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Journal of Inorganic Biochemistry 76 (1999) 197–209

JOURNAL OF Inorganic Biochemistry

Synthesis and characterization of isolable thiolatocobalamin complexes relevant to coenzyme B_{12}-dependent ribonucleoside triphosphate reductase

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Received 5 March 1999; received in revised form 2 August 1999; accepted 16 August 1999

Abstract

The syntheses, isolation and characterization of cyclohexylthiolatocobalamin (C_6H_{11}SCbl), glutathionylcobalamin (GluSCbl), and cysteinylcobalamin (CysSCbl) are reported in 75, 55, and 65% yield, respectively. Characterization was achieved using elemental analyses, L-SIMS (liquid secondary ion mass spectrometry), UV–visible spectroscopy and, for the more stable C_6H_{11}SCbl and GluSCbl, our recently established ^{1}H NMR method (which emphasizes the readily interpreted aromatic region of the cobalamin's ^{1}H NMR spectrum). Preliminary evidence is presented for clean homolysis of the RS–Co bond in C_6H_{11}SCbl, GluSCbl, and CysSCbl to give RS$^{\cdot}$ and $^{\cdot}$Co(II)Cbl radical pairs analogous to those that are intermediates in ribonucleoside triphosphate reductase (RTPR). A summary is provided which emphasizes the seven variables identified to date, underlying the successful syntheses and isolation of thiolatocobalamins, variables which make the one-step syntheses of RSCbls considerably more complex than they initially appear. Also briefly discussed are the analogous protein–S–Cbl complexes that are seen as side-products in RTPR, and the probability that such side-products are formed when HOCbl·HX is used as a possible 'active-site inhibitor' complex with B_{12}-dependent enzymes.

Keywords: Thiolatocobalamin complexes; Ribonucleoside triphosphate reductase; Coenzyme B_{12}

1. Introduction

Ribonucleotide reductases convert ribonucleotides to their deoxyribonucleotide analogs and are, therefore, essential for DNA synthesis and cell growth, both normal or malignant, in all living organisms (RTPR lead references: [1–6]; see also [7–12]). The coenzyme B_{12} (adocobalamin or AdoCbl)-dependent ribonucleoside triphosphate reductase (RTPR) is one of the structurally simplest and smallest of the ribonucleotide reductases and, therefore, RTPR has become a focus for efforts aimed at elucidating the intimate mechanism of action of the ribonucleotide reductase enzymes. In pioneering work, Stubbe and co-workers [7] have demonstrated the existence of a protein–S$^{\cdot}$ $^{\cdot}$Co(II)Cbl radical pair (where S$^{\cdot}$ represents a protein side-chain cysteinyl radical) during the reactions of RTPR by techniques which include EPR, UV–visible spectroscopy [8,9], and site-directed mutagenesis [10]. They have also provided evidence that the protein-S$^{\cdot}$ thiyl radical, generated from cysteine 408 in RTPR, abstracts H$^{\cdot}$ from the ribonucleotide substrate [8,10–12].

A number of mercaptocobaloximes (for alkylthiolato-(pyridinato)cobaloximes see [13–15]; [16,17]), Costa-type RS–Co complexes [18,19] and other B_{12}-model compounds [20–23] have been synthesized as RS–B_{12} models to determine the properties of the Co–S bond. A literature survey shows that RS–B_{12} models can be made by several different routes. The (alkylthiolato)cobaloximes (R′S–Co(dmg)$_2$(L), R′ = C_6H_5, CH_3, C_2H_5; L = py (CH_3)$_2$N-*p*-$C_6H_5NH_2$) were synthesized by ligand substitution using sodium thiolate and chloro(pyridinato)cobaloxime with an equimolar amount of base [15]. Alternatively, (alkylthiolato)(pyridinato)cobaloximes and (arylalkylthiolato)(pyridinato)cobaloximes can be made from alkylcobaloximes (R′–Co(dmg)$_2$(L′), R′ = Me, Et, i-Pr, Ph, n-Bu, s-octyl; L′ = py, 4-(t-Bu)-py, 4-CN-py) and diaryl disulfides via a radical pathway, either photochemically ($\lambda_{exc} > 275$ nm, 12–35°C) or thermally (105°C for alkyl and 55°C for arylalkyl, the exact temperature depending on the

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0162-0134/99/$ - see front matter
PII S0162-0134(99)00128-2

initial arylalkylcobaloxime Co–C bond strengths) [24–26]. Another interesting class of RS–B_{12} models has been synthesized from a series of cobalt pentadentate salen analogs possessing a thioether pendant group where sulfur is intramolecularly attached to cobalt [20–23].

