A simple, convenient method to synthesize cobalamins: synthesis of homocysteinylcobalamin, *N*-acetylcysteinylcobalamin, 2-*N*-acetylamino-2-carbomethoxyethanethiolatocobalamin, sulfitocobalamin and nitrocobalamin†

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Glutathionylcobalamin, nitrocobalamin and sulfitocobalamin are important cobalamin metabolites isolable from human tissues. Herein we demonstrate that a procedure used to synthesize and isolate γ-glutamylcysteinylcobalamin and glutathionylcobalamin in aqueous solution in high yield and purity can be used to synthesize other novel, biologically relevant thiolatocobalamins, including D,L-homocysteinylcobalamin, *N*-acetyl-L-cysteinylcobalamin (Na^+ salt) and 2-*N*-acetylamino-2-carbomethoxy-L-ethanethiolatocobalamin, as well as other non-alkylcobalamins, such as sulfitocobalamin (Na^+ salt) and nitrocobalamin. This uncomplicated, general procedure will assist researchers in identifying unknown cobalamin metabolites isolated from biological samples, and researchers interested in studying the uptake and intracellular cobalamin processing mechanisms utilizing non-alkylcobalamin derivatives that are not yet commercially available. The X-ray structure and XAS spectrum of *N*-acetyl-L-cysteinylcobalamin are also presented.

Introduction

Corrinoid-dependent enzymes are widespread in nature and play key roles in human, animal and microbial metabolism.[1] Two known B_{12}-dependent enzymes exist in humans: methylcobalamin (MeCbl)-dependent methionine synthase and adenosylcobalamin (AdoCbl)-dependent methylmalonyl-coenzyme A mutase.[2–4] The clinical hallmarks of B_{12} deficiency are megaloblastic anemia ('pernicious anemia'), and/or neuropathies.[5] Fig. 1 gives the structure of the two coenzyme forms of vitamin B_{12} and related B_{12} derivatives (cobalamins) found in humans. Upon reaching cells, cobalamin derivatives are converted to MeCbl and AdoCbl by currently ill-defined mechanisms. Many studies have been carried out over the past several decades involving the extraction and identification of cobalamins from mammalian cells, tissue and blood, in addition to other biological samples such as foods and seaweed. Adenosylcobalamin, methylcobalamin and aquacobalamin ($H_2OCbl^+ \leftrightarrow HOCbl + H^+$; $pK_a = 7.8$)[6] are the major cobalamin metabolites isolated from biological samples.[7–10] Sulfitocobalamin (SO_3Cbl^-) is also isolable from mammalian cells and foods,[9–19] and there are also reports of the isolation of nitrocobalamin (NO_2Cbl) from biological sources.[11,20] Whether or not cyanocobalamin is truly 'naturally occurring' is controversial; some studies report small amounts of this derivative, especially in smokers.[7,21]

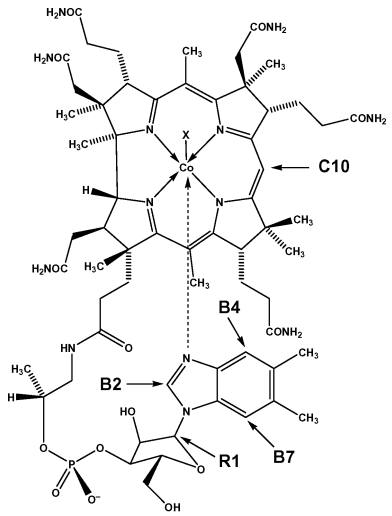

Fig. 1 Structures of naturally occurring cobalamins: X = 5′-deoxyadenosyl, adenosylcobalamin (coenzyme B_{12}); X = CH_3, methylcobalamin; X = H_2O/OH^-, aquacobalamin/hydroxycobalamin; X = SO_3^{2-}, sulfitocobalamin; X = NO_2^-, nitrocobalamin; X = CN^-, cyanocobalamin; X = glutathione, glutathionylcobalamin. The positions of the protons that resonate in the aromatic region in the ^1H NMR spectrum of cobalamins (B2, B4, B7, R1 and C10) are also shown.

Thiol derivatives of B_{12}, thiolatocobalamins, were first identified in the 1960s, but have not attracted much attention until recently.[18,19,22–44] Glutathionylcobalamin (GSCbl) is an important

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cobalamin metabolite in mammals,[31–33] and is more active than other cobalamins in promoting methionine synthase activity in rabbit spleen extracts. We proposed that GSCbl (or a closely related thiolatocobalamin adduct) is a precursor of the two coenzyme forms of vitamin B_{12}, AdoCbl and MeCbl.[41] Formation of GSCbl from H_2OCbl^+ and glutathione is irreversible ($K_{obs} \sim 3 \times 10^8$ M^{-1} at pH 7.4, 25 °C) and rapid ($t_{1/2} \sim 3$ s for [GSH] = 5 mM, pH 7.4, 37 °C).[45] An alternative role for GSCbl was also recently proposed, in which the formation of GSCbl prevents B_{12} from being scavenged by xenobiotics.[24] Finally, McCaddon and co-workers suggested that GSCbl and related thiolatocobalamins might be more effective than currently available pharmaceutical B_{12} forms (CNCbl and H_2OCbl^+) in treating B_{12}-related conditions associated with oxidative stress such as Alzheimer's disease.[34,35] However, the apparent clinical benefit of combining *N*-acetyl-L-cysteine with H_2OCbl^+ in patients with Alzheimer's disease or mild cognitive impairment requires formal clinical trials to prove or refute such observations.[34,46]

