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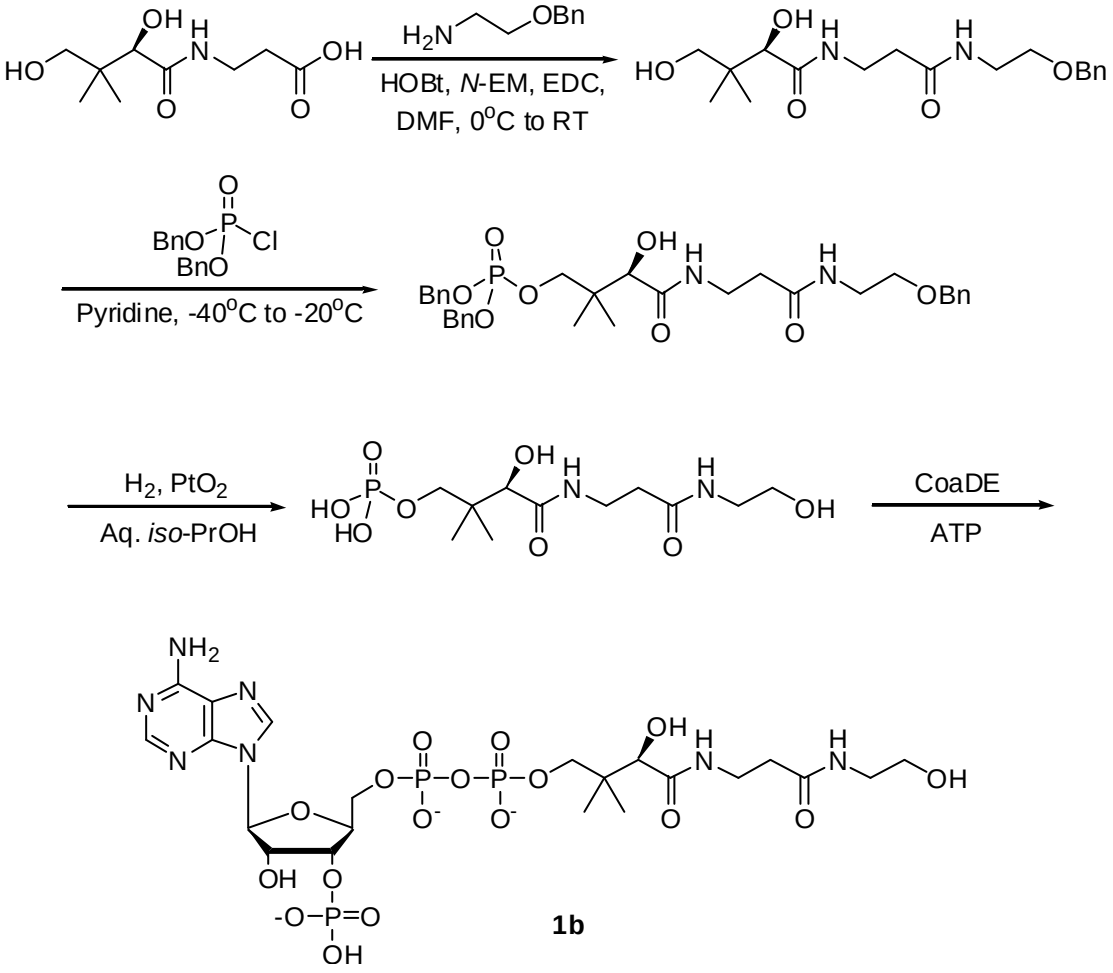
for

The Selectivity for Cysteine over Serine in Coenzyme A Biosynthesis

Erick Strauss* and Tadhg P. Begley*

Synthesis of OxyCoA (1b)



