SUPPLEMENTARY MATERIAL

for

Formation of the dimethylbenzimidazole ligand of Coenzyme B_{12} under physiological conditions by a facile oxidative cascade.

Lori Maggio-Hall, Pieter C. Dorrestein,
Tadhg P. Begley* and Jorge C. Escalante-Semerena*

Abbreviations: HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; EI-MS, electron impact mass spectrometry; DMB, 5,6-dimethylbenzimidazole; BOC_2O, Di-tert-butyl dicarbonate, $NaBH_3(CN)$, sodium cyanoborohydride.

General protocols for the isolation and characterization of 5,6-dimethylbenzimidazole (4) and 1-(5,6-dimethyl-2,3-dihydro-2*H*-benzamidazol-2-yl)-butane-1,2,3,4 tetrol phosphate(12): The reactions were analyzed by TLC using 90:10 $CHCl_3$: MeOH (Silica gel 60 F_{254} Plates). The spots were visualized by UV-vis (254 nm) or with Ceric ammonium molybdenate. **4** has an Rf of 0.2, and the 4,5-dimethyl-benzene-1,2-diamine (**10**) has an Rf of 0.5 under these conditions. From an aqueous reaction mixture, **4** and **10** were readily extracted with 3 volumes of $CHCl_3$ (>95% efficiency for both), dried over Na_2SO_4 or $MgSO_4$ and evaporated under reduced pressure. When necessary, further purification was achieved using flash chromatography (silica) with 90:10 $CHCl_3$:MeOH or a gradient from 98:2 to 90:10 $CHCl_3$:MeOH.

HPLC of the reaction mixtures: Three different HPLC systems were used in this study.

HPLC 1: HPLC was performed using a Luna C18 analytical column (Phenomenex, Torrance, CA) and a Waters HPLC system equipped with a model 600 solvent delivery system and a 996 photodiode array detector. Data were analyzed using Waters Millenium™ software. The solvent system is comprised of a short descending NaCl gradient (20 mM to 0 mM in 4 minutes) is followed by a longer ascending methanol gradient (0 to 100% in 40 minutes)[1].

HPLC 2: Compound 12 was purified on a Luna C18(2) semi-preparative column and analyzed by MS and NMR spectroscopy. For this purpose, the length of the methanol gradient was extended to 60 min, with a flow rate of 4.5 ml min^{-1}. Compound 12 purified by this method was dried down under vacuum using a SpeedVac concentrator (Savant, Farmingdale, NY**)**.

HPLC 3: For analytical purposes, a LiChrospher RP18 column (Supelco, 5µM 250mm*4.6mm) and a Hewlett Packard series 1100 HPLC equipped with a diode array detector was used. The solvent gradient was 0-2 min 10% water, 40% Methanol, 50%

[1] Maggio-Hall, L. A., and Escalante-Semerena, J. C. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 11798-11803.

25mM diisopropylethylamine-acetate (DE-AC), pH 6, 2-14 min. 10% water, 40-90% MeOH and 50-0% DE-AC, 14-18 min. 10% water, 90% MeOH, 0% DE-AC, 18-25 min. 10% water, 90-40% MeOH, 0-50% DE-AC. **10** eluted after 11.5 min. and **4** eluted after 16.3 min.

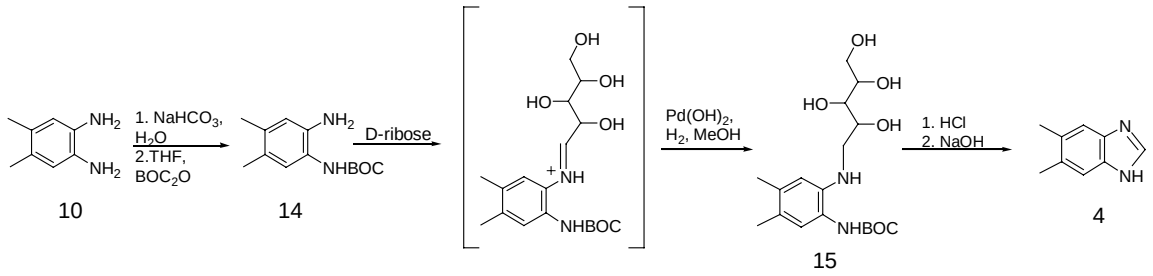

Figure 1. Synthesis of **15**.

