

Synthesis, Synchrotron X-ray Diffraction, and Kinetic Studies on the Formation of a Novel Thiolatocobalamin of Captopril: Evidence for *cis-trans* Isomerization in the β -Axial Ligand.

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The orally administered therapeutic captopril is widely used for treating hypertension, congestive heart failure, and cardiovascular disease. However, a number of undesirable side effects are associated with high doses of captopril. By coordinating a therapeutic to the upper ($=\beta$) axial site of the naturally occurring macrocycle cobalamin (vitamin B₁₂), the absorption and cellular uptake of the therapeutic can be significantly enhanced. We report the synthesis of captopril-cobalamin, a derivative of vitamin B₁₂ in which captopril is bound via its thiol group at the β -axial site of cobalamin. Characterization of captopril-cobalamin by ¹H NMR spectroscopy and X-ray diffraction shows that captopril-cobalamin exists in both solution and the solid state as a mixture of geometric isomers. Kinetic studies on the formation of captopril-cobalamin have been carried out, and the data fits a model in which the thiol form (RSH, $k_1 = 40.9 \pm 1.2 \text{ M}^{-1} \text{ s}^{-1}$) and the thiolate form of captopril (RS⁻, $k_2 = 660 \pm 170 \text{ M}^{-1} \text{ s}^{-1}$) react rapidly with aquacobalamin.

Introduction

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a zinc-containing dipeptidyl carboxypeptidase^{1,2} on the luminal surface of vascular endothelium and the epithelial cells of several organs, including heart, lung, and kidneys.^{3–5} ACE cleaves the carboxyl end of the inactive decapeptide angiotensin I (Ang I) to form angiotensin II (Ang II), a potent vasoconstrictor. ACE also cleaves and inactivates the vasodilator bradykinin,⁶ and these actions result in raised blood pressure.² ACE inhibitors are widely used to treat high blood pressure, congestive heart failure, and cardiovascular disease.⁷ They can also attenuate endothelial dysfunction and reduce organ damage including diabetes-associated kidney disease.^{7,8}

Captopril (1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline, Scheme 1) is the prototypic ACE inhibitor.¹ It inactivates ACE by binding via its sulphhydryl group to the zinc center.⁵ Its effects are short-lived and several daily doses are required.⁹ Furthermore, common side effects include a dry cough,⁹ rash, and taste disturbances which are attributed to the thiol group.¹⁰ Administering captopril can also lead to a zinc and/or copper deficiency,¹¹ since captopril binds strongly to both these metal cations.^{11–14} These adverse effects and pharmacokinetic limitations stimulated the development of enalapril and other ACE inhibitors lacking the sulphhydryl moiety.¹⁵ However, sulphhydryl ACE inhibitors may be advantageous in reducing cardiac impairment.¹⁶

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(1) Kadin, H. *Analytical profile of Drug substances*; Florey, K., Ed.; Academic Press: New York, 1982; Vol. 11, pp 79–137.

(2) Erdos, E. G. *FASEB J.* 2006, 20, 1034–1038.

(3) Ryan, J. W.; Ryan, U. S.; Schultz, D. R.; Whitaker, C.; Chung, A.; Dorer, F. E. *Biochem. J.* 1975, 146, 497–499.

(4) Lavoie, J. L.; Sigmund, C. D. *Endocrinology* 2003, 144, 2179–2183.

(5) Williams, T. A.; Barnes, K.; Kenny, A. J.; Turner, A. J.; Hooper, N. M. *Biochem. J.* 1992, 288, 875–881.

(6) Ceconi, C.; Francolini, G.; Olivares, A.; Comini, L.; Bachetti, T.; Ferrari, R. *Eur. J. Pharmacol.* 2007, 577, 1–6.

(7) Werner, C.; Baumhaekel, M.; Teo, K. K.; Schmieder, R.; Mann, J.; Unger, T.; Yusuf, S.; Boehm, M. *Clin. Res. Cardiol.* 2008, 97, 418–431.

(8) Hanif, K.; Snehlati; Pavar, M. C.; Arif, E.; Fahim, M.; Pasha, M. A. Q.; Pasha, S. J. *Cardiovasc. Pharmacol.* 2009, 53, 145–150.

(9) Richart, T.; Staessen, J. A.; Birkenhager, W. H. *Drugs* 2007, 67, 1379–1382.

(10) Havelka, J.; Boerlin, H. J.; Studer, A.; Greminger, P.; Tenschert, W.; Luescher, T.; Siegenthaler, W.; Vetter, W.; Walger, P.; Vetter, H. *Br. J. Clin. Pharmacol.* 1982, 14, 71S–76S.

(11) Christie, G. L.; Hughes, M. A.; Rees, S. B.; Williams, D. R. *Inorg. Chim. Acta* 1988, 151, 215–225.

(12) Hughes, M. A.; Smith, G. L.; Williams, D. R. *Inorg. Chim. Acta* 1985, 107, 247–252.

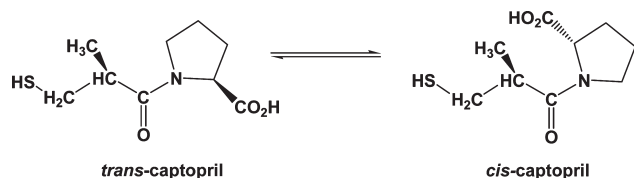
(13) Atzei, D.; De Filippo, D.; Rossi, A.; Lai, A.; Saba, G.; Bucci, R. *Spectrochim. Acta, Part A* 1992, 48, 911–919.

(14) Torreggiani, A.; Di Foggia, M.; Manco, I.; De Maio, A.; Markarian, S. A.; Bonora, S. *J. Mol. Struct.* 2001, 565, 347–352.

(15) Patchett, A. A.; Harris, E.; Tristram, E. W.; Wyratt, M. J.; Wu, M. T.; Taub, D.; Peterson, E. R.; Ikeler, T. J.; Ten Broeke, J. *Nature* 1980, 288, 280–283.

(16) Evangelista, S.; Manzini, S. *J. Int. Med. Res.* 2005, 33, 42–54.

Scheme 1



Sulfhydryl ACE inhibition also stimulates nitric oxide activity and reduces oxidative stress in endothelial cells and hypertensive patients.^{17,18}

Cobalamins (CbIs) comprise a central cobalt atom surrounded by a tetrapyrrole macrocycle called the corrin ring and incorporating an intramolecularly coordinated nucleotide which binds to the central cobalt at a lower (α) axial site via a 5,6-dimethylbenzimidazole. The upper (β) axial ligand is variable and can be labile if the ligand is inorganic (Figure 1); that is, bound to the Co via an atom other than carbon. Cobalamins are only synthesized by microorganisms such as bacteria, yeast, and algae.^{19,20} In higher animals they are essential for hematopoiesis and growth and maintenance of the nervous system.²¹ Cobalamins assimilated from the diet are transported to cells by specific carrier proteins.²¹ They are then internalized and converted intracellularly to two derivatives with coenzyme function, adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl).

Thiol derivatives of CbIs, thiolatocobalamins ($X = \text{RS}$, Figure 1), have interested us for many years. Examples include derivatives of glutathione,²² cysteine,²³ cyclohexylthiol,²³ D,L-homocysteine,²⁴ N-acetyl-L-cysteine,²⁴ 2-N-acetylamino-2-carbomethoxy-L-ethanethiol,²⁴ γ -glutamylcysteine,^{25a} and pentafluorothiophenol.²⁶ Glutathionylcobalamin (GSCbl) is a naturally occurring RSCbl.^{27–30} Interest in RSCbIs was heightened with the observation by one of us (A.M.) that patients with cognitive impairment respond better to a thiol/aquacobalamin combination compared with

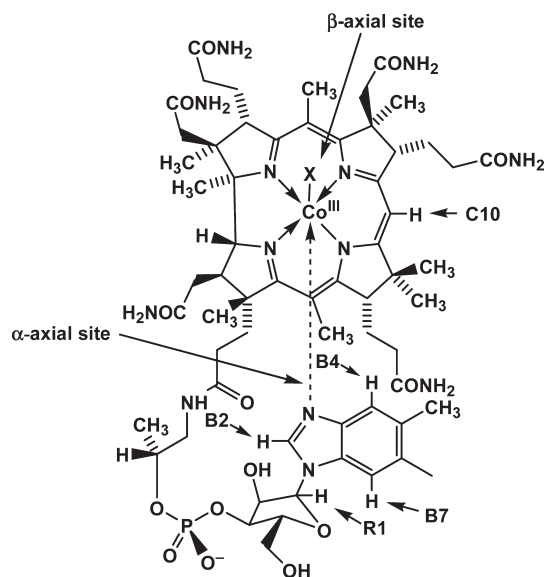


Figure 1. Structure of cobalamins (vitamin B₁₂ derivatives). X = CN, Me, Ado, RS, and so forth.