Scheme S1*: Synthesis of OxyCoA (**1b**)*

***O*-Benzyl oxypantetheine.** Sodium pantothenate (1.45 g, 6.0 mmol) was dissolved in DMF (60 mL), and HOBt (892 mg, 6.6 mmol), *N*-ethylmorpholine (72 µL, 0.6 mmol) and *O*-benzyl ethanolamine (998 mg, 6.6 mmol) were added, and the mixture was cooled to 0°C (ice bath). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC-HCl) (1.27 g, 6.6 mmol) was added, and the mixture was stirred at 0°C for 1 h. After warming to RT the mixture was stirred overnight (18 h). The solution was then diluted with ethyl acetate (250 mL), and the organic layer was sequentially washed with 1 M HCl (2x50 mL), 1 M $NaHCO_3$ (2x50 mL) and brine (1x15 mL), and was dried over Na_2SO_4, filtered and the solvent removed in vacuo. The crude mixture was purified by flash chromatography (silica gel; CH_2Cl_2/ methanol, 95:5 to 92:8) to give the product as a clear oil (444 mg, 21%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (s, 3H), 0.97 (s, 3H), 2.39 (dt, 1H), 3.40-3.47 (m, 2H), 3.44 (d, 2H), 3.50-3.56 (m, 2H), 3.90 (s, 1H), 4.49 (s, 2H), 6.01 (b, 1H), 7.24-7.37 (arom, 5H).

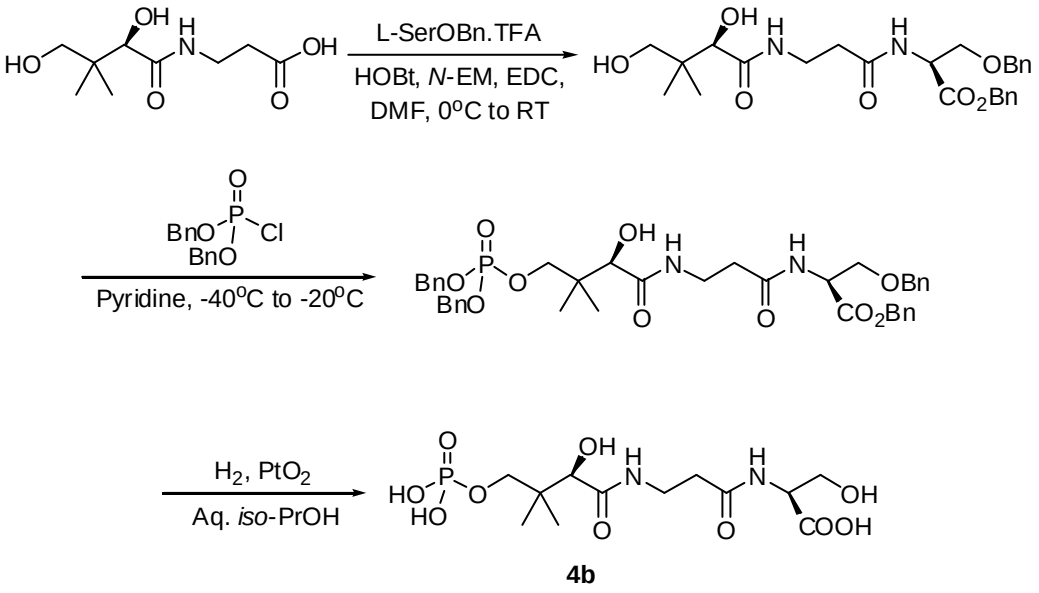
***O*-Benzyl oxypantetheine 4-*O*,*O*-dibenzylphosphate.** Dibenzylchlorophosphate was prepared *in situ* by reacting dibenzylphosphite (tech., 85%) (1.24 g, 3.8 mmol) and *N*-chlorosuccinimide (504 g, 3.8 mmol) in dry benzene (5 mL) for 2 h at RT. The reaction mixture was filtered to remove the succinimide and the filtrate added dropwise to a solution of *O*-benzyl oxypantetheine (414 mg, 1.17 mmol) in dry pyridine (5.5 mL) at -40°C (dry ice/acetonitrile). After stirring at -40°C for 2h, the mixture was placed in a -20°C freezer overnight. The reaction was allowed to warm to room temperature and was subsequently quenched with water (4 mL). The solvent was removed *in vacuo* and ethyl acetate (30 mL) was added. The resulting suspension was washed with 1 M H_2SO_4 (2x6 mL), 1 M $NaHCO_3$ (2x6 mL) and saturated Na_2SO_4 (1x6 mL), dried (Na_2SO_4), and the solvent was removed. The product was purified by flash column chromatography (silica gel; CH_2Cl_2/methanol, 97:3 to 95:5) to give the product as a colorless oil (345 mg, 48% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.75 (s, 3H), 1.04 (s, 3H), 2.38(dt, 2H), 3.42-3.54 (m, 7H), 3.85 (s, 1H), 4.00 (dd, 1H), 4.95-5.05 (m, 4H), 7.25-7.35 (arom, 15H).