Synthesis of 14: $NaHCO_3$ (1.04g.) was slowly added to a solution of **10** (680 mg) in 40 ml of distilled water resulting in gas evolution and the formation of a suspension. After stirring for 10 min., BOC_2O (dissolved in 30 ml THF) was added to this aqueous suspension. The resulting two-phase system was vigorously stirred for 12 hours. The organic layer was separated and the aqueous layer was extracted with THF. The THF was removed by rotary evaporation and the product was purified by flash chromatography (silica gel, 6*24 cm) using 98:2 $CHCl_3$: MeOH to give **14 as** a white solid. Yield 82%, ^{1}HNMR ($CDCl_3$, 400 MHz) 1.49 (s,9H), 2,12 (s,3H), 2.13 (s,3H), 3.58 (br.s., 2H), 6.19 (br. s., 1H), 6.56 (s, 1H), 7.01 (s, 1H). TLC (90: 10 $CHCl_3$:MeOH, Rf) 0.7, (98: 2 $CHCl_3$:MeOH, Rf) 0.4.

Synthesis of the N-BOC-protected-5-(2-amino-4,5-dimethyl-phenylamino)-pentane-1,2,3,4-tetraol (15): D-ribose (79 mg) and $Pd(OH)_2$ (5 mg, Pd/C did not work) were added to **14** (102 mg), dissolved into 7 ml of MeOH. This solution was purged with hydrogen gas for 2 hours followed by filtration through a pad (8 mm) of celite. Rotary evaporation gave a colorless oil. **15** was then purified by silica chromatography (silica, 4*12 cm) using 98:2 $CHCl_3$:MeOH followed by 90:10 $CHCl_3$:MeOH after unreacted **14** eluted, to yield 85 mg of a white solid. ^{1}HNMR (D_2O, 400 MHz) 1.28 (s, 9H), 1.96 (s, 3H), 2.03 (s, 3H), 2.94 (dd, 1H, J=2.8, J=12.8), 3.28 (dd, 1H, J=3.2, J=13.2), 3.45 (dd, 1H, J=4.8, J=12), 3.53 (t, 1H, J=6), 3.62 (m, 2H), 3.75, (m, 1H), 6.60 (s, 1H), 6.75 (s, 1H). EI-MS $(M+1)^+$ 371.2. EI-MS^2 371.2: 371.2, 315.2, 271.2. TLC (90: 10 $CHCl_3$:MeOH, Rf) 0.3, (98: 2 $CHCl_3$:MeOH, Rf) 0.1.