aquacobalamin³¹ alone.^{32–34} RSCbIs also appear to have superior antioxidant properties compared with other Cbl forms.³⁵

Absorption and delivery of therapeutics or imaging agents to cells can be enhanced by coordination to the β -axial site of CbIs.^{36–41} In addition, the β axial ligand of cobalamin remains intact until released intracellularly by enzymes or nucleophiles such as glutathione.⁴¹ We wish to report the synthesis, characterization, and kinetic studies on the formation of the thiolatocobalamin derivative of captopril, “captopril-cobalamin” (CapSCbl). By binding captopril to Cbl, the absorption, cellular uptake and tissue penetration may potentially be enhanced in addition to limiting adverse reactions arising from its sulfhydryl group. X-ray diffraction and NMR spectroscopy evidence is provided for the formation of two isomers of CapSCbl which differ in the stereochemistry of the captopril ligand.

Experimental Section

Hydroxycobalamin hydrochloride (HOCbl·HCl, 98% stated purity by manufacturer) was purchased from Fluka. The percentage of water in HOCbl·HCl ($\cdot n\text{H}_2\text{O}$) (batch-dependent, typically 10–15%) was determined by converting HOCbl·HCl to dicyanocobalamin, $(\text{CN})_2\text{Cbl}^-$ (0.10 M KCN, pH 10.5, $\epsilon_{368} = 30.4 \text{ mM}^{-1} \text{ cm}^{-1}$).⁴² Captopril (98%) was purchased from Acros. MES (99%), TES (99%), CAPS (99%), and CHES (99.5%) buffers were from either Sigma or

(17) Zhang, X.; Xie, Y.-W.; Nasjletti, A.; Xu, X.; Wolin, M. S.; Hintze, T. H. *Circulation* **1997**, *95*, 176–182.

(18) Pasini, A. F.; Garbin, U.; Nava, M. C.; Stranieri, C.; Pellegrini, M.; Boccioletti, V.; Luchetta, M. L.; Fabrizzi, P.; Cascio, L.; Cominacini, L. *Am. J. Hypertens.* **2007**, *20*, 443–450.

(19) Beck, W. S. *Handbook of Vitamins*, 3rd ed.; Rucker, R. B., Suttie, J. W., McCormick, D. B., Machlin, L. J., Eds.; Marcel Dekker: New York, 2001; p 470.
(20) Perlman, D. *Advances in Applied Microbiology*; Umbreit, W. W., Ed.; Academic Press: New York, 1959; Vol. 1, p 87.

(21) Banerjee, R. *Chemistry and Biochemistry of B₁₂*; John Wiley & Sons, Inc.: New York, 1999; pp 350–351.

(22) Brasch, N. E.; Xia, L. *U.S. Patent Applic.* 20040054128, **2004**.

(23) Brasch, N. E.; Hsu, T.-L. C.; Doll, K. M.; Finke, R. G. *J. Inorg. Biochem.* **1999**, *76*, 197–209.

(24) Suarez-Moreira, E.; Hannibal, L.; Smith, C. A.; Chavez, R. A.; Jacobsen, D. W.; Brasch, N. E. *Dalton Trans.* **2006**, 5269–5277.

(25) (a) Suto, R. K.; Brasch, N. E.; Anderson, O. P.; Finke, R. G. *Inorg. Chem.* **2001**, *40*, 2686–2692. (b) Randaccio, L.; Geremia, S.; Stener, M.; Toffoli, D.; Zangrando, E. *Eur. J. Inorg. Chem.* **2002**, *1*, 93–103.

(26) Hsu, T.-L. C.; Brasch, N. E.; Finke, R. G. *Inorg. Chem.* **1998**, *37*, 5109–5116.

(27) Pezacka, E.; Green, R.; Jacobsen, D. W. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 443–50.

(28) Pezacka, E. H.; Green, R.; Jacobsen, D. W. *FASEB J.* **1990**, *4*, A2126.

(29) Jacobsen, D.; Lee-Denison, C.; Luce, K.; Green, R. *Fed. Proc.* **1987**, *46*, 1005.

(30) Hannibal, L.; Axhemi, A.; Glushchenko, A. V.; Suarez-Moreira, E.; Brasch, N. E.; Jacobsen, D. W. *Clin. Chem. Lab. Med.* **2008**, *46*, 1739–1746.

(31) The names hydroxycobalamin (HOCbl) and aquacobalamin (H_2OCbl^+) are often used interchangeably.

(32) McCaddon, A. *Wales Patent Applic.* WO2002GB01843, **2002**.

(33) McCaddon, A.; Davies, G. *Int. J. Geriatr. Psych.* **2005**, *20*, 998–1000.

(34) McCaddon, A. *Nutr. J.* **2006**, *5*, 6.

(35) Birch, C. C.; Brasch, N. E.; McCaddon, A.; Williams, J. H. H. *Free Radical Biol. Med.* **2009**, *47*(2), 184–188.

(36) Bagnato, J. D.; Eilers, A. L.; Horton, R. A.; Grissom, C. B. *J. Org. Chem.* **2004**, *69*, 8987–8996.

(37) Chalasani, K. B.; Russell-Jones, G. J.; Yandrapu, S. K.; Diwan, P. V.; Jain, S. K. *J. Control. Release* **2007**, *117*, 421–429.

(38) Mundwiler, S.; Spingler, B.; Kurz, P.; Kunze, S.; Alberto, R. *Chem.—Eur. J.* **2005**, *11*, 4089–4095.

(39) Petrus, A. K.; Vortherms, A. R.; Fairchild, T. J.; Doyle, R. P. *ChemMedChem* **2007**, *2*, 1717–1721.

(40) Smeltzer, C. C.; Cannon, M. J.; Pinson, P. R.; Munger, J. D., Jr.; West, F. G.; Grissom, C. B. *Org. Lett.* **2001**, *3*, 799–801.

(41) Hogenkemp, H. P. C.; Collins, D. A.; Grissom, C. B.; West, F. G., in ref., p 385.

(42) Barker, H. A.; Smyth, R. D.; Weissbach, H.; Toohey, J. I.; Ladd, J. N.; Volcani, B. E. *J. Biol. Chem.* **1960**, *235*, 480–488.

Acros. KNO_3 (99%) and CH_3COOH (glacial, HPLC grade) were purchased from Acros and Fisher, respectively. D_2O (99.8 atom % D) and CD_3OD (99.8 atom % D) were purchased from Acros. TSP (3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid, sodium salt) was purchased from Sigma. Water was purified using a Barnstead Nanopure Diamond water purification system or HPLC grade water was used. All captopril solutions were prepared directly before use.

pH measurements were made at room temperature with an Orion Model 710A pH meter equipped with a Mettler-Toledo Inlab 423 or 421 electrode. The electrode was filled with 3 M KCl/saturated AgCl solution, pH 7.0. The electrodes were standardized with standard BDH buffer solutions at pH 2.01, 4.01, 6.98, and 10.0. Solution pH was adjusted using HCl, HNO_3 , acetic acid, or NaOH solutions as necessary.

^1H NMR spectra were recorded on a Bruker 400 MHz spectrometer equipped with a 5 mm probe at 25 ± 1 °C. Solutions were prepared in either D_2O , buffered (MES, TES) D_2O , or CD_3OD . TSP (3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid, sodium salt) was used as an internal standard.

UV-visible spectra were recorded on a Cary 5000 spectrophotometer equipped with a thermostatted cell changer (25.0 ± 0.1 °C), operating with WinUV Bio software (version 3.00). Rate data for rapid reactions were collected using an Applied Photophysics RX.2000 Rapid Mixing Stopped-Flow unit in conjunction with the Cary 5000 spectrophotometer (25.0 ± 0.1 °C).

Electrospray mass spectra were recorded on a Thermo-Finnigan LCQ Duo ion trap mass spectrometer at the mass spectrometry facility in the Department of Chemistry, Colorado State University.