The synthesis of pure, isolable thiolato*cobalamins* (RSCbls) is a different story. Reactions of aquacobalamin (H_2OCbl^+) with thiols in solution have been investigated previously [27,28]. A wide variety of thiols and sulfides, including 2-mercaptoethanol [29–33], homocysteine [28], cysteine [27,28,34–38], ethanethiol [27], cysteamine [39], sodium sulfide, and hydrogen sulfide [36,40], react with H_2OCbl^+ to form putative thiol-cobalamin adducts as intermediates; these reactions also reduce Co(III) to Co(II)Cbl at a rate which increases with higher pH and thiol concentrations [29–38]. In contrast to alkylthiols, other sulfur-containing ligands such as thiosulfato [41], thiocyanato [41,42] and sulfito [36] yield stable complexes. No interaction between H_2OCbl^+ and disulfides or thioethers was found [27]. In addition, an intramolecular thiolate–B_{12} complex has been synthesized by Hogenkamp's group [43], and a related complex has been investigated recently by Brown and Zou [44]. However, until recently [45] there was only one known isolable thiolatocobalamin (RSCbl), glutathionylcobalamin (GluSCbl) [27,28,46–50], a naturally occurring RSCbl believed to be a biosynthetic precursor to AdoCbl and MeCbl [49]. Even GluSCbl, which is stable enough for column chromatography and HPLC [48,49], was not isolated in a form free from the NaOAc elutant used for its column chromatography. In short, isolable, pure RSCbl complexes are rare [45].

Isolable thiolatocobalamins are of interest, however, for a number of reasons:

(i) Preliminary experiments [51] have demonstrated that RSCbls homolyze cleanly to RS$^\cdot$ + $^\cdot$Co(II)Cbl radicals, and are therefore ideal and novel precursors for the study of RS$^\cdot$ + $^\cdot$Co(II)Cbl radical pairs analogous to those seen in RTPR [7], or for studies of H$^\cdot$ abstraction by RS$^\cdot$ from RTPR substrate-like molecules. Other traditional methods to generate RS$^\cdot$, including pulse radiolysis of thiols and the reaction of AIBN with thiols, generate ambiguity as to which specific radical species is responsible for the H$^\cdot$ abstraction.

(ii) The conditions for formation, stabilization and the properties of thiolatocobalamins are relevant to the RTPR protein side-chain-bound, cysteinyl-Cbls formed as side-products during inactivation of RTPR by 2′-2′-difluorocytidine [52] (in fact, the use of a discrete RSCbl model complex from the present studies provided the crucial spectroscopic properties that helped the Stubbe group to identify unequivocally their protein–S–Cbl complexes; see Fig. 2 elsewhere [52]).

(iii) The bond dissociation energy (BDE) of RS–Cbls is unknown, but it provides a chemical precedent that the maximal stabilization of the RS$^\cdot$ $^\cdot$Co radical pair in the active site of RTPR can achieve, or must avoid, in various stages of the catalytic cycle.

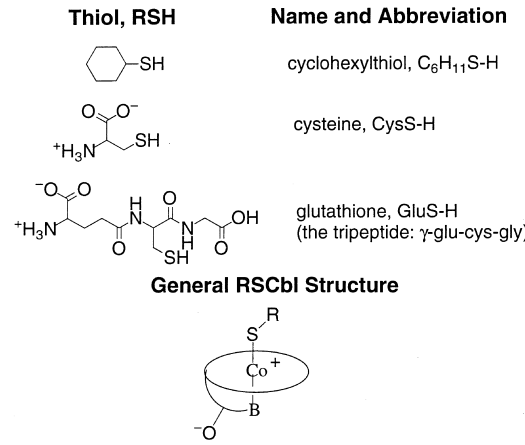


Fig. 1. Structures of the thiols and their corresponding thiolatocobalamin complexes.