In this paper we describe the synthesis of three novel thiolatocobalamins: D,L-homocysteinylcobalamin (HcyCbl), the sodium salt of *N*-acetyl-L-cysteinylcobalamin (Na[NACCbl]), and 2-*N*-acetylamino-2-carbomethoxy-L-ethanethiolatocobalamin (NACMECbl). The thiol ligand structures are shown in Fig. 2. HcyCbl is of interest, since it is potentially a naturally occurring cobalamin metabolite. In the MeCbl-dependent methionine synthase reaction, a methyl group is transferred from methyltetrahydrofolate to homocysteine *via* MeCbl to generate methionine and tetrahydrofolate. Impairment of this reaction leads to elevated serum levels of homocysteine, which is associated with an increased risk of cardiovascular, peripheral vascular and cerebrovascular diseases.[47–52] In addition, moderately raised serum homocysteine levels are more prevalent in patients suffering from neurological diseases such as Alzheimer's disease,[53–56] and appear to predict cognitive decline.[57,58] Na[NACCbl] is also of interest, given the benefits of co-administrating H_2OCbl^+ and *N*-acetyl-L-cysteine to Alzheimer's patients,[34,46] and the possibility that $NACCbl^-$, rather than the individual components themselves, is responsible for the beneficial effects of this approach. The X-ray structure and XAS spectrum of Na[NACCbl]·$18H_2O$ are also reported.

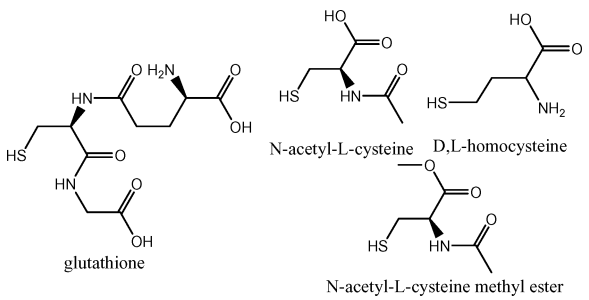

Fig. 2 Structure of the thiol ligands.

We were also interested to see whether our procedure could be used to synthesize other non-thiol cobalamin derivatives, such as the sodium salt of sulfitocobalamin (Na[SO_3Cbl]) and nitrocobalamin (NO_2Cbl). Although both are isolable from biological samples,[11–17,20] and are well studied and structurally characterized,[23,29,38,43,59–69] neither are commercially available. NO_2Cbl is also of interest, since if nitrosylcobalamin (NOCbl) is formed in biological systems to any appreciable extent, NO_2Cbl rather than NOCbl will be isolated after typical (aerobic) isolation and purification procedures.[70,71]

Finally, we note that there are numerous studies concerning the isolation and identification of cobalamin metabolites from biological samples which report 'unknown' analogues of cobalamin.[72–77] To assist in their identification, simple procedures are required to synthesize potential candidates with different β-axial ligands, allowing spectroscopic and chromatographic comparison of unknown cobalamin metabolites with known standards. In addition, pure, well characterized cobalamin derivatives will assist researchers investigating intracellular cobalamin processing. Simple synthetic procedures are also useful when expensive, radioactive cobalamin derivatives are desired for studies on the uptake and/or conversion of cobalamin derivatives in humans, animals and cell models.[40,78–80]

Experimental

Hydroxycobalamin hydrochloride (HOCbl·HCl), 98% (stated purity by manufacturer) was purchased from Fluka. The percentage of water in HOCbl·HCl (·nH_2O) (batch-dependent, typically 10–15%), was determined by converting HOCbl·HCl to dicyanocobalamin, $(CN)_2Cbl^-$ (0.10 M KCN, pH 10.0, $\varepsilon_{368\,nm} = 30.4$ mM^{-1} cm^{-1} [81]). NaN_3 (99%), MES, KNO_3 (99%) and D,L-homocysteine were from ACROS Organics and glutathione (GSH, 98%; *i.e.*, in its reduced form) was purchased from Aldrich. NaOAc (99%), $NaNO_2$ (>98%), $NaSO_3$ (>98%) and *N*-acetyl-L-cysteine were obtained from Sigma. Water was purified using a Barnstead Nanopure Diamond water purification system and/or HPLC grade water. All thiol solutions were prepared directly before use.

pH Measurements were made with a Corning Model 445 pH meter in conjunction with a Mettler-Toledo Inlab 423 electrode at room temperature. 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 4.01 and 6.98. Solution pH was adjusted using HCl or NaOH solutions as necessary.

^{1}H NMR spectra were recorded on an Inova 500 MHz or a Bruker 400 MHz spectrometer equipped with a 5 mm probe at room temperature (22 ± 1 °C). Solutions were prepared in D_2O or MES buffer (pD 5.50) and TSP (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt) was used as an internal standard. UV-Vis 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). Electrospray mass spectra were recorded using a BRUKER Esquire-LC mass spectrometer. Mass spectra for all cobalamins were recorded in the positive mode, except for sulfitocobalamin, which was recorded in the negative mode.

For experiments conducted under anaerobic conditions, solutions were degassed using three freeze–pump–thaw cycles and argon using standard Schlenk techniques. Air-free manipulations were carried out on a Schlenk line or in an MBRAUN Labmaster 130(1250/78) glovebox.

Synthesis of *N*-acetyl-L-cysteine methyl ester

N-Acetyl-L-cysteine methyl ester was prepared according to a modified published procedure.[82] Briefly, CH_3OH (1.6 ml, 39 mmol)

and concentrated HCl (180 μl) were added slowly to a solution of *N*-acetyl-L-cysteine (NAC, 640 mg, 3.9 mmol) in chloroform (9 ml). The reaction mixture was refluxed for 3 h at 66 °C, taken to dryness by rotary evaporation and the product recrystallized from ethyl ether. Yield: 405 mg (59%). ^{1}H NMR: (δ, ppm) 1.29 (t CH), 2.07 (s, MeCO), 2.97 (d CH_2), 3.78 (s MeO).

Determination of the thiol concentrations

The total reduced thiol concentration was determined using the Ellman's procedure.[83] One part of the thiol solution was diluted with five parts of Ellman's reagent (32 μg ml^{-1} dithionitrobenzoic acid and 1.25 mM EDTA in 0.5 M TRIS, pH 8.5) and the absorbance determined at 412 nm ($\varepsilon = 13.6$ mM^{-1} cm^{-1}).