FT-IR spectra were recorded using a Bruker Tensor 27 Infrared Spectrophotometer. Samples were prepared by grinding with KBr using mortar and pestle and crushed in a mechanical die press to form translucent pellets.

For experiments under anaerobic conditions, solutions were degassed using 3 freeze-pump-thaw cycles under argon using standard Schlenk techniques.^{43,44} Air-free experiments were carried out on a Schlenk line or in a MBRAUN Labmaster 130 (1250/78) glovebox.

Synthesis of CapSCbl. A solution of captopril (347 μL , 213 mM, 74 μmol , 1.2 mol equiv) in H_2O (pH adjusted to 4.3) was added dropwise to a solution of $\text{HOCbl}\cdot\text{HCl}$ (96.8 mg, 62 μmol) in H_2O (1 mL, pH adjusted to 6.6) with stirring. The final pH of the reaction mixture was 4.2. The reaction was allowed to proceed for 3 h at 0 °C in the dark. The product precipitated upon dripping into a chilled acetone solution (-20 °C), and was filtered, washed with chilled acetone (50 mL, -20 °C), diethyl ether (10 mL, -20 °C), and dried under vacuum (50 °C, 5×10^{-2} mbar) overnight. Yield: 86 mg (88%). ^1H NMR spectroscopy (D_2O , δ ppm): major peaks at 7.19 (s, B7), 6.94 (s, B2), 6.40 (s, B4), 6.28 (d, R1), and 6.09 (s, C10) ppm and minor peaks at 6.97 (s, B2) and 6.08 (s, C10) ppm. The purity assessed by ^1H NMR spectroscopy⁴⁵ was $\sim 98\%$. UV-vis spectroscopy: $\lambda_{\text{max}} = 333, 372, 428, 530,$ and 560 nm. ES-MS (m/z): 1589.3 (calcd for $[\text{CapSCbl} + 2\text{Na}]^+$, $\text{C}_{71}\text{H}_{101}\text{CoN}_{14}\text{Na}_2\text{O}_{17}\text{PS} = 1589.6$); 1567.3 (calcd for $[\text{CapSCbl} + \text{H} + \text{Na}]^+$, $\text{C}_{71}\text{H}_{102}\text{CoN}_{14}\text{NaO}_{17}\text{PS} = 1567.6$); 1545.2 (calcd for $[\text{CapSCbl} + 2\text{H}]^+$, $\text{C}_{71}\text{H}_{103}\text{CoN}_{14}\text{O}_{17}\text{PS} = 1545.6$); 1351.4 (calcd for $[\text{Cbl} + \text{Na}]^+$, $\text{C}_{62}\text{H}_{88}\text{CoN}_{13}\text{NaO}_{14}\text{P} = 1351.6$); 806.3 (calcd for $[\text{CapSCbl} + 3\text{Na}]^{2+}$, $\text{C}_{71}\text{H}_{101}\text{CoN}_{14}\text{Na}_3\text{O}_{17}\text{PS} = 806.3$); 795.2 (calcd for $[\text{CapSCbl} + \text{H} + 2\text{Na}]^{2+}$, $\text{C}_{71}\text{H}_{102}\text{CoN}_{14}\text{Na}_2\text{O}_{17}\text{PS} = 795.3$); 784.2 (calcd for $[\text{CapSCbl} + \text{Na} + 2\text{H}]^{2+}$, $\text{C}_{71}\text{H}_{103}\text{CoN}_{14}\text{NaO}_{17}\text{PS} = 784.3$); 773.2 (calcd for

$[\text{CapSCbl} + 3\text{H}]^{2+}$, $\text{C}_{71}\text{H}_{104}\text{CoN}_{14}\text{O}_{17}\text{PS} = 773.3$); 687.3 (calcd for $[\text{Cbl} + 2\text{Na}]^{2+}$, $\text{C}_{62}\text{H}_{88}\text{CoN}_{13}\text{Na}_2\text{O}_{14}\text{P} = 687.3$); 665.2 (calcd for $[\text{Cbl} + 2\text{H}]^{2+}$, $\text{C}_{62}\text{H}_{90}\text{CoN}_{13}\text{O}_{14}\text{P} = 665.5$). FT-IR (KBr, cm^{-1}): The peak at 2567 cm^{-1} corresponding to the S-H stretching vibration of captopril⁴⁶ disappeared upon binding to the cobalamin.

Crystallization of CapSCbl. For CapSCbl-1, an aliquot of captopril solution (460 mM, 20 μL , 1.4 mol equiv) was added to a solution of $\text{HOCbl}\cdot\text{HCl}$ (64 mM, 100 μL). NaCl (15 mg) was added to this solution, and the solution stirred until all the NaCl was dissolved. The reaction mixture was kept in the dark, and the solvent was allowed to evaporate slowly. After a week, suitable crystals for X-ray diffraction studies were obtained. When anaerobic conditions were used, the complex crystallized in the CapSCbl-2 form. An aliquot of captopril (454 mM, 40 μL , 2.5 mol equiv) was added to a solution of $\text{HOCbl}\cdot\text{HCl}$ (72 mM, 100 μL) and NaCl (15 mg) added with stirring. Crystals were obtained after a week upon slow evaporation of the solvent.

X-ray Diffraction Studies on CapSCbl. Crystals of CapSCbl-1 and CapSCbl-2 were removed from the crystallization solution with nylon loops mounted on copper CrystalCapS pins (Hampton Research), placed in paraffin oil, and flash frozen in liquid nitrogen. X-ray diffraction experiments were carried out on beamlines BL9-2 and BL11-1 at the Stanford Synchrotron Radiation Laboratory (SSRL). The CapSCbl-1 data were collected on a MarMosaic-325 CCD detector using X-rays produced by a 16 pole wiggler insertion device, with a wavelength of 0.85503 Å (14500 eV) from a liquid nitrogen cooled double crystal monochromator. Two data sets were collected, both consisting of 90 1° images with a crystal to detector distance of 95 mm and covering the same range of ϕ angle. The first high resolution pass had an exposure time of 20 s, and the second pass had an exposure time of 3 s and a beam attenuation of 75%, to record the strong low resolution reflections discarded from the first pass because of overloading of the CCD detector. The data were processed with the program XDS⁴⁷ and scaled together with the program XSCALE.⁴⁷ Bijvoet pairs were not merged, and an absorption correction was not applied. A total of 34803 reflections were measured to a nominal resolution of 0.84 Å, resulting in a final unique data set of 12716 reflections with a merging R -factor of 0.049.

The CapSCbl-2 data were collected at BL11-1 using a MarMosaic-325 CCD detector. X-rays were produced by a 26 pole wiggler insertion device, with a wavelength of 0.79987 Å (15500 eV) from a water cooled side scattering asymmetric cut Si(111) single crystal monochromator. A single data set consisting of 180 1° images was collected, with a crystal to detector distance of 95 mm and an exposure time of 15 s. The data were processed with the program XDS⁴⁷ and scaled with the program XSCALE.⁴⁷ A total of 102343 reflections were measured to approximately 0.73 Å, giving 33693 unique reflections with a merging R -factor of 0.050.

The CapSCbl-1 structure was solved by Patterson methods as implemented in the program SHELXS⁴⁸ to locate the cobalt, phosphorus, and some of the corrin nitrogen atoms. The remaining light atoms were located by difference Fourier synthesis using SHELXS. The structure was refined by full matrix least-squares methods using the program SHELXL.⁴⁸ All of the cobalamin non-hydrogen atoms were refined with anisotropic thermal parameters, and hydrogen atoms were added in idealized positions and refined in riding positions. A correction for the anomalous scattering from cobalt at 14500 eV was applied during refinement. Additional difference electron density peaks were modeled as water molecules. The captopril moiety

(43) Zhao, R.; Lind, J.; Merenyi, G.; Eriksen, T. E. *J. Am. Chem. Soc.* **1994**, *116*, 12010–12015.

(44) Zhao, R.; Lind, J.; Merenyi, G.; Eriksen, T. E. *J. Chem. Soc., Perkin Trans. II* **1997**, 569–574.

(45) Brasch, N. E.; Finke, R. G. *J. Inorg. Biochem.* **1999**, *73*, 215–219.

(46) Wang, S.-Li; Lin, S.-Y.; Chen, T.-F.; Chuang, C.-H. *J. Pharm. Sci.* **2001**, *90*, 1034–1039.

(47) Kabsch, W. *J. Appl. Crystallogr.* **1993**, *26*, 795–800.