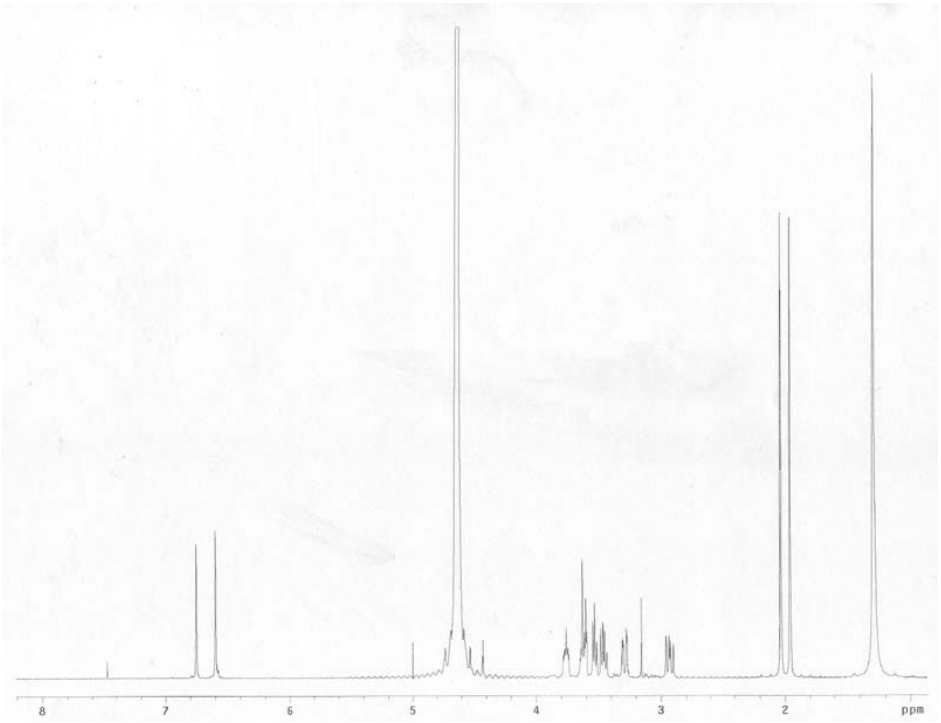

Figure 2. ^{1}HNMR of **15.**

Formation of 4 from 15: 20 mg of **14**, in 20 ml of 1M HCl, was stirred until TLC analysis (90:10 $CHCl_3$:MeOH) indicated that the reaction had gone to completion (4-8 hours). The reaction mixture was then titrated with 1M NaOH until the pH was 7-8. This solution was stirred for 5 hours at RT, extracted with 3*40ml of chloroform, and the solvent was removed by rotary evaporated. Two products, **10** and **4** were identified by MS, HPLC and NMR from the chloroform extract.

Synthesis of 4 and 12 from 10 and 11. Reaction mixtures contained **10** (10 mM) and ribose-5'-phosphate (**11**, 10 mM, The reaction also works using D-ribose,) in 50 mM 2-[4-morpholino]ethanesulfonic acid (MES) buffer pH 6. Reactions were incubated from 4 to 72 h at 37ºC and analyzed by HPLC (HPLC conditions 1). The products **4** and **12** were purified by semi-preparative HPLC (HPLC conditions 2) or purified by extraction and flash chromatography on silica. The reaction is also observed in unbuffered aqueous solutions.

4: UV-vis (λ_{max}, nm) 245, 280 and 290. EI-MS 146(100%) 145(68%) 131(59%). ESI-MS (M^+) 147 ^{1}HNMR ($CDCl_3$) 7.9 (s, 1H), 7.4 (s, 2H), 2.1(s, 6H).

12: ^{1}HNMR(D_2O): 2.21 (s, 6H), 3.55 (quin, 1H), 3.80 (m, 2H), 4.10 (dd, 1H), 5.12 (d, 1H), 7.30 (s,2H).ESI-MS(M/z, M^-): 345.2 ESI-MS^2 345.2 (M/z, M^-): 327.1, 247.1 217.1, 187.1, 169.0, 150.9. ESI-MS(M/z, M^+): MS^2 347.2(M/z, M^+): 329.1, 311.0, 267.1, 249.0, 231.1, 189.0 .