Synthesis of thiolatocobalamins

All syntheses were carried out under red-light-only conditions, due to the potential light sensitivity of thiolatocobalamins.[43] Unless specifically stated otherwise, all syntheses were carried out under aerobic conditions.

N-Acetyl-L-cysteinylcobalamin, sodium salt (Na[NACCbl])

A solution of *N*-acetyl-L-cysteine (263 μml, 284 mM, 74.7 μmol, 1.1 mol equiv.) in MES buffer (0.1 M, pH ~6) was added drop-wise to a solution of HOCbl·HCl (107 mg, 67.9 μmol) in MES buffer (0.80 ml, 0.1 M, pH ~6) with stirring, and the reaction was allowed to react for 30 min at 0 °C. The product precipitated upon dripping into a chilled acetone solution (−20 °C), and was filtered, washed with chilled acetone (20 ml, −20 °C) and diethyl ether (10 ml, −20 °C). The product was dried at 50 °C under vacuum (2×10^{-2} mbar) overnight. Yield: 90 mg (87%). The purity assessed by conversion to dicyanocobalamin[81] was 95 ± 2%. ^{1}H NMR (D_2O, δ, ppm): 6.10 (s C10), 6.28 (d R1), 6.40 (s B4), 6.96 (s B2), 7.20 (s B7). The cobalamin purity assessed by ^{1}H NMR spectroscopy[84] was ~98%. ES-MS, *m/z*: 1492.3 (calcd for $[NACCbl + 2\,H]^+$, $C_{67}H_{98}CoN_{14}O_{17}PS = 1492.6$); 1513.9 (calcd for $[NACCbl + H + Na]^+$, $C_{67}H_{97}CoN_{14}NaO_{17}PS = 1514.6$); 746.5 (calcd for $[NACCbl + 3\,H]^{2+}$, $C_{67}H_{99}CoN_{14}O_{17}PS = 746.8$); 757.7 (calcd for $[NACCbl + Na + 2\,H]^{2+}$, $C_{67}H_{98}CoN_{14}NaO_{17}PS$ = 757.8).

2-*N*-Acetylamino-2-carbomethoxy-L-ethanethiolatocobalamin (NACMECbl)

The procedure was similar to that for Na[NACCbl], except that a solution of *N*-acetyl-L-cysteine methyl ester (NACME, 249 μml, 250 mM, 62.2 μmol, 1.2 mol equiv.) in MES buffer was added to a HOCbl·HCl solution (81.5 mg, 51.8 μmol) in MES buffer, and the mixture left for 30 min to react at 0 °C. Yield: 66.3 mg (85%). The purity assessed by the dicyanocobalamin test[81] was 96 ± 2%. ^{1}H NMR (D_2O, δ, ppm): 6.10 (s C10), 6.30 (d R1), 6.40 (s B4), 6.95 (s B2), 7.21 (s B7). The cobalamin purity assessed by ^{1}H NMR spectroscopy was ~98%. ES-MS, *m/z*: 1506.6 (calcd for $[NACMECbl + H]^+$, $C_{68}H_{100}CoN_{14}O_{17}PS = 1506.6$); 1527.9 (calcd for $[NACMECbl + Na]^+$, $C_{68}H_{99}CoN_{14}NaO_{17}PS = 1528.6$); 753.7 (calcd for $([NACMECbl + 2\,H]^{2+}$, $C_{68}H_{101}CoN_{14}O_{17}PS = 753.8$); 765.0 (calcd for $[NACMECbl + Na + H]^{2+}$, $C_{68}H_{100}CoN_{14}NaO_{17}PS = 764.8$).

D,L-Homocysteinylcobalamin (HcyCbl)

The synthesis of this derivative was carried out under strictly anaerobic conditions. An anaerobic solution of D,L-Hcy (395 μml, 191 mM, 75.4 μmol, 1.2 mol equiv.) in MES buffer (0.1 M, pH ~6) was added drop wise to an anaerobic solution of HOCbl·HCl (98.7 mg, 62.8 μmol) in MES buffer (0.80 ml, 0.1 M, pH ~6) with stirring, and the reaction allowed to proceed for 5 min at 0 °C. The product precipitated upon dripping into a degassed chilled acetone solution (−20 °C), and was filtered, washed with chilled acetone (20 ml, −20 °C). The product was dried at 50 °C under vacuum (2×10^{-2} mbar) overnight. Yield: 64.4 mg (70%). The purity assessed by conversion to dicyanocobalamin was 97%. ^{1}H NMR (D_2O, δ, ppm): 6.10 (s C10), 6.28 (d R1), 6.38 (s B4), 6.95 (s B2), 7.20 (s B7). The cobalamin purity assessed by ^{1}H NMR spectroscopy was ~98%. ES-MS, *m/z*: 1464.8 (calcd for $[HcyCbl + H]^+$, $C_{66}H_{98}CoN_{14}O_{16}PS = 1464.6$); 1486.7 (calcd for $[HcyCbl + Na]^+$, $C_{66}H_{97}CoN_{14}NaO_{16}PS = 1486.6$); 732.5 (calcd for $[HcyCbl + 2\,H]^{2+}$, $C_{66}H_{99}CoN_{14}O_{16}PS = 732.8$); 743.4 (calcd for $[HcyCbl + Na + H]^{2+}$, $C_{66}H_{98}CoN_{14}NaO_{16}PS = 743.8$).

Synthesis of non-thiol cobalamins

Sulfitocobalamin, sodium salt (Na[SO_3Cbl]). A Na_2SO_3 solution (266 mM, 519 μl, 138.1 μmol, 1.3 mol equiv.) was added drop-wise to a HOCbl·HCl solution (162.3 mg, 103.3 μmol) in MES buffer (pH 6.0, 1.60 ml), and the mixture left to react at 0 °C (ice bath) for 2 h. The product precipitated upon dripping into acetone (20 ml, −20 °C), and was washed with acetone (20 ml), diethyl ether (−20 °C) and dried overnight under vacuum. Yield: 145.7 mg (98%). The purity assessed by conversion to dicyanocobalamin[81] was 99 ± 2%. ^{1}H NMR (D_2O, δ, ppm): 5.92 (s C10), 6.15 (d R1), 6.33 (s B4), 6.84 (s B2), 7.07 (s B7).[64] The cobalamin purity assessed by ^{1}H NMR spectroscopy was 98%. ES-MS, *m/z*: 1408.5 (calcd for $[SO_3Cbl]^-$, $C_{62}H_{89}CoN_{13}O_{17}PS^- = 1409.5$).