(48) Sheldrick, G. M.; Schneider, T. R. *Methods Enzymol.* **1997**, *277*, 319–343.

Table 1. Crystal Data and Structure Refinement Parameters for CapSCbl-1 and CapSCbl-2

parameter	CapSCbl-1	CapSCbl-2
empirical formula	C ₇₁ H ₁₀₁ N ₁₅ O ₁₇ PCoS.14 H ₂ O	C ₇₁ H ₁₀₁ N ₁₅ O ₁₇ PCoS.12 H ₂ O
FW/g mol ⁻¹	1811	1774
T/K	100	100
wavelength/Å	0.85503	0.79987
space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
a/Å	16.05	16.05
b/Å	21.23	24.97
c/Å	24.70	63.96
V/Å ³	8416.3(2)	25633.1(5)
Z	4	12
absorption coefficient/mm ⁻¹	0.31	0.31
F(000)	3080	10872
limiting indices	-16 ≤ h ≤ 16 -24 ≤ k ≤ 24 -28 ≤ l ≤ 28	-20 < h ≤ 13 -33 ≤ k ≤ 29 -84 ≤ l ≤ 70
reflections collected/unique	34803/12716	102343/33693
R _{merge} and R _{sym}	0.049, 0.043	0.050, 0.077
refinement method	full-matrix least-squares on F ²	full-matrix least-squares on F ²
data/restraints/parameters	10382/97/1063	25827/1969/3072
GOF on F ²	2.139	1.588
Flack parameter	0.0684	ND ^a
R factors (F > 4σ(F))	R1 = 0.1126, wR2 = 0.2981	R1 = 0.1445, wR2 = 0.3885
R factor (all data)	R1 = 0.1281	R1 = 0.1628
largest difference peak and hole/e Å ⁻³	+0.67 and -0.54	+ 5.5 and -2.3

appeared to have two distinct conformations corresponding to the *trans* and *cis* conformations with respect to the amide bond. Both conformations were modeled in the structure, and the occupancies refined with SHELXS. The atoms of the mercapto-2-methylpropionyl moiety (S70, C71, C72, C74 and O75), along with the amide nitrogen of the proline moiety (N76), were all refined in a single position with anisotropic thermal parameters, with the exception of C73, and the remaining atom of the proline were refined isotropically in the *cis* and *trans* configurations. When the two captopril configurations moieties were refined fully anisotropically, these prolyl atoms proved to be highly anisotropic, with a tendency to refine as nonpositive definite. Because the entire captopril moiety could not be refined anisotropically, this gave rise to a goodness of fit (GOF) greater than 2. The final crystallographic *R*-factor, R1 was 0.1124 for 10382 reflections with $F_o > 4\sigma_F$. Additional data collection and refinement statistics are given in Table 1.

The CapSCbl-2 structure was also solved by Patterson methods as implemented in the program SHELXS⁴⁸ and refined with SHELXL.⁴⁸ A correction for the anomalous scattering from cobalt at 15500 eV was applied during refinement. All non-hydrogen atoms were refined with anisotropic thermal parameters, hydrogen atoms were added in idealized positions and refined in riding positions, and difference peaks were modeled as water molecules. There are three independent captopril-cobalamin molecules in the CapSCbl-2 asymmetric unit. Two of these molecules had the ligand in the *trans* conformation and the third was in the *cis* conformation. Once all the atoms of the cobalamin and the captopril had been added, residual peaks between 0.5 and 1.0 Å from the Co, N, S, and P atoms were observed in $F_o - F_c$ electron density maps, appearing as if there was a second captopril-cobalamin molecule "shadowing" the first. This unaccounted for density resulted in a high crystallographic *R*-factor, and in an effort to get a better fit to the experimental data, some of these residual peaks were modeled as "alternates" of the real atom positions for the three cobalt atoms, the three sulfur atoms, and the three phosphorus atoms. This lowered the *R*-factor but gave rise to additional residual peaks in these "shadow" structures. Since there was already a high quality structure of the captopril-cobalamin complex obtained from the CapSCbl-1 crystal form, it was decided to remove these "alternate" positions and leave the CapSCbl-2 structure in its current state. The final crystallographic *R*-factor, R1 was 0.1445 for

25827 reflections with $F_o > 4\sigma_F$, and using data between 10.0 and 0.76 Å resolution. Additional data collection and refinement statistics are given in Table 1.

Kinetic Measurements on the Formation of CapSCbl from Aquacobalamin/Hydroxycobalamin and Captopril. All solutions were prepared in 0.050 M buffer at a total ionic strength of 0.50 M (KNO₃). Captopril solutions were freshly prepared. The rates of reactions at pH 4.5–8.0 were determined under aerobic conditions using stopped-flow spectroscopy and were independent of the wavelength at which they were measured. The rates of the reactions at pH 8.5–9.5 were measured under anaerobic conditions using the Cary 5000 spectrophotometer and air-free cuvettes. Above pH 8.5, data were collected at an isosbestic wavelength for the conversion of CapSCbl to cob(II)alamin (500 nm at pH 9.0; 494 nm at pH 9.5). Data fits were carried out using the program Microcal Origin version 7.5 or 8.0.

Attempted Separation of the Two CapSCbl Isomers by HPLC. HPLC analyses were conducted on an Agilent 1100 series HPLC system equipped with a degasser, quaternary pump, photodiode array detector (resolution of 2 nm) using a semipreparative Alltech Alltima C₁₈ column (5 μm, 100 Å, 10 mm × 300 mm) thermostatted to 25 °C with a flow rate of 3 mL/min. Peaks were monitored for their absorbance at 254 and 350 nm. A mobile phase consisting of acetic acid in water (pH 3.5, 0.1% v/v), **A**, and acetic acid in CH₃CN (0.1% v/v), **B**, were used in the following method: 0–2 min, isocratic elution of 95:5 **A**:**B**; 2–14 min, linear gradient to 85:15 **A**:**B**; 14–19 min, linear gradient to 82:18 **A**:**B**; 19–32 min, linear gradient to 65:35 **A**:**B**; 32–33 min, linear gradient to 40:60 **A**:**B**; and 33–35 min, linear gradient to 95:5 **A**:**B**. Under these conditions, cobalamins (Cbls) elute slowly, with the retention time of Cbl standards (CNCbl, MeCbl, AdoCbl, and H₂OCbl⁺) varying from 21.7 min (H₂OCbl⁺) to 31.7 min (CH₃Cbl).

Results and Discussion

Synthesis and Characterization of CapSCbl. CapSCbl was synthesized in high yield and purity by the addition of an aqueous solution of captopril (1.2 mol equiv, pH 4.3) dropwise to an aqueous solution of HOCbl·HCl (pH 6.6). The pH of the final product solution was 4.2. The procedure is similar to that used to synthesize other thiolatocobalamins²⁴ but with minor modifications. The

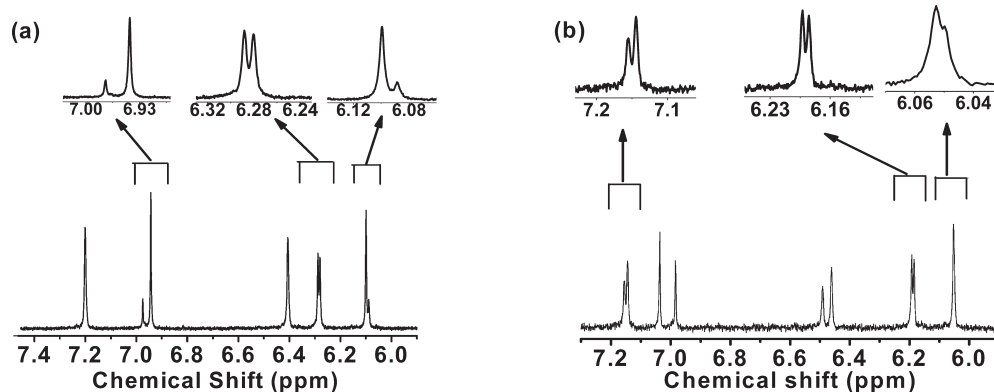


Figure 2. Aromatic region of the ^1H NMR spectrum of CapSCbl (25 ± 1 °C). (a) In D_2O , pD 5.5, MES buffer. Five major peaks are at 7.19 (1H, s), 6.94 (1H, s), 6.40 (1H, s), 6.28 (1H, d) and 6.09 (1H, s) ppm, attributable to the B7, B2, B4, R1, and C10 protons (see Figure 1 for the numbering scheme), respectively, of the Cbl macrocycle. Two minor peaks are observed at 6.97 (s) and 6.08 (s) ppm. (b) In CD_3OD . Peaks were observed at (7.14 + 7.15) (1H), (6.98 + 7.04) (1H), (6.46 + 6.49) (1H), 6.19 (1H, d), and (6.053 + 6.050) (1H) ppm.

pH of the final product solution plays a key role in the synthesis and was kept in the range 3.5–4.5. Lowering or raising the pH beyond this range causes the CapSCbl product to decompose to H_2OCbl^+ as determined by ^1H NMR and UV–vis spectroscopy. The synthesis was also attempted in 0.10 M MES buffer at pH 6.0 (both the $\text{HOCbl}\cdot\text{HCl}$ and captopril solutions adjusted to pH 6.0); however, the final product contained considerable H_2OCbl^+ in addition to the desired CapSCbl product.