Oxypantetheine 4′-phosphate. A solution of *O*-benzyl oxypantetheine 4′-*O*,*O*-dibenzylphosphate (345 mg, 563 µmol) in isopropanol/water (4:1, 7.5 mL) was hydrogenated for 6.5 h at RT and atmospheric pressure in the presence of PtO_2 (280 mg) as catalyst. The catalyst was removed by centrifugation, washed with isopropanol/water (4:1), and the solvent removed from the combined washings *in vacuo*. The residue was dissolved in water and lyophilized to give the product as a clear glass (186 mg, 97%). The product was dissolved in H_2O, titrated to pH ~6.5 with 1 M NaOH and stored as frozen

aliquots of a stock solution (200 mM) at -20°C. ^{1}H NMR (400 MHz, D_2O): δ 0.69 (s, 3H), 0.76 (s, 3H), 2.30 (t, 2H), 3.10 (t, 2H), 3.30 (t, 2H), 3.40 (dd, 1H), 3.43 (t, 2H), 3.61 (dd, 1H), 3.81 (s, 1H).

OxyCoA (1b). Reaction mixtures (600 μL) contained oxypantetheine 4′-phosphate (15 mM), ATP (28 mM), $MgCl_2$ (10 mM), CoaD (60 μg) and CoaE (90 μg) in Tris-HCl buffer (50 mM, pH 7.6). Reactions were initiated by addition of the biosynthetic enzymes, incubated for 2 hours at 37°C, stopped by transferring the reaction to 95°C for 5 minutes, and the precipitated protein was removed by centrifugation (13,000 rpm for 5 min). The supernatants of two identical reaction mixtures were combined and loaded onto a single DEAE-cellulose column (1x25 cm) pre-equilibrated with NH_4HCO_3 (50 mM), and the column was eluted at 1.5 mL/minute with a 600 mL gradient of NH_4HCO_3 (50 mM to 300 mM). The chromatography was monitored at A_{254}. The product eluted as the last fraction from the column at ~180 mM NH_4HCO_3. The product-containing fractions were combined and lyophilized, dissolved in water and lyophilized again. This was repeated until a constant weight of product was achieved. Yield: 13 mg (tetra-ammonium salt) (94%). ^{1}H NMR (400 MHz, D_2O): δ 0.55 (s, 3H), 0.68 (s, 3H), 2.26 (t, 2H), 3.05 (t, 2H), 3.26 (t, 2H), 3.34 (dd, 1H), 3.42 (t, 2H), 3.62 (dd, 1H), 3.81 (s, 1H), 4.02-4.06 (m, 3H), 4.37-4.40 (m,2H), 5.97 (d, 1H), 8.06 (s, 1H), 8.35 (s, 1H).

Synthesis of 4′-phosphopantothenoylserine (4b)



Scheme S2. *Synthesis of 4'-phosphopantothenoylserine,* ***4b***.

***O*-Benzyl serine benzyl ester.** *N*-(tBoc) *O*-benzyl serine (500 mg, 1.69 mmol) was dissolved in 1 M NaOH (1.7 mL), and the solvent was removed *in vacuo*. The residue was dissolved in DMF (6.5 mL), and benzyl bromide (202 μL, 1.70 mmol) added. The reaction mixture was heated at 70°C overnight, and the amount of solvent reduced *in vacuo*. Ethyl acetate (50 mL) was added, and the solution washed with brine (4x10 mL), dried over Na_2SO_4, and filtered. The solvent was removed *in vacuo* to give the intermediate product as a clear oil (508 mg), which was dissolved in trifluoroacetic acid/CH_2Cl_2 (1:1) (15 mL) and stirred at 0°C for 1 h. The solvent was removed *in vacuo* the give the final product as the trifluoroacetate salt (557 mg, 83%).