Nitrocobalamin (NO_2Cbl). The procedure for the synthesis of nitrocobalamin was similar to that for Na[NACCbl], except that a $NaNO_2$ solution (61.1 mM, 100 μml, 61.1 μmol, 1.2 mol equiv.) was added drop-wise to a HOCbl·HCl solution (80.0 mg, 50.9 μmol) in MES buffer (pH 6.0, 0.90 ml), and the mixture left to react at 0 °C (ice bath) for 2 h. The product precipitated upon dripping into acetone (5 ml), and was washed with acetone (20 ml) and dried overnight under vacuum at 60 °C. Yield: 151 mg (92%). The purity assessed by conversion to dicyanocobalamin[81] test was 99 ± 2%. ^{1}H NMR (D_2O, δ, ppm) 6.20 (s C10), 6.28 (d R1), 6.42 (s B4), 6.74 (s B2), 7.20 (s B7).[70] The cobalamin purity assessed by ^{1}H NMR spectroscopy was 98%. ES-MS analysis, *m/z*: 1375.7 (calcd for $[NO_2Cbl + H]^+$, $C_{62}H_{90}CoN_{14}O_{16}P = 1376.6$); 1398.6 (calcd for $[NO_2Cbl + Na]^+$, $C_{62}H_{89}CoN_{14}NaO_{16}P = 1398.6$); 689.0 (calcd for $[NO_2Cbl + 2\,H]^{2+}$, $C_{62}H_{91}CoN_{14}O_{16}P = 688.8$); 699.9 (calcd for $[NO_2Cbl + Na + H]^{2+}$, $C_{62}H_{90}CoN_{14}NaO_{16}P = 699.8$).

X-Ray diffraction studies on *N*-acetyl-L-cysteinylcobalamin (sodium salt)

An aliquot of a NAC solution (182 mM, 40 μL, in HPLC grade H_2O) was added to a solution of HOCbl·HCl (100 μL, 36 mM, in H_2O), NaCl (1.4 mg) added, and the solution mixed until everything dissolved. The solution was kept at room temperature

Table 1 Crystal data and structure refinement parameters for Na[NACCbl]·18H_2O

Parameter	Value
Empirical formula	$NaC_{67}H_{132}N_{14}O_{35}PCoS$
FW/g mol^{-1}	1838
T/K	100
Wavelength/Å	0.817975
Space group	$P2_12_12_1$
a/Å	16.153
b/Å	21.076
c/Å	25.449
V/Å^3	8663.8(8)
Z	4
Absorption coefficient/mm^{-1}	0.31
F(000)	3208
Limiting indices	$-19 \leq h \leq 19$, $-25 \leq k \leq 25$, $-32 \leq l \leq 32$
Reflections collected/unique	53901/17617
R_{merge} and R_{sym}	0.105, 0.119
Refinement method	Full-matrix least squares on F^2
Data/restrains/parameters	17617/0/1143
GOF on F^2	0.986
R factors ($I > 4\sigma(I)$)	$R1 = 0.1030$, $wR2 = 0.2645$
R factor (all data)	$R1 = 0.1082$
Largest difference peak and hole/e Å^3	+1.3 and −1.1

in a closed glass vial protected from light. Small crystals were observed after 24 h. Suitable crystals for X-ray diffraction studies were obtained after leaving the sample in a fridge at 4 °C for 5 d.

A crystal (~0.3 × 0.1 × 0.1 mm) was mounted under paraffin oil in a nylon loop and flash frozen in liquid nitrogen. Diffraction experiments were carried out on beamline BL11-1 at the Stanford Synchrotron Radiation Laboratory (SSRL). Data were collected on an ADSC Q-315 CCD detector using X-rays produced by a 26 pole wiggler insertion device, with a wavelength of 0.81798 Å (15160 eV) from a side scattering bent asymmetric cut Si(111) crystal monochromator. Two data sets were collected, both consisting of 90 1° images with a crystal to detector distance of 97 mm and covering the same range of phi angle. The first high-resolution pass had an exposure time of 15 s and the second pass had an exposure time of 1 s to record the strong low resolution reflections discarded from the first pass due to overloading of the CCD detector. The data were processed with the program XDS and scaled together with the program XSCALE.[85] Bijvoet pairs were not merged and no absorption correction was applied. A total of 53901 reflections were measured to a nominal resolution of 0.77 Å, resulting in a final unique dataset of 17617 reflections with a merging R factor of 0.105.

The structure was solved by Patterson methods to locate the cobalt, phosphorus and sulfur atoms, then the lighter atoms located by difference Fourier synthesis, as implemented in the program SHELXS.[86] The structure was refined by full matrix least-squares methods using SHELXL. All 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 15160 eV was applied during refinement. Additional difference electron density peaks were modelled as water molecules. One of the hydroxyl oxygen atoms of the ribose moiety appeared to have two distinct conformations and these were modelled with 50% occupancy. The final crystallographic R factor, $R1$, was 0.1023 for 16106 reflections, with $F_o > 4\sigma F$. Additional data collection and refinement statistics are given in Table 1.

CCDC reference number 615032.