UV–vis wavelength maxima of cobalamins are dependent on the β -axial ligand of the Cbl.⁴⁹ CapSCbl has wavelength maxima identical to other thiolatocobalamins (Table S1 and Figure S1, Supporting Information); hence captopril is coordinated to Cbl via the S atom. CapSCbl was also characterized by ES-MS. Peaks with splitting in agreement with computer simulated spectra were observed for $[\text{CapSCbl} + 2\text{H}]^+$, $[\text{CapSCbl} + \text{H} + \text{Na}]^+$, and $[\text{CapSCbl} + x\text{H} + y\text{Na}]^{2+}$ ($x, y = 0, 1, 2$ or 3; Experimental Section). Importantly, the ES-MS spectrum of CapSCbl clearly shows that no additional Cbl species are present in the product (Figure S2, Supporting Information). The FT-IR spectrum of captopril showed a peak at 2567 cm^{-1} corresponding to the SH stretching vibration.⁴⁶ This peak disappeared upon the binding of captopril to H_2OCbl^+ , providing further support for captopril coordinating via the thiol S in CapSCbl.

^1H NMR chemical shifts of Cbls in the aromatic region are also dependent on the β -axial ligand of Cbls.⁴⁵ The most interesting structural feature of the CapSCbl complex first became apparent upon recording the ^1H NMR spectrum of CapSCbl, Figure 2a. Five major signals attributable to the B2, B4, B7 protons of the α -5,6-dimethylbenzimidazole nucleotide, the C10 proton of the corrin ring and R1 proton of the ribose are observed at 6.94 (s), 6.40 (s), 7.19 (s), 6.09 (s), and 6.28 (d) ppm, respectively. ^1H NMR chemical shifts for CapSCbl and other RSCbls are listed in Supporting Information, Table S1. There is excellent agreement between the chemical shifts of CapSCbl and other RSCbls. However, unlike other RSCbls, CapSCbl also has two additional minor peaks ($\sim 18\%$) at 6.08 and 6.97 ppm, with peak integrals suggesting that CapSCbl exists in two isomeric forms,

since the sum of the peak integrals of the (6.08 + 6.09) and (6.94 + 6.97) peaks equals that of the 6.28, 6.40, and 7.19 ppm peaks within experimental error (0.97, 1.00, 1.05, 1.02, 1.00, respectively).

The captopril ligand itself exists as *cis* and *trans* isomers in solution with respect to the peptide bond involving the proline amide group (*cis*-captopril: *trans*-captopril $\sim 60:40$, 25 °C, pH 7.4, Scheme 1).^{50–52} Captopril also exists as *cis* and *trans* isomers in the solid state,⁴⁶ and ACE is inhibited by the *trans* isomer only.^{46,53,54} To provide further evidence for the *cis*-*trans* isomerization of the captopril ligand in CapSCbl, the effect of temperature and pD on the ^1H NMR spectrum of CapSCbl was investigated. However, unlike captopril,^{50,51} the relative amounts of the two species remained unchanged with pD (pD 3.4, 7.4, and 9.4), and raising the temperature to 75 °C simply resulted in broadening of all resonances. Control experiments showed that peak broadening is also observed in the NMR spectra of other cobalamins (CNCbl and H_2OCbl^+ , 25 versus 75 °C). Attempts were also made to separate the two stereoisomers by HPLC (see Experimental Section); however despite using mild elution conditions to increase the retention time of cobalamins to promote separation, a single peak was observed at 27.3 min with a UV–visible spectrum corresponding to CapSCbl ($\lambda_{\text{max}} = 333$ and 372 nm).

Importantly, the existence of *cis* and *trans* isomers for CapSCbl was confirmed by X-ray diffraction studies and in the solid state at least, the *trans* isomer is the major species (see below). In solution, the ratio of the two isomers is solvent dependent. Figure 2b gives the aromatic region of the ^1H NMR spectrum of CapSCbl in CD_3OD . Nine peaks are observed at (6.053 + 6.050), 6.19 (d), (6.46 + 6.49), (6.98 + 7.04), and (7.14 + 7.15) ppm, integrating as 0.96, 1.00, 1.05, 0.99, and 1.06 protons, respectively. In CD_3OD , the ratio of the two isomers

(50) Mariappan, S. V. S.; Rabenstein, D. L. *J. Org. Chem.* **1992**, *57*, 6675–6678.

(51) Rabenstein, D. L.; Isab, A. A. *Anal. Chem.* **1982**, *54*, 526–529.

(52) Nishikawa, T.; Abe, R.; Sudo, Y.; Yamada, A.; Tahara, K. *Anal. Sci.* **2004**, *20*, 1395–1398.

(53) Hassall, C. H.; Kroehn, A.; Moody, C. J.; Thomas, W. A. *J. Chem. Soc., Perkin Trans. I* **1984**, 155–164.

(54) Thorsett, E. D.; Harris, E. E.; Aster, S. D.; Peterson, E. R.; Snyder, J. P.; Springer, J. P.; Hirshfield, J.; Tristram, E. W.; Patchett, A. A. *J. Med. Chem.* **1986**, *29*, 251–260.

(49) Pratt, J. M. *Inorganic Chemistry of Vitamin B₁₂*; Academic Press: London, 1972; pp 45–67.

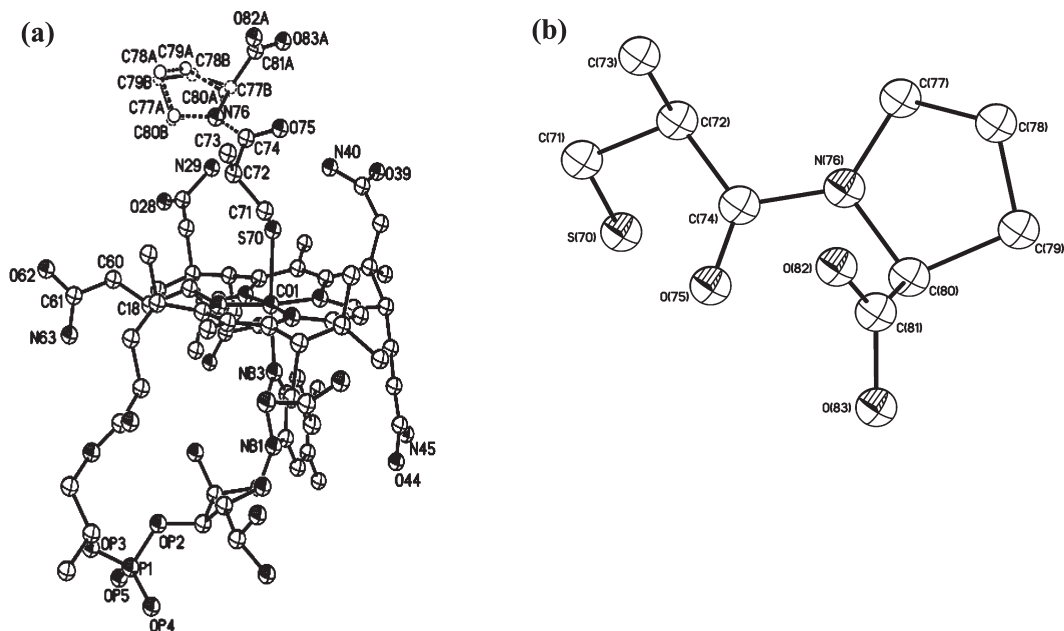


Figure 3. (a) Thermal ellipsoid plot (30%) of captopril-cobalamin. Only the cobalt atom, some of the atoms of the captopril ligand and some of the corrin atoms have been labeled for clarity and (b) close view of the captopril ligand showing complete atom labeling.