(iv) Our studies reveal that the reaction H_2OCbl^+ + protein–SH → protein–S–Cbl + H_2O + H^+ appears to explain reports of analomously high binding to B_{12}-dependent enzymes when H_2OCbl^+ (or HOCbl·HX) is used as the cobalamin cofactor [53–59]. Professor Stubbe's laboratories have, in independent studies of RTPR, recently come to the same conclusion [52,60,61].

(v) Finally, the fifth and very fundamental reason is that RSCbl complexes provide a new source of RS$^\cdot$ and, significantly, also Co$^\cdot$ radicals; RSCbl complexes should, therefore (and as discussed elsewhere [1]), have significant applications in other areas of science, for example, in olefin free-radical polymerizations and polymer molecular-weight control.

Our recent paper [45] reports a survey of methods for the synthesis and isolation of pentafluorophenylthiolatocobalamin (C_6F_5SCbl), with the best synthesis giving about 95% pure product in 81% isolated yield. Pentafluorothiophenol (C_6F_5SH) was chosen for initial studies of the best routes for the synthesis of thiolatocobalamins because of (a) its anticipated stronger RS–Cbl bond energy, hence (b) its anticipated greater ease of isolation and stability [45], and (c) its very valuable ^{19}F NMR handle, allowing us to characterize the C_6F_5SCbl product more fully and to easily detect side-reactions involving the C_6F_5S group. However, even though $C_6F_5S^\cdot$ has superior H$^\cdot$ abstraction abilities [62], it is by no means clear that it is a good model for the cysteinyl side-chain –S$^\cdot$ in RTPR. Hence, other isolable RSCbl complexes are important and, therefore, are the focus of the present paper, including cysteinylcobalamin.

Herein we report the synthesis, isolation, and characterization of cyclohexylthiolatocobalamin ($C_6H_{11}SCbl$), glutathionylcobalamin (GluSH), and the metastable cysteinylcobalamin (CysSCbl). The structures of the thiols, and the resultant RSCbl complexes, are provided in Fig. 1. The seven important variables identified to date in the synthesis of thiolatocobalamins are also summarized, along with a discussion

[1] See footnote 11 in Ref. [45].

to link this work into the broader literature of B_{12}-dependent enzymes such as ribonucleoside triphosphate reductase.

2. Results and discussion

2.1. Synthesis and characterization

2.1.1. Cyclohexylcobalamin ($C_6H_{11}SCbl$)

Initially, it was unclear whether or not excess $C_6H_{11}SH$ would be required for the complete conversion of HOCbl·HX to $C_6H_{11}SCbl$; hence, prior to any synthesis attempts, 1 equiv. HOCbl·HCl (5.35×10^{-5} M) was titrated with $C_6H_{11}SH$ (0–8.64×10^{-5} M, 0–1.6 mol equiv.) in MeOH at room temperature:

$$\mathrm{HOCbl\cdot HCl + C_6H_{11}SH \overset{\text{MeOH}}{\underset{\text{r.t.}}{\rightleftharpoons}} C_6H_{11}SCbl + H_2O + HCl} \quad (1)$$

The reaction's progress was monitored by UV–visible spectroscopy and the corresponding set of spectra are given in Fig. 2. The inset of Fig. 2 shows a plot of change in absorbance versus the ratio of [$C_6H_{11}SH$] to [HOCbl·HCl] at three wavelengths (560, 532 and 354 nm). It is clear from this plot that $C_6H_{11}SCbl$ is fully formed after 1 equiv. of $C_6H_{11}SH$ has been added to 1 equiv. HOCbl·HCl and even under dilute conditions, i.e., excess thiol is not required. However, scaling up this procedure for synthetic purposes ($10^{-5}\rightarrow10^{-2}$ M HOCbl·HCl + $C_6H_{11}SCbl$) resulted in incomplete (about 50%) product formation (i.e., ~1:1 HOCbl·HCl:$C_6H_{11}SCbl$ was formed) when 1 equiv. RSH was added to 1 equiv. HOCbl·HCl. This can be explained by considering the solution pH; in the