For crystallographic data in CIF or other electronic format see DOI: 10.1039/b610158e

Measurement of the Co(III) absorption spectrum

The cobalt absorption edges for the Na[NACCbl] sample were measured on SSRL beamline BL9–2 from the frozen crystal used for data collection (irradiated sample) and from a freshly mounted frozen crystal which had not been exposed (unirradiated sample), using X-rays produced by a 16 pole wiggler insertion device through a flat Rh coated collimating mirror, a liquid nitrogen cooled double Si(111) crystal monochromator and a toroidal focussing mirror. The spectra were collected in fluorescence mode between 7500 and 7900 eV using a Canberra/Eurisys Si drift detector with a total acquisition time of 440 s. The spectra were normalized by dividing the sample fluorescence at each point by the fluorescence at the inflection point of the first EXAFS peak (at 7.784 eV). The first derivative of the spectra were calculated using the program AUTOCHOOCH[87] which is based on the Kramers–Kronig transformation algorithm and the inflection point or threshold energy taken as the minimum of this transformation.

Results and discussion

Synthesis and characterization of D,L-homocysteinylcobalamin, *N*-acetyl-L-cysteinylcobalamin (Na^+ salt) and 2-*N*-acetylamino-2-carbomethoxy-L-ethanethiolatocobalamin

Previously one of us reported procedures for synthesizing and isolating the thiolatocobalamins γ-glutamylcysteinylcobalamin and glutathionylcobalamin in aqueous solution in high yield and purity by the addition of a small excess of thiol to a highly concentrated solution of aquacobalamin, followed by the addition of

Table 2 UV-Vis and ^{1}H NMR spectroscopy data for cobalamins at pH/pD 5.5 (0.10 M MES buffer); electronic spectra were recorded at 25.0 °C and NMR spectra were collected at room temperature

	UV-Vis spectroscopy data				^{1}H NMR spectroscopy data: chemical shift (ppm)				
Cobalamin	λ_{max}/nm				B7	B2	B4	R1	C10
H_2OCbl^+		349	411	525	7.18	6.54	6.47	6.26	6.30
GSCbl[a]	333	372	428	534	7.19	6.95	6.39	6.28	6.09
$NACCbl^-$	333	372	428	534	7.19	6.95	6.40	6.28	6.09
NACMECbl	333	372	428	534	7.19	6.95	6.40	6.28	6.09
HcyCbl	333	372	428	534	7.20	6.95	6.38	6.28	6.10
SO_3Cbl^-	312	365	418	517	7.17	6.94	6.43	6.25	6.02
NO_2Cbl		354	413	532	7.20	6.74	6.42	6.28	6.20

[a] ^{1}H NMR chemical shifts from ref. 84.

acetone to precipitate the product after completion of the reaction.[27,43] A similar procedure was also used to synthesize pentafluorophenylthiolatocobalamin and cyclohexylthiolatocobalamin in methanol.[25,26] Herein we report that this procedure can be utilized to synthesize the novel and biologically relevant thiolatocobalamin derivatives HcyCbl, Na[NACCbl], and NACMECbl in high purity (>95%) and in good yield (>70%). To obtain a pure product, it was necessary to synthesize HcyCbl under anaerobic conditions. All other syntheses were carried out under aerobic conditions. It is not entirely clear why the synthesis of HcyCbl must be carried out under anaerobic conditions while the other thiolatocobalamins are successfully synthesized in the presence of air. It has previously been reported that the close proximity of a ligand NH_3^+ group to the Co–S bond could be responsible for the poor stability of the related cysteinylcobalamin and cysteinylglycinylcobalamin derivatives.[43] The products were characterized by ES-MS, ^{1}H NMR spectroscopy, and UV-Vis spectroscopy. Fig. 3 gives the ^{1}H NMR spectrum of the aromatic region for HcyCbl. The expected five signals attributable to the B2, B4, B7 protons of the α-dimethylbenzimidazole nucleotide, the C10 proton of the corrin ring and R1 proton of the ribose are observed at 6.95, 6.38, 7.20, 6.10 and 6.28 (d), respectively.[84]

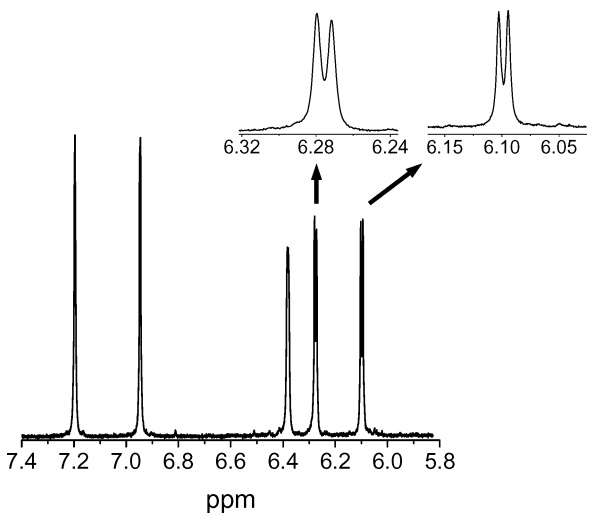

Fig. 3 ^{1}H NMR spectrum of D,L-homocysteinylcobalamin in D_2O, pD 5.9 (0.1 M MES buffer), 22 ± 1 °C. Five major signals are observed at 7.20, 6.95, 6.38, 6.28, and 6.10 ppm, corresponding to the resonances of the B7, B2, B4, R1 and C10 protons (see Fig. 1).

Similar spectra were observed for the other thiolatocobalamins. Since we used a racemic mixture (D,L-Hcy) of the ligand, two stereoisomers of HcyCbl are formed, making the peak at 6.10 ppm appear as a doublet when it actually corresponds to two separate species. ^{1}H NMR chemical shifts in the aromatic region and UV-Vis wavelength maxima for the new derivatives are summarized in Table 2, together with previously reported values for glutathionylcobalamin.[84] From this table it can be seen that there is excellent agreement between the chemical shifts and wavelength maxima for all thiolatocobalamins.

The percentage of cobalamin impurities present in the products can also be estimated from the aromatic region of the ^{1}H NMR spectrum, and was found to be ~2% for HcyCbl, Na[NACCbl], and NACMECbl. The percentage of non-corrinoid products (salts) in the product can be determined by converting the thiolatocobalamin to dicyanocobalamin after drying the product at 50 °C under vacuum ($\varepsilon_{367\,nm} = 30.4\,mM^{-1}\,cm^{-1}$),[81] and was found to be ≤5%.