(major: minor isomer) is $\sim 57:43$, not $82:18$ as observed in D_2O (Figure 2a). Upon taking an NMR sample of CapSCbl in CD_3OD to dryness and redissolving it in D_2O , a 1H NMR spectrum was observed identical to that obtained by directly dissolving the dry CapSCbl product in D_2O . Similarly, taking a sample of CapSCbl in D_2O to dryness and redissolving it in CD_3OD gave a 1H NMR spectrum identical to that observed in Figure 2b. These experiments demonstrate that the extra peaks observed in addition to the five expected for a cobalamin are indeed a consequence of *cis/trans* isomerization, rather than solvent-induced decomposition. Further evidence of the solvent dependent *cis/trans* isomerization is obtained by adding increasing amounts of D_2O (up to 55% v/v) to a sample of CapSCbl in CD_3OD , which changes the ratio of the peaks in addition to the chemical shifts in the aromatic region of the 1H NMR spectrum. (Figure S3, Supporting Information). The final spectrum resembles the spectrum of CapSCbl in D_2O only. *cis* and *trans* isomers with respect to the captopril ligand has also been demonstrated in silver complexes of captopril using 1H and ^{13}C NMR spectroscopy.⁵⁵

Given the potential interest in CapSCbl as therapeutic, preliminary studies were carried out to investigate its stability in solution. In air, CapSCbl decomposed slightly to produce $\sim 5\%$ H_2OCbl^+ after 24 h and $\sim 9\%$ H_2OCbl^+ after 1 week (TES buffer, pD 7.4, RT; 1H NMR spectroscopy experiment). Under slightly acidic, aerobic conditions, decomposition of CapSCbl occurred more readily ($\sim 13\%$ H_2OCbl^+ after 24 h and $\sim 18\%$ H_2OCbl^+ after 1 week, MES buffer, pD 6.1). At pD 4.3, $\sim 12\%$ H_2OCbl^+ was observed after 24 h. Decomposition of CapSCbl was almost negligible under anaerobic conditions at all these pH conditions; hence, decomposition of CapSCbl in aerobic solution is a con-

sequence of the well established B_{12} -catalyzed aerial oxidation of thiols.^{56–60}

X-ray Structure of CapSCbl. CapSCbl crystallizes in two orthorhombic crystal forms depending upon the conditions during synthesis. When the complex is crystallized in the presence of air (CapSCbl-1), the crystals adopt the standard $P2_12_12_1$ space group with one molecule per asymmetric unit (cell parameters $a = 16.05$, $b = 21.23$, $c = 24.70$). Conversely, when the complex is crystallized in an inert atmosphere (CapSCbl-2), the crystals have a larger $P2_12_12_1$ unit cell (cell parameters, $a = 16.05$, $b = 24.97$, $c = 63.96$), with three molecules per asymmetric unit. To test the reproducibility of these observations, multiple crystals of aerobic and anaerobic crystals, from several different preparations with varying mol equiv of captopril, were mounted, flash cooled, and their diffraction patterns measured. Without exception, the aerobic crystals always gave the standard small orthorhombic CapSCbl-1 cell and the anaerobic crystals gave the larger CapSCbl-2 cell. (Note that the CapSCbl-2 structure was observed with 1.4 equiv captopril under anaerobic conditions.) Analysis of the unit cell parameters shows that the two cells are related, with the CapSCbl-2 unit cell being a subgroup of the more common CapSCbl-1 cell (see discussion of crystal packing below).

Figure 3a gives a thermal ellipsoid plot of CapSCbl-1. The captopril molecule is shown in Figure 3b for clarity. The captopril ligand is bound to the cobalamin through the sulfur atom as expected, with a Co–S bond distance of 2.282(3) Å. The Co–S and axial base Co–NB3 bond distances are slightly but significantly different (that is,

(56) Jacobsen, D. W.; Pezacka, E. H.; Brown, K. L. *J. Inorg. Biochem.* **1993**, *50*, 47–63.

(57) Peel, J. L. *Biochem. J.* **1963**, *88*, 296–307.

(58) Schrauzer, G. N.; Sibert, J. W. *Arch. Biochem. Biophys.* **1969**, *130*, 257–266.

(59) Schrauzer, G. N. *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 417–426.

(60) Jacobsen, D. W.; Troxell, L. S.; Brown, K. L. *Biochemistry* **1984**, *23*, 2017–2025.

(55) Isab, A. A.; Wazeer, M. I. M. *Spectrochim. Acta, Part A* **2006**, *65*, 191–195.

Table 2. Comparison of the Co Coordination Sphere for Thiolatocobalamins^{24,25}

	Co–S	Co–NB3	Co–N21	Co–N22	Co–N23	Co–N24	corrin fold
CapSCbl-1 ^a	2.282(3)	2.106(5)	1.880(4)	1.906(4)	1.886(4)	1.893(3)	14.9
CapSCbl-2 ^{a,b}	2.261(4)	2.094(9)	1.879(9)	1.921(9)	1.913(8)	1.886(8)	14.2
NACCbl ²⁴	2.250(2)	2.058(6)	1.878(5)	1.916(5)	1.929(5)	1.884(5)	17.5
γ -GluCysCbl ^{25a}	2.267(2)	2.049(6)	1.885(5)	1.902(6)	1.914(6)	1.891(5)	24.2
(NH ₂) ₂ CSCbl ^{25b}	2.300(2)	2.032(5)	1.884(5)	1.916(4)	1.926(5)	1.895(4)	14.5

^aThis work. ^bThe average for the three independent CapSCbl molecules in the asymmetric unit.

not identical within experimental error) from other thiolatocobalamin structures, Table 2. The four in-plane Co–N bond distances are very similar for all the thiolatocobalamins (Table 2).^{24,25} The cobalt ion of CapSCbl-1 sits in the plane of the four corrin nitrogen atoms (the displacement = -0.027 Å). Comparison of the CapSCbl-1 structure with that of γ -GluCysCbl^{25a} and *N*-acetyl-L-cysteinylCbl (NACCbl)²⁴ is interesting, in that whereas the upward corrin fold⁵⁶ in γ -GluCysCbl was found to be the largest yet observed in a cobalamin structure (24.2°), that in NACCbl (17.5°) and CapSCbl-1 (14.9°) is similar to other Co–S containing cobalamins. This is intriguing given that the Co–S and Co–N bond lengths (Table 2) show very little variation between these three structures. In γ -GluCysCbl and NACCbl there is a potential hydrogen bonding interaction between N40 and the cysteinyl sulfur, and it was suggested that this might contribute to the larger upward fold of the corrin ring.^{20,21} Similar interactions could also exist in CapSCbl-1, since the orientation of the N40 and S70 atoms are close to optimal for hydrogen bonding, with a C71–S70–N40 angle of about 115° and a N–S distance of 3.38 Å.

The most interesting observation was the presence of both the *trans* and *cis* configurations of the captopril ligand. In the early stages of refinement, the captopril ligand was built into the electron density in a *trans* configuration about the C74–N76 bond. Residual peaks in the electron density indicated the possibility of a lower occupancy *cis* configuration, and this alternate conformation was also built in and refined. The *trans* configuration appears to be favored over the *cis* in an almost 70:30 ratio, on the basis of the refined occupancy factors. In the *trans* configuration, the carboxylate moiety projects into the solvent channel and makes two hydrogen bonding contacts with the O44 and N45 atoms of a neighboring cobalamin, and a third interaction with a solvent molecule, whereas in the *cis* configuration, the carboxylate functionality projects toward the ring-contracted side of the cobalamin molecule. Interestingly, the electron density for the amide group on carbon C18 appears to be disordered over two possible positions, one major conformation (about 80%) which points away from the *cis*-captopril carboxylate (shown in Figure 3) and the other weaker configuration pointing toward it. There would be a severe steric clash between this second conformation and the carboxylate of the *cis*-captopril so presumably the amide group can only adopt this conformation with the *trans*-captopril.