***O*-Benzyl *N*-pantothenoyl serine benzyl ester.** Sodium pantothenate (362 mg, 1.5 mmol) was dissolved in DMF (15 mL), and HOBt (223 mg, 1.65 mmol), *N*-ethyl morpholine (185 μL, 1.54 mmol) and *O*-benzyl serine benzyl ester (trifluoroacetate salt) (557 mg, 1.39 mmol) added, and the mixture cooled to 0°C (ice bath). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (316 mg, 1.65 mmol) was added, and the mixture stirred at 0°C for 1 h. After warming to RT the mixture was stirred overnight (18 h). The solution was diluted with ethyl acetate (75 mL), and the organic layer was sequentially washed with 1 M HCl (2x15 mL), 1 M $NaHCO_3$ (2x15 mL) and brine (1x15 mL), and was dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. The crude mixture was purified by flash chromatography (silica gel; CH_2Cl_2/methanol 90:10) to give the product as a clear oil (540 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (s, 3H), 0.99 (s, 3H), 2.37-2.45 (m, 1H), 2.52-2.57 (m, 1H), 3.37-3.41 (m, 2H), 3.46 (s, 2H), 3.62-3.70 (m, 2H), 3.90-3.93 (m, 1H), 3.94 (s, 1H), 4.42 (q, 2H), 4.71-4.75 (m, 1H), 5.14 (q, 2H), 6.47 (b, 1H), 7.17 (b, 1H), 7.24-7.35 (arom, 10H).

***O*-Benzyl *N*-(pantothenoyl 4′-*O,O*-dibenzylphosphate) serine benzyl ester.** The phosphorylation was performed as for *O*-benzyl oxypantetheine 4′-*O*,*O*-dibenzylphosphate, using *O*-Benzyl *N*-pantothenoyl serine benzyl ester (540 mg, 1.11 mmol) as starting material. The crude product was purified by flash chromatography (silica gel; CH_2Cl_2/methanol 94:6) to give the product as a clear oil (199 mg, 24%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.76 (s, 3H), 1.03 (s, 3H), 2.34-2.44 (m, 1H), 2.49-2.52 (m, 1H), 3.47-3.56 (m, 2H), 3.58-3.64 (dq, 2H), 3.85-3.87 (m, 1H), 3.89 (s, 1H), 4.00-4.04 (m, 1H), 4.42 (q, 2H), 4.74-4.76 (m, 1H), 4.98-5.03 (m, 4H), 5.08-5.17 (m, 2H), 6.74 (b, 1H), 7.16 (b, 1H), 7.24-7.38 (arom, 20H).

4′-Phosphopantothenoylserine (4b). A solution of *O*-benzyl *N*-(pantothenoyl *4′-O,O*-dibenzylphosphate) serine benzyl ester (195 mg, 261 μmol) in isopropanol/water (4:1) (7.5 mL) was hydrogenated for 6.5 h at RT and atmospheric pressure in the presence of PtO_2 (280 mg) as catalyst. The catalyst was removed by centrifugation, washed with isopropanol/water (4:1), and the solvent removed from the combined washings *in vacuo*. The residue was dissolved in water and lyophilized to give **4b** as a clear glass (126 mg, 97%). The product was dissolved in H_2O, titrated to pH ~6.5 with 1 M NaOH and stored as frozen aliquots of a stock solution (200 mM) at -20°C. ^{1}H NMR (400 MHz, D_2O): 0.68 (s, 3H), 0.76 (s, 3H), 2.39 (t, 2H), 3.32 (t, 2H), 3.36 (dd, 1H), 3.58 (dd, 1H), 3.64-3.77 (dq, 2H), 3.83 (s, 1H), 4.30 (m, 1H).

HPLC conditions

Separation of CoA's was effected using 100 mM potassium phosphate, pH 6.6 with increasing amounts of methanol as eluant, according to the following gradient profile: 0-4 min., 2% MeOH; 4-6 min., 2-4% MeOH; 6-8 min., 4% MeOH; 8-10 min., 4-8% MeOH; 10-15 min., 8% MeOH; 15-18 min., 8-20% MeOH; 18-22 min., 20% MeOH; 22-23 min., 20-25% MeOH; 23-25 min., 25% MeOH.

For separation of *4′*-phosphopantothenoylserine (**4b**) 100 mM potassium phosphate, pH 6.6 was used with increasing amounts of methanol as eluant, according to the following gradient profile: 0-10 min., 0% MeOH; 10-15 min., 0-20% MeOH; 15-20 min., 20% MeOH; 20-22 min., 20-30% MeOH; 22-25 min., 30% MeOH.