X-Ray structure of Na[NACCbl]·18H_2O

Crystals suitable for X-ray diffraction studies were obtained for the sodium salt of *N*-acetyl-L-cysteinylcobalamin from a concentrated solution of HOCbl·HCl, with 2 molar equivalents of NAC and 1% NaCl in water. Crystal data and structure refinement parameters are given in Table 1. Na[NACCbl]·18H_2O crystallizes in the orthorhombic space group $P2_12_12_1$ with one molecule per asymmetric unit. Analysis of the crystal packing by comparison of the ratios of the $c : a$ and $b : a$ unit cell dimensions show that these crystals are typical of cluster I packing ($c : a = 1.575$, $b : a = 1.305$).[69] The crystal structure of the cobalamin molecule has been described exhaustively in the literature and the current Na[NACCbl]·18H_2O structure (Fig. 4A) does not deviate markedly from the cobalamin structures known to date. The individual cobalamin molecules are oriented in the crystal such that the plane of the corrin ring is roughly parallel to the *ab* plane of the unit cell. However, the neighbouring cobalamin molecules are not perfectly parallel with each other, which gives rise to layers of zig-zagged planes when viewed perpendicular to the *bc* plane, separated by layers of solvent molecules. The axial base and the *N*-acetyl-L-cysteinyl ligand extend into these solvent layers. When viewed down the 2-fold screw along the *c* axis, long solvent channels are clearly evident. This solvent structure has been modelled in Na[NACCbl] as 18 water molecules. The majority of the solvent molecules are directly hydrogen bonded to either

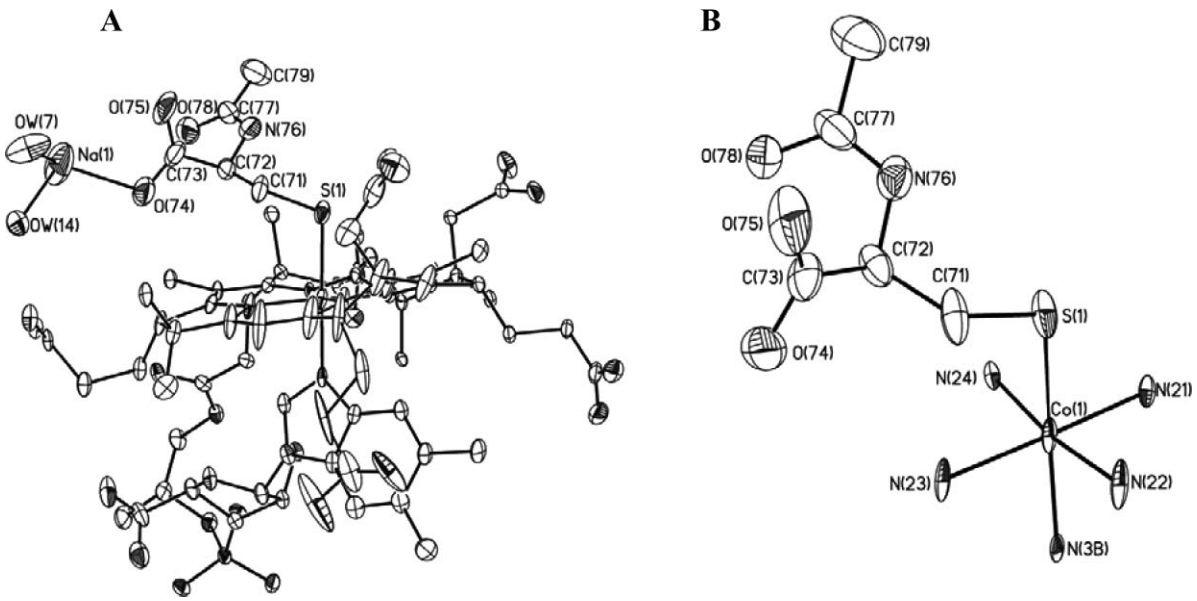

Fig. 4 Thermal ellipsoid plot (30%) of Na[*N*-acetyl-L-cysteinylcobalamin]·18H_2O. (A) View of the entire cobalamin complex. The *N*-acetyl-L-cysteine is bound to the Co through the sulfur atom (Co–S bond distance = 2.250(3) Å) and the carboxylate O of the NAC ligand is bound to a Na^+ cation. Two waters bound to the Na^+ cation are also shown. Other solvent molecules are not shown for clarity. (B) Close-up view of the *N*-acetyl-L-cysteine ligand.

Table 3 Comparison of the Co coordination sphere in a number of Co–S containing cobalamins

	Na[NACCbl]	Na[γ-GluCysCbl][a]	$[(NH_2)_2CSCbl]Cl$[b]	[NCS]Cbl[c]	$NH_4[SO_3Cbl]$[b]
Co–S	2.25	2.27	2.22	2.25	2.23
Co–N3B	2.06	2.05	2.01	1.99	2.13
Co–N21	1.88	1.89	1.85	1.89	1.87
Co–N22	1.92	1.90	1.90	1.92	1.91
Co–N23	1.93	1.91	1.91	1.92	1.89
Co–N24	1.88	1.89	1.88	1.90	1.89
Corrin fold/°	17.5	24.2	14.9	14.9	16.3

[a] From ref. 43. [b] From ref. 88. Also see ref. 59. [c] From ref. 69.

an oxygen or a nitrogen atom, although one water molecule is hydrogen bonded to other water molecules only. There is evidence for the presence of a sodium ion at a distance of 2.61 Å from one of the oxygen atoms (O74) of the carboxylate group of the cysteinyl ligand. Although it was initially modelled as a water molecule, it was found to be within 3 Å of five other oxygen atoms (O74 and four water molecules, at an average distance of 2.87 Å), hence it was changed to a sodium ion in the final stages of the refinement. This is consistent with the presence of a negative charge at the cysteinyl carboxylate, and the observation of a sodium ion near the glutamyl carboxylate in the γ-GluCysCbl structure.[43] There are significant intermolecular contacts in the crystal lattice, and although there are five direct nitrogen–oxygen hydrogen bonds linking neighbouring cobalamin molecules, the majority of the interactions involve water-mediated hydrogen bonds. Although the final *R* factor of 10.24% is typical of the cobalamin structures,[43] this could possibly be explained by a combination of inherent disorder in the crystal lattice due to the relative lack of direct contacts *versus* water-mediated interactions, and the presence of disorder in the solvent structure itself. Attempts were made during the latter stages of the refinement to model the disorder solvent but this gave negligible improvement to the overall *R* factors.