Crystal packing in cobalamins is typically analyzed by comparison of the ratios of the *c/a* and *b/a* unit cell dimensions.^{61–63} CapSCbl-1 crystals are typical of cluster I packing (*c/a* = 1.589, *b/a* = 1.323). In the CapSCbl-1 crystal, the individual cobalamin molecules are oriented such that the plane of the corrin ring is roughly aligned with the *ab* plane of the unit cell, approximately 18° off being truly parallel. When viewed perpendicular to the *bc* plane, the neighboring cobalamin molecules are not perfectly parallel with each other, giving rise to layers of zig-zagged planes, these planes being roughly parallel to the *b*-axis. The separation between these layers along the *c*-axis is 13 Å, with the layers separated by solvent molecules. The corrin rings in one layer stack directly on top of a corrin ring two layers above or below, as a consequence of the 2-fold screw axis, with the corrin ring in the intervening layer displaced parallel to the *ab* plane by approximately 4 Å. This type of structural pattern is typical for cobalamins and has been described in detail in the literature.^{62,64} As observed in other cobalamin structures,^{62,64} when viewed down the *c* axis, long solvent-filled channels are clearly evident, with smaller side pockets also filled with ordered water molecules projecting off this main channel. The solvent structure has been modeled in CapSCbl-1 as 14 water molecules. The axial base and the captopril ligand extend into the solvent layers.

The majority of the solvent molecules are hydrogen bonded directly to either an oxygen or a nitrogen atom on the cobalamin molecule. There are significant intermolecular contacts in the crystal lattice, and although there are five direct nitrogen–oxygen hydrogen bonds linking neighboring cobalamin molecules, the majority of the interactions involve water-mediated hydrogen bonds. Similar H-bonded interactions have been observed and described for several cobalamin molecules including AdoCbl.^{62,64} In particular, five water molecules in the side pocket of both the CapSCbl-1 and the CapSCbl-2 structures have an identical structural disposition to, and make the same H-bonding interactions as, highly conserved water molecules reported in other cobalamin structures.⁶²

In the CapSCbl-2 structure, there are three cobalamin molecules in the asymmetric unit, and the crystal packing resembles that in the CapSCbl-1 crystals, in that they are arranged in the same zigzag pattern when viewed perpendicular to the *bc* plane, although because of the switch of the *b* and *c* axes, the cobalamin planes are parallel to the *c*-axis. Superposition of the CapSCbl-2 structure onto the

(61) Garau, G.; Geremia, S.; Marzilli, L. G.; Nardin, G.; Randaccio, L.; Tauzher, G. *Acta Crystallogr., Part B* **2003**, *59*, 51–59.

(62) Randaccio, L.; Geremia, S.; Nardin, G.; Wuerger, J. *Coord. Chem. Rev.* **2006**, *250*, 1332–1350.

(63) Randaccio, L.; Geremia, S.; Nardin, G.; Sjölof, M.; Srnova, I. *Inorg. Chem.* **1999**, *38*, 4087–4092.

(64) Bouquiere, J. P.; Finney, J. L.; Savage, H. F. J. *Acta Crystallogr.* **1994**, *B50*, 566–578.

CapSCbl-1 structure (based upon one of the CapSCbl-2 molecules) shows that the other two molecules in the CapSCbl-2 asymmetric unit are in similar but not quite identical positions to symmetry-related molecules in the CapSCbl-1 unit cell. As mentioned above, one of the cobalamin molecules has the captopril in a *cis* conformation where the carboxylate moiety points down toward the cobalamin ring, making the β -side of the corrin ring slightly more compact. This seems to facilitate the movement of a molecule in the plane directly above in toward the first molecule by nearly 1 Å since the interaction between the amide group on carbon C43 and the captopril carboxylate is lost. Conversely, because the captopril carboxylate now clashes sterically with C54 and C60 (in the latter case the amide group swings down below the corrin plane), the pyrrolidine ring has moved upward away from the corrin by about 1.5 Å in a direction toward another cobalamin molecule in the plane above. As a consequence this molecule has moved away by approximately 1 Å. The resultant effect seems to be an increase in the angular displacement of the molecules in this plane; that is, the zigzag nature of these planes is expanded slightly. It is not possible at this stage to draw conclusions as to why this expanded cell is favored over the more typical smaller cell under anaerobic conditions. Clearly the small cell must be an energetically favored arrangement of cobalamin molecules since it is observed in a large number of crystals of cobalamin complexed with a multitude of ligands, some bulkier than captopril, including *N*-acetylcysteine²⁴ and γ -glutamylcysteine.^{25a} Yet under anaerobic conditions the cobalamin molecules of CapSCbl do not pack in quite the same manner, and this leads to a switch to the larger unit cell. However it cannot be simply a consequence of the lack of oxygen, since crystals of the nitroxycobalamin complex, grown under similar conditions, had the small unit cell.⁶⁵ The presence of both *cis* and *trans* conformations of captopril could also potentially explain these differences. Furthermore, two of the axes in the two crystal forms are identical, with the new *c*-axis in CapSCbl-2 resulting from an approximate tripling of the CapSCbl-1 *b*-axis.

Kinetic Studies on the Formation of CapSCbl. The kinetics of the reaction between aquacobalamin/hydroxycobalamin ($\text{H}_2\text{OCbl}^+/\text{HOcbl}$) and captopril were studied as a function of captopril concentration at pH 4.5–9.5 under pseudo first-order conditions ($[\text{captopril}] \geq 10 \times [\text{H}_2\text{OCbl}^+/\text{HOcbl}]$). Figure 4 gives UV–vis spectra recorded after the addition of 10 equiv of captopril (5.00×10^{-4} M) to an aerobic solution of $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ (5.0×10^{-5} M) at pH 7.75 (0.050 M HEPES buffer, $I = 0.50$ M (KNO_3), 25.0 °C). From this figure it can be seen that $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ is cleanly converted to CapSCbl ($\lambda_{\text{max}} = 372, 430, 532, \text{ and } 561$ nm) with sharp isosbestic points at 340, 365, 449, and 540 nm. The reaction was also followed by stopped-flow spectroscopy. The best fit of the absorbance at 350 nm versus time to a first-order rate equation is shown in the inset to Figure 4, and gives $k_{\text{obs}} = (9.91 \pm 0.62) \times 10^{-3} \text{ s}^{-1}$. Figure 5 summarizes the rate data obtained at pH 7.72 (0.050 M HEPES buffer, $I = 0.50$ M (KNO_3), 25.0 °C) as a function

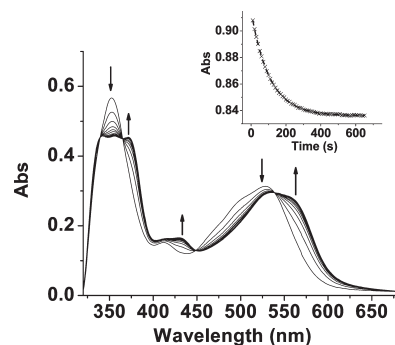


Figure 4. UV–vis spectra recorded after the addition of 10 equiv captopril (5.00×10^{-4} M) to an aerobic solution of $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ (5.0×10^{-5} M) at pH 7.75, 0.050 M HEPES buffer ($I = 0.50$ M, KNO_3), 25.0 °C. Inset: The best fit of absorbance data (obtained by stopped-flow spectroscopy) at 350 nm versus time to a first-order rate equation, giving $k_{\text{obs}} = (9.91 \pm 0.62) \times 10^{-3} \text{ s}^{-1}$ (pH = 7.72, 0.050 M HEPES buffer, $I = 0.50$ M (KNO_3)).

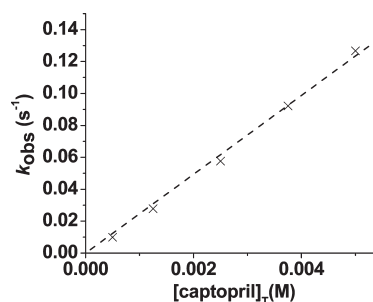


Figure 5. Plot of observed rate constant, k_{obs} , versus total captopril concentration for the reaction $\text{H}_2\text{OCbl}^+/\text{HOcbl} + \text{CapSH}/\text{CapS}^- \rightarrow \text{CapSCbl} (+ \text{H}^+)$ at pH 7.72 (0.050 M HEPES buffer, 25.0 °C, $I = 0.50$ M (KNO_3)). Data have been fitted to a line passing through origin, giving $k_{\text{obs}}/[\text{captopril}]_{\text{T}} = 24.7 \pm 0.5 \text{ M}^{-1} \text{ s}^{-1}$.

of captopril concentration (5.00×10^{-4} – 5.00×10^{-3} M). The data can be fitted to a line passing through the origin which indicates that the reaction is irreversible. From the slope of the line, $k_{\text{obs}}/[\text{captopril}]_{\text{T}}$ was found to be $24.7 \pm 0.5 \text{ M}^{-1} \text{ s}^{-1}$.

