. For symmetrical substrate like DMB, the reaction trace shows one product whereas in case of 5-MeBza, the current method does not resolve the possible isomers that might be forming indicating single peak for the product of reaction unlike 5-OHBza, 5-OMeBza where we can see the formation of two isomers in the HPLC traces. **E)** HPLC traces of reaction all the benzimidazolyl substrates with *Eubacterium barkeri (Eb)Eb*CobT.

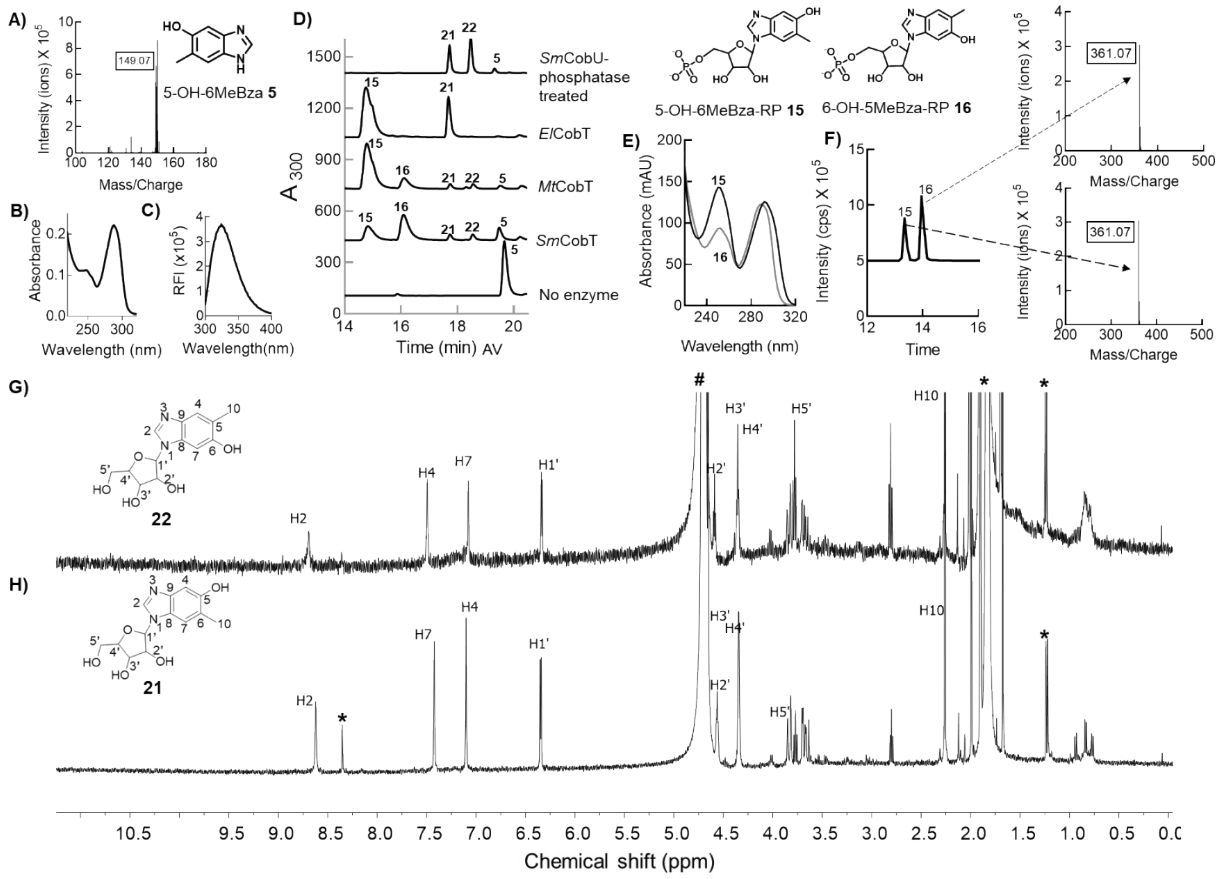


Figure S12 (related to Figure 3): Characterization of 5-OH-6MeBza **5,** 5-OH-6MeBza-RP **15** and 6-OH-5MeBza-RP **16**. A) Mass spectrum, (B) UV-visible absorption and **(C)** fluorescence emission spectra of 5-OH-6-MeBza **5** (D) HPLC chromatogram showing the two Bza-RP products formed (**15** and **16**) from reaction of 5-OH-6MeBza with CobT homologs *Sm*CobT, *Mt*CobT, and *El*CobT. Also included is the chromatogram for their dephosphorylated riboside derivatives **21** and **22** when the *Sm*CobT reaction mixture is treated with alkaline phosphatase (E) UV-vis profile and (F) LC-MS analysis of the two Bza-RP isomers showing identical mass spectra (H) ^{1}H-NMR of the two dephosphorylated riboside isomers **21**and **22**. # - H_2O peak, * - residual peaks of ammonium acetate buffer protons at 1.91 ppm along with some impurities arising during the HPLC purification process.

6. References

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