The *N*-acetyl-L-cysteine ligand is bound to the cobalamin through the sulfur atom as expected (Fig. 4B), with a Co–S bond distance of 2.250(3) Å, similar to the bond lengths observed in other Co–S containing cobalamin structures determined previously (Table 3).[43,59,69,88] Furthermore, the four in-plane Co–N bonds and the axial base Co–N bond are all very similar to those reported elsewhere. Comparison of the Na[NACCbl]·18H_2O structure with that of γ-GluCysCbl is rather interesting, in that whereas the upward corrin fold in the latter was found to be the largest yet observed in a cobalamin structure (24.2°), the corrin fold angle in Na[NACCbl]·18H_2O of 17.5° is significantly less, although still slightly larger than some of the other Co–S containing cobalamins. This is rather intriguing given that the Co–S and Co–N bond lengths show very little variation between these two structures, although it was observed in the γ-GluCysCbl that there was a hydrogen bonding interaction between N40 and the cysteinyl sulfur (N–S distance of 3.41 Å), and it was suggested that this could contribute to the larger upward fold of the corrin ring.[43] A similar interaction could exist in Na[NACCbl]·18H_2O, although the orientation of the N40 and S1 atoms are not entirely optimal for hydrogen bonding, in that the C71–S1–N40 angle is close to 90° and the N–S distance is 3.55 Å. However, there could still be a weak interaction and it is conceivable that the strength

of this intramolecular hydrogen bond may have an impact on the amount of pucker in the corrin ring.

Co(III) X-ray absorption spectrum

The cobalt X-ray absorption spectroscopy (XAS) spectrum from 7650 to 7850 eV for the Na[NACCbl] crystal used in the X-ray diffraction experiments (irradiated NACCbl) and a second Na[NACCbl] crystal from the same crystal batch which had not previously been exposed to X-rays (unirradiated NACCbl) are given in Fig. 5. The overall shape of the XANES region of the unirradiated Na[NACCbl] crystal is reminiscent of the XANES spectra for unirradiated CNCbl and H_2OCbl^+.[89] The XANES spectrum of the crystal used for the X-ray diffraction data collection shows a significant drop in the intensity of the first maximum, as was also previously observed for CNCbl and H_2OCbl^+ upon prolonged exposure to X-rays.[89] In this study, the samples were exposed to X-rays during the collection of the XAS spectra and for extended periods in between as a simulation of typical X-ray exposure during X-ray structure data collection. In the case of Na[NACCbl], the irradiated sample was produced during normal synchrotron X-ray diffraction data collection (the total exposure time was between 25–30 min, including initial test images), and the XANES spectrum of the irradiated sample was measured subsequently to determine whether any damage had occurred. To our knowledge, the current results with Na[NACCbl] is the first reported example showing that irradiation damage does indeed occur to cobalamins during X-ray diffraction data collection. The XANES spectrum of GSCbl has also been previously reported,[29] and resembles the irradiated Na[NACCbl] XANES spectrum. In this study, XAS and EXAFS spectra were recorded 6–9 times, hence it is possible that photoreduction of the sample did occur. It has been proposed that irradiation of cobalamins may lead to mixed Co(III)/Co(II) oxidation states,[89] which may ultimately be responsible for the abnormally long Co–N bond lengths observed for Cbl-bound methylmalonyl-CoA mutase and glutumate mutase as a consequence of the formation of six-coordinate Cbl(II) species.[89] It is well established in protein crystallography that X-ray exposure during diffraction data collection generates free radicals which cause extensive radiation damage to the protein molecule, including cleavage of disulfide bonds, decarboxylation of acid residues, changes in crystal packing and photoreduction of metal centers in metalloproteins.[90,91]

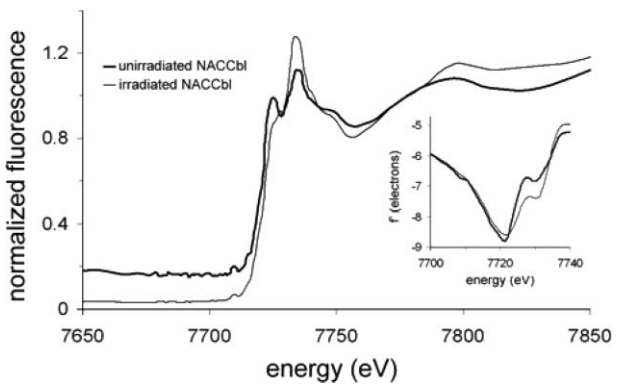

Fig. 5 X-Ray absorption spectrum of unirradiated (bold) and radiated Na[NACCbl]·18H_2O between 7500 and 7900 eV. The inset shows the first derivative of the spectrum, with the negative peak position (7721.3 and 7721.4 eV for the irradiated and unirradiated samples respectively) corresponding to the position of the inflection point or threshold energy.