Rate constants for the reaction between $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ and captopril for $8.45 < \text{pH} < 9.48$ were determined under anaerobic conditions. At pH 8.45 the reaction was monitored at 350 nm; however at higher pH conditions the concentration of the thiolate (RS^-) form of captopril becomes significant. CapS^- reduces CapSCbl to cob(II)alamin,⁶⁶ which is oxidized back to $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ in the presence of air. In other words, B_{12} -catalyzed aerial oxidation of the thiol to form a disulfide occurs.^{56–60,66} The reaction of interest was therefore monitored at an isosbestic wavelength for the reduction of CapSCbl to cob(II)alamin. Plots of k_{obs} versus $[\text{captopril}]_{\text{T}}$ were once again linear and passed through the origin (Supporting Information, Figure S4). We were unable to collect any meaningful data above pH 9.5, since the subsequent reduction of CapSCbl by the thiolate (RS^-) form of captopril to cob(II)alamin is considerably faster than the reaction of HOcbl with captopril to form CapSCbl.

Figure 6 summarizes the dependence of the second-order rate constant for the formation of CapSCbl,

(65) Hannibal, L.; Smith, C. A.; Jacobsen, D. W.; Brasch, N. E. *Angew. Chem., Int. Ed.* **2007**, *46*, 5140–5143.

(66) Xia, L.; Cregan, A. G.; Berben, L. A.; Brasch, N. E. *Inorg. Chem.* **2004**, *43*, 6848–6857.

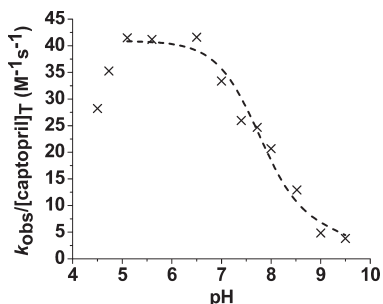
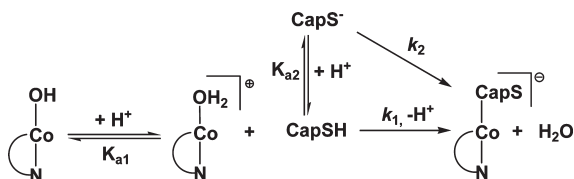


Figure 6. Plot of $k_{\text{obs}}/[\text{captopril}]_{\text{T}}$ versus pH for the reaction of $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ with captopril to form CapSCbl (0.050 M buffer, 25.0 °C, $I = 0.50$ M (KNO_3)). Data in the pH range 5.1–9.5 was fitted to eq 1 in the text, giving $k_1 = 40.9 \pm 1.2 \text{ M}^{-1} \text{ s}^{-1}$ and $k_2 = 660 \pm 170 \text{ M}^{-1} \text{ s}^{-1}$.

Scheme 2



$k_{\text{obs}}/[\text{captopril}]_{\text{T}}$, as a function of pH. It has been previously established that only H_2OCbl^+ can undergo ligand substitution at the β -axial site, with HOcbl being inert to substitution.^{67–69} The proposed mechanism is given in Scheme 2, in which H_2OCbl^+ reacts with the thiol (k_1 pathway) or thiolate forms (k_2 pathway) of captopril to form CapSCbl. From Scheme 2 it can be shown that

$$\begin{aligned} k_{\text{obs}}/[\text{CapS}]_{\text{T}} &= k_1(\text{fractionH}_2\text{OCbl}^+)(\text{fractionCapSH}) \\ &\quad + k_2(\text{fractionH}_2\text{OCbl}^+)(\text{fractionCapS}^-) \\ &= (k_1[\text{H}^+]^2 + k_2K_{\text{a}2}[\text{H}^+]) / (K_{\text{a}1} + [\text{H}^+])^2 (K_{\text{a}2} + [\text{H}^+]) \end{aligned} \quad (1)$$

where $K_{\text{a}1}$ and $K_{\text{a}2}$ are the acid dissociation constants corresponding to the equilibria shown in Scheme 2. The best fit of the data for $\text{pH} \geq 5.1$ to eq 1 fixing $\text{p}K_{\text{a}1} = 7.76$ ⁶⁶ and $\text{p}K_{\text{a}2} = 9.8$ ^{1,51,70,71} gives $k_1 = 40.9 \pm 1.2 \text{ M}^{-1} \text{ s}^{-1}$ and $k_2 = 660 \pm 170 \text{ M}^{-1} \text{ s}^{-1}$. The large experimental error associated with k_2 arises since this pathway becomes significant only for values approaching $\text{p}K_{\text{a}2}$ ($= 9.8$). We were unable to measure rate data for $\text{pH} > 9.5$. The $\text{p}K_{\text{a}}$ of the $-\text{COOH}$ group of captopril is 2.9–3.7^{1,51} and is probably responsible for the decrease in the second-order

rate constant, $k_{\text{obs}}/[\text{captopril}]_{\text{T}}$, in the pH 4.5–5 region. It was not possible to obtain rate data at $\text{pH} < 4.5$ to probe this further, however, because of H^+ -catalyzed decomposition of CapSCbl.⁷²

Detailed kinetic studies have been previously reported on the formation of glutathionylcobalamin from $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ and glutathione under the same conditions as this study (25.0 °C, $I = 0.50$ M (KNO_3)).⁶⁶ As for the aquacobalamin/captopril system, $k_{\text{obs}}/[\text{GSH}]_{\text{T}}$ decreases with increasing pH, since H_2OCbl^+ (not HOcbl), reacts with glutathione. Analysis of the rate data for this system was somewhat complicated by the fact that the $\text{p}K_{\text{a}}$'s for deprotonation of the amine and thiol groups of glutathione overlap. The rate constant for the reaction of H_2OCbl^+ with the thiol (RSH) form of captopril or glutathione are similar (40.9 ± 1.2 and $18.5 \text{ M}^{-1} \text{ s}^{-1}$, respectively). A comparison of rate constants for reaction of H_2OCbl^+ with the thiolate (RS^-) forms of captopril or glutathione is less informative because of large error in the former value (660 ± 170 and $163 \pm 8 \text{ M}^{-1} \text{ s}^{-1}$, respectively).⁶⁶

To summarize, reacting aquacobalamin with captopril results in formation of the corresponding thiolatocobalamin complex, captopril-cobalamin, in high yield (88%) and purity (98%). Characterization of the product by ^1H NMR spectroscopy revealed that two isomeric forms of the captopril ligand exist for captoprilcobalamin in solution, with the isomer ratio being solvent dependent. X-ray diffraction studies confirmed this, and showed that at least in the solid state, the *trans* isomer is preferentially formed. Finally, kinetic studies on the formation of captoprilcobalamin from $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ and captopril showed that both the thiol and thiolate forms of captopril react with H_2OCbl^+ , whereas HOcbl does not react with captopril.

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Supporting Information Available: UV–vis and ^1H NMR spectroscopy data for thiolatocobalamins at $\text{pD} 6.0$ (Table S1), UV–vis spectrum of CapSCbl in H_2O (Figure S1), ES-MS (+ve mode) spectrum of CapSCbl (Figure S2), ^1H NMR spectra of CapSCbl in $\text{CD}_3\text{OD}/\text{D}_2\text{O}$ mixtures (Figure S3), plot of observed rate constant versus captopril concentration for the reaction of $\text{H}_2\text{OCbl}^+/\text{HOcbl}$ with captopril at $\text{pH} 8.99$ (Figure S4). This material is available free of charge via the Internet at <http://pubs.acs.org>. The CCDC deposition numbers for CapSCbl-1 and CapSCbl-2 are 740752 and 740753, respectively. These can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

(67) Randall, W. C.; Alberty, R. A. *Biochemistry* **1967**, *6*, 1520–1525.

(68) Marques, H. M. S. *Afr. J. Chem.* **1991**, *44*, 114–117.

(69) Marques, H. M. *Dalton Trans.* **1992**, 2019–2027.

(70) Remko, M. *Chem. Pap.* **2007**, *61*, 133–141.

(71) Hu, T.-M.; Chou, T.-C. *AAPS J.* **2006**, *8*, E485–E492.

(72) Schumacher, L.; Mukherjee, R.; Brasch, N. E., paper in preparation.