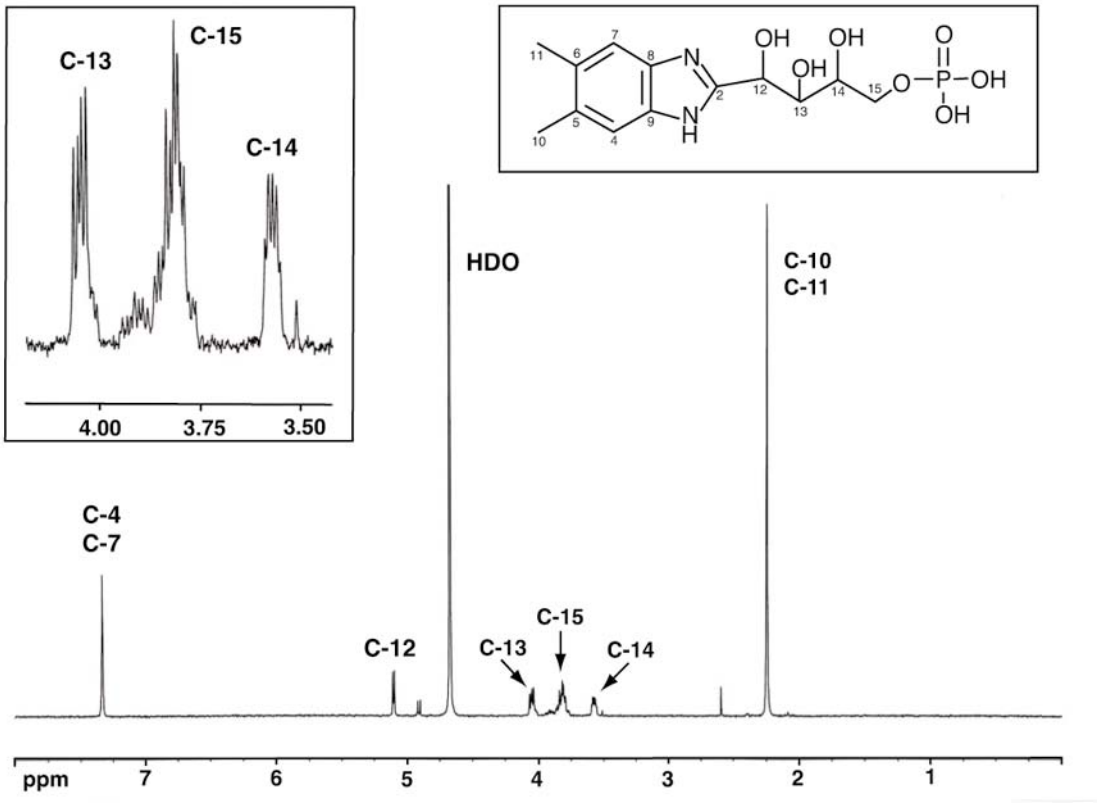

Figure 3. ^{1}HNMR of compound **12**.

Synthesis of DMB from [1-^{13}C]-ribose. An aqueous solution of **10** (5mM) and D-[1-^{13}C]ribose (5mM) was stirred at 37^0C for 72 hours in an open flask, the reaction mixture was then extracted with chloroform, the extracts were dried ($MgSO_4$) and the solvent was removed by rotary evaporation.

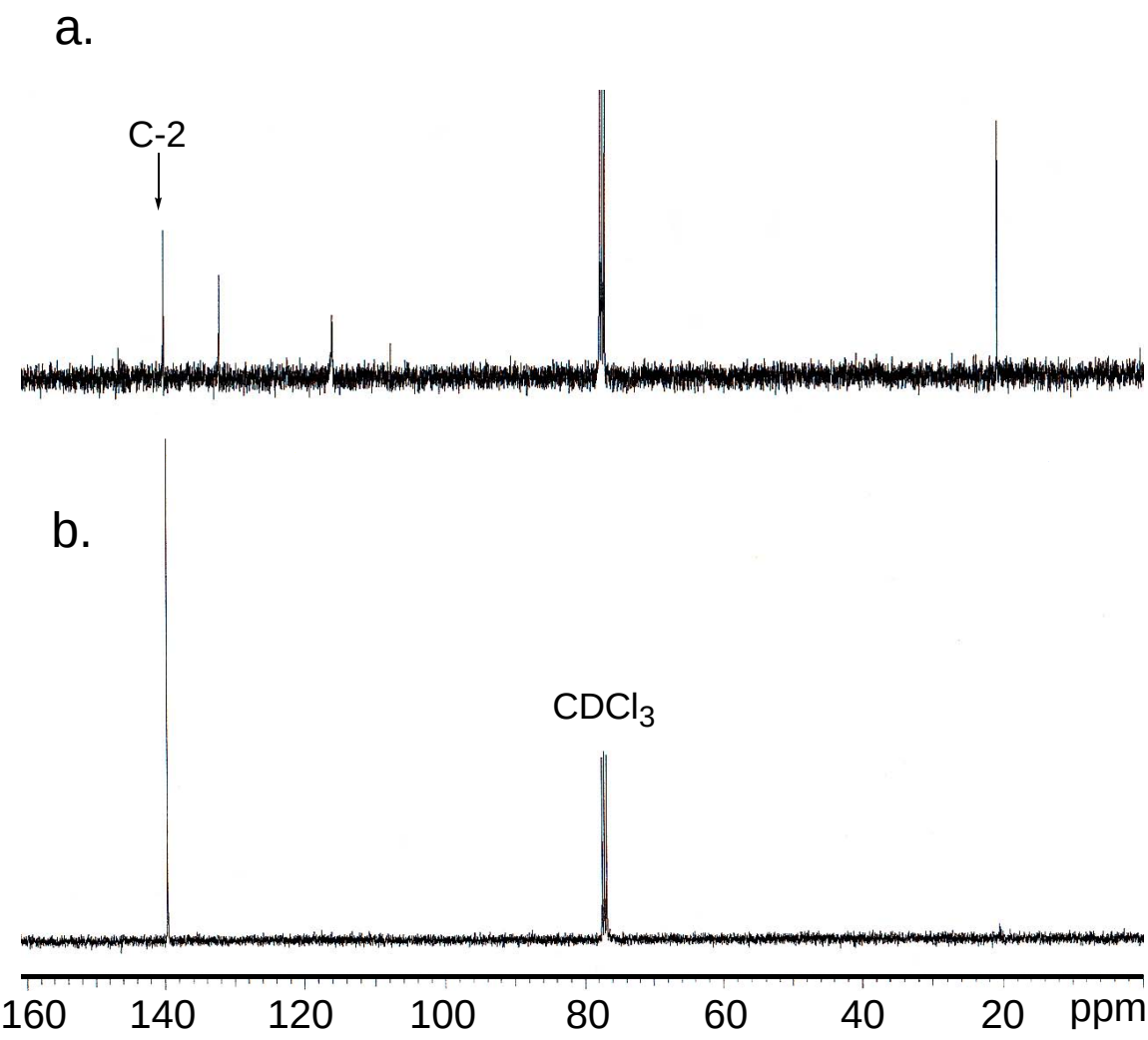

Figure 4. ^{13}CNMR analysis of **4**. a. unlabeled **4** as obtained from Aldrich. b. **4** formed by reacting [^{13}C-1]-ribose with **10**.

Synthesis of 2,5,6-trimethyl-benzimidazole: 3-hydroxy-2-butanone (10mM) in water was reacted with **10** (10mM) for 72 hours at 37^{0}C. The reaction was then extracted with chloroform, rotary evaporated and dried in vacuo. Yield 95% ^{1}HNMR ($CDCl_3$, 400 MHz) 2.32 (s, 6H), 2.55 (s, 3H), 7.24 (s, 2H) identical to Aldrich spectrum. TLC 90: 10 $CHCl_3$:MeOH, Rf) 0.35.

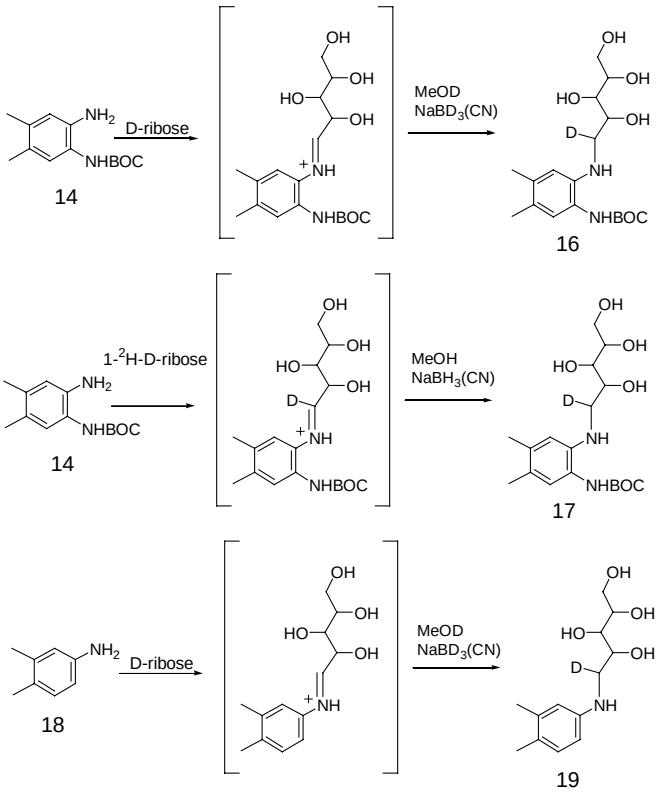

Figure 5. Synthesis of **16, 17** and **19**.

Synthesis of 16: D-ribose (100 mg) and $NaBD_3(CN)$ (50mg) were added to a solution of **14** (50 mg), dissolved in 10 ml of MeOD. After stirring at RT for 12 hours, the solvent was removed by rotary evaporation and **16** was purified by silica chromatography (4*12 cm) using first 98:2 $CHCl_3$:MeOH followed by 90:10 $CHCl_3$:MeOH once **14** eluted. After rotary evaporation, **16** was isolated as a white solid. ^{1}HNMR (D_2O, 400 MHz) 1.28 (s, 9H), 1.96 (s, 3H), 2.03 (s, 3H), 2.92 (d, 0.72H, J=12.8), 3.28 (s, 0.28H), 3.45 (dd, 1H, J=4.8, J=12), 3.53 (t, 1H, J=6), 3.62 (m, 2H), 3.75, (q, 1H), 6.60 (s, 1H), 6.75 (s, 1H). ESI-MS $(M+1)^+$ 372.3. ESI-MS^2 372.3: 372.3, 316.2, 272.4. TLC (90: 10 $CHCl_3$:MeOH, Rf) 0.3, (98: 2 $CHCl_3$:MeOH, Rf) 0.1. This synthesis is analogous to the synthesis of **19**, for which the stereochemistry has been described. We repeated this reaction and obtained almost identical stereoselectivities for both reductive amination products, **16** and **19**[2].

[2] Lingens, B.;Schild, T. A.; Vogler B. Renz, P.; Biosynthesis of vitamin B12 Transformation of riboflavin ^{2}H-labeled in the 1'R position or 1'S position into 5,6-dimethylbenzimidazole. *Eur. J. Biochem.* 207, 981-985 (1992).

Synthesis of 17: 1-^{2}H-D-ribose (0.5 mg) and $NaBD_3(CN)$ (5 mg) were added to a solution of **14** (2 mg), dissolved in 1 ml of MeOD. After stirring at RT for 24 hours, the solvent was removed by rotary evaporation and **17** was purified by silica chromatography (1*12 cm) using first 98:2 $CHCl_3$:MeOH followed by 90:10 $CHCl_3$:MeOH once **14** eluted. After rotary evaporation, **17** was isolated as a white solid. ^{1}HNMR (D_2O, 400 MHz) 1.28 (s, 9H), 1.96 (s, 3H), 2.03 (s, 3H), 2.92 (d, 0.28H, J=12.8), 3.28 (s, 0.72H), 3.45 (dd, 1H, J=4.8, J=12), 3.53 (t, 1H, J=6), 3.62 (m, 2H), 3.75, (q, 1H), 6.60 (s, 1H), 6.75 (s, 1H). ESI-MS $(M+1)^{+}$ 372.3. ESI-MS^{2} 372.3: 372.3, 316.2, 272.4. TLC (90: 10 $CHCl_3$:MeOH, Rf) 0.3, (98: 2 $CHCl_3$:MeOH, Rf) 0.1.

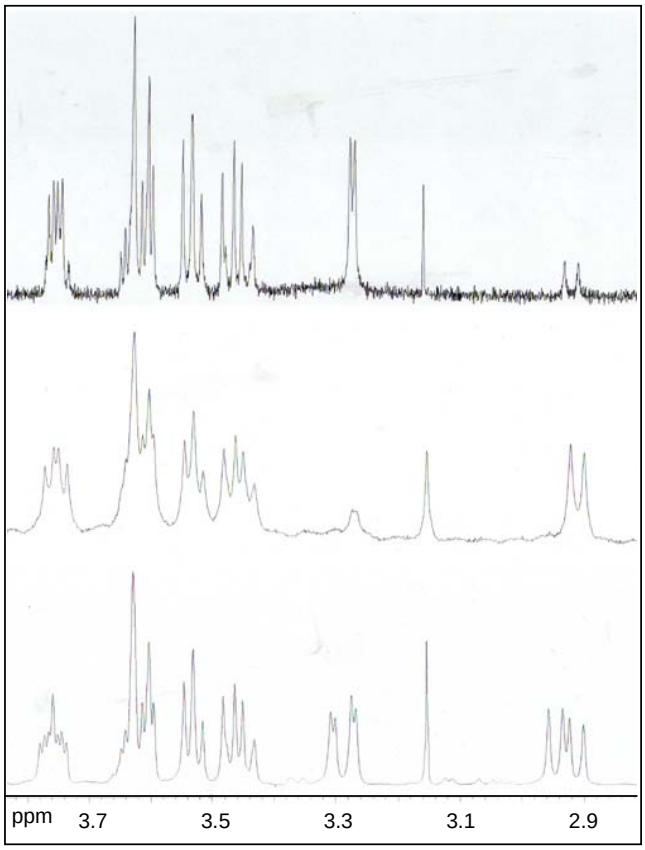

Figure 6. partial ^{1}HNMR spectrum for: Top, **17**. Middle, **16** Bottom, **15** (non-deuterated). The C_1 protons resonate at 3.3 and 2.9 ppm.