A change in the threshold energy has been interpreted as additional evidence for significant photoreduction by the X-ray beam.[89] The threshold energy (or inflection point) of the absorption edge, calculated from the first derivative of the edge spectra (see inset) using the Kramers–Kronig algorithm implemented in AUTOCHOOCH, is the same within experimental error (7721.3 ± 0.2 and 7721.4 ± 0.2, respectively) for the unirradiated and irradiated Na[NACCbl] crystals. This result combined with the reasonable Co–S and Co–N bond lengths suggest that radiation damage of Na[NACCbl] probably has a minimal effect on the Co oxidation state and the first coordination sphere ligands during X-ray data collection in this particular case. The threshold frequency has also previously been used as an indicator of the oxidation state of the cobalt center, with the threshold frequency supposedly increasing with increasing oxidation state.[92] However, the value of 7721.3 eV obtained for unirradiated Na[NACCbl] is closer to that reported for Cbl(I) (7721.0 ± 0.2) rather than Cbl(II) (7722.0 ± 0.2), or the X-ray insensitive AdoCbl (7722.0 ± 0.2) and MeCbl (7722.5 ± 0.2),[89] suggesting that the threshold frequency may not always be a good indicator of the oxidation state of the cobalt center. Values of 7721.8 ± 0.25 and 7721.0 ± 0.2 have been previously reported for GSCbl and cysteinylcobalamin, respectively.

Synthesis and characterization of sulfitocobalamin and nitrocobalamin

Although equilibria and rate constants for the formation of a wide range of non-alkylcobalamin derivatives in aqueous solution ligated *via* S, O or N atoms to the cobalt(III) have been reported,[61] very few studies report the actual isolation of these compounds in a pure form. Excess ligand is used to ensure the reaction goes to completion, requiring a subsequent column chromatography desalting step (typically Amberlite XAD-2 or XAD-4) to remove the remaining unreacted ligand prior to isolation of the product. A Sephadex C-25 column chromatography step may also be required to separate the product from unwanted cobalamin side-products. Our method employs *very high*, *practically saturated*, *concentrations of aquacobalamin* in addition to high ligand concentrations, so that the desired product is rapidly and completely formed using essentially molar equivalents of ligand. The requirement of a lower mole equivalent of ligand for complete formation of the product at higher aquacobalamin concentrations is readily understood from the definition of the formation constant for the complex. Since the product solution is so concentrated, the product is easily isolated in high yield by the addition of cold acetone. In addition, since high concentrations of ligand are also used, the percentage of ligand which is converted to other forms during the reaction can be significantly reduced, so that excess ligand is not required to compensate for side reactions involving the ligand. For example, the percentage of reduced thiol which undergoes B_{12}-catalyzed aerial oxidation to its inactive disulfide form (disulfides do not react with aquacobalamin[44]) during the synthesis of thiolatocobalamins from aquacobalamin is considerably less if the synthesis is carried out at high concentrations, and can remove

the need for a large excess of the reduced thiol and/or anaerobic conditions.

Sulfitocobalamin (SO_3Cbl^-) is a biologically important B_{12} metabolite,[11–17] and there are several reports concerning the synthesis and isolation of this derivative.[15,37,93] These methods require a large excess of sulfite to obtain complete formation of SO_3Cbl^-, requiring subsequent de-salting of the product using column chromatography. B_{12}-catalyzed oxidation of sulfite to sulfate can also result in the product being contaminated by the byproduct sulfatocobalamin.[20] It was of interest to see if our procedure could be applied to the synthesis and isolation of pure $Na[SO_3Cbl]$ in high yield, and indeed this cobalamin was readily synthesized in the presence of air using 1.2 mol equiv. sulfite without the need for either anaerobic conditions or subsequent de-salting by column chromatography to remove excess sulfite. The ^1H NMR spectrum of $Na[SO_3Cbl]$ is given in Fig. S1 in the electronic supplementary information (ESI)† and clearly demonstrates that the product is pure (~98%). Chemical shift values and UV-Vis wavelength maxima for $Na[SO_3Cbl]$ are summarized in Table 2. The percentage of salts in the product was found to be ~1% by conversion to dicyanocobalamin.

Nitrocobalamin (NO_2Cbl) is also a biologically important vitamin B_{12} metabolite.[11,20] NO_2Cbl is the aerobic decomposition product of nitrosylcobalamin, NOCbl,[70,71] and it has been postulated that NOCbl is a naturally occurring B_{12} derivative.[11,94–97] Evidence suggesting the biological relevance of reactions between nitric oxide and cobalamins include the observation that addition of hydroxycobalamin can diminish the biological effect of NO,[98,99] and the inhibition of vitamin B_{12}-dependent methionine synthase and methylmalonyl-CoA mutase by NO both *in vitro* and *in vivo*.[94–96] Indeed, Goldberg and co-workers isolated a new form of cobalamin in addition to NO_2Cbl from chicken brain under anaerobic conditions which they suggest is nitrosylcobalamin.[11] To our knowledge, there is only one report concerning the isolation of crystals of NO_2Cbl using excess nitrite; no yield was given.[100] NO_2Cbl was prepared using our general procedure in high purity (99%) and yield (92%) by the addition of 1.2 equiv. sodium nitrite to aquacobalamin followed by the subsequent addition of acetone. Chemical shift values and UV-Vis wavelength maxima for NO_2Cbl are given in Table 2. The aromatic region of the ^1H NMR spectrum for NO_2Cbl is given in Fig. S2 in the ESI,† and shows this derivative has ~2% cobalamin impurities. The percentage of non-corrinoid impurities determined by conversion to dicyanocobalamin was ~1%.

Summary

To summarize, the novel compounds HcyCbl, Na[NACCbl], and NACMECbl have been synthesized for the first time using our previously reported, remarkably simple procedure for the synthesis of thiolatocobalamins. We have also demonstrated that this method can be utilized to prepare other non-thiol containing cobalamin derivatives such as $Na[SO_3Cbl]$ and NO_2Cbl in high yield and purity, thus removing the need for a subsequent column chromatography de-salting step to remove excess ligand and/or anaerobic conditions to prevent B_{12}-catalyzed oxidation of air-sensitive ligands. We anticipate that this procedure can be readily adapted for the synthesis of a wide variety of non-alkyl cobalamin derivatives, and will thus prove to be extremely useful to those interested in synthesizing cobalamin derivatives to assist in the identification of unknown cobalamins isolated from biological samples, and those interested in testing the biological activities of different cobalamin derivatives in studies designed to elucidate uptake and intracellular B_{12} processing mechanisms.

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