Stereochemistry of DMB formation: 1 mg of **16**, in 2 ml of 1M HCl, was stirred until TLC analysis (90:10 $CHCl_3$:MeOH) indicated that the deprotection to **3** had gone to completion (4-8 hours). The reaction mixture was then titrated with 1M NaOH until the

pH was 7-8. This solution was stirred for 5 hours at RT and extracted with 2*4ml of chloroform, rotary evaporated and the products identified by HPLC (HPLC conditions 3) and characterized by ESI-MS.

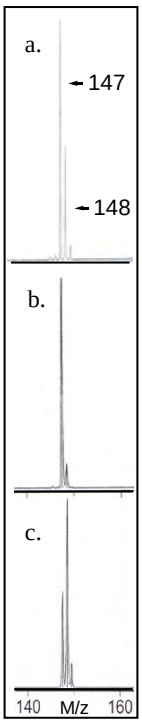

Figure 7. ESI-mass spectrum of **4**: a. Synthesized from **17** b. obtained from Aldrich ($M+H^+$=147). c. Synthesized from **16**.

H/D exchange at C-2 of 4: A 50 ml reaction mixture in D_2O was prepared containing 10mM D-ribose and 10 mM **10**. At time points 5, 10, 25, 31, and 49 hours a 10 ml aliquot was removed, extracted with chloroform, rotary evaporated and analyzed by ^{1}HNMR, The C-2 protons at 7.9 ppm were integrated (at t=0 min. this integrates to 1 proton, and decreases over time) with respect to the aromatic C5 and C8 protons of DMB at 7.4 ppm (two protons). Similarly, **4** (5 mM) dissolved in D_2O was extracted and analyzed after 0, 5, 7, 10, 25, 31, 49,and 72 hours.

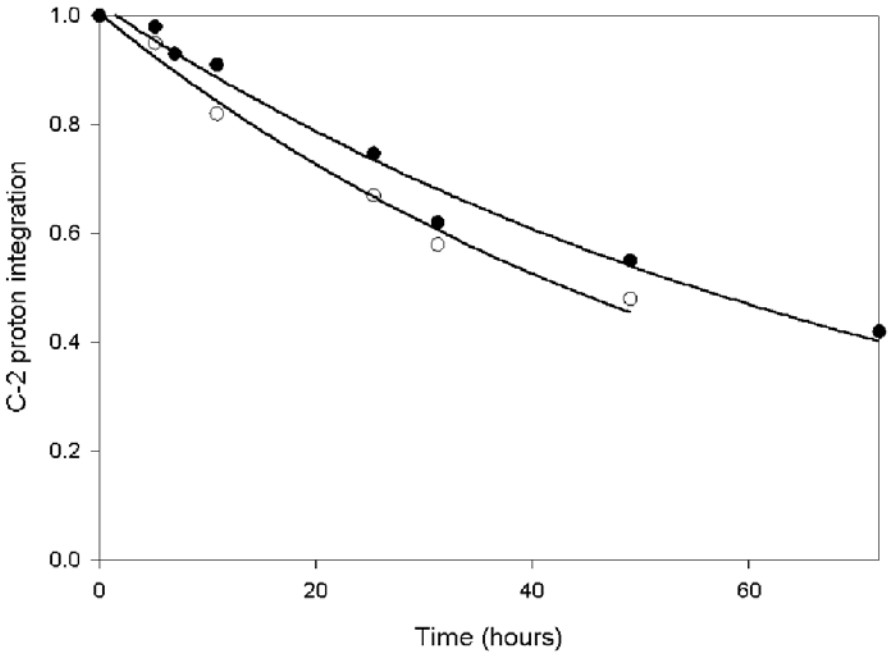

Figure 8. The time dependent proton exchange of **4** at C-2: closed circles **4** in D_2O, open circles reaction of D-ribose with **10** in D_2O. The solid lines are the fit to a first order exponentional decay function with the rates 0.013±0.001 hr^{-1} and 0.016±0.001 hr^{-1}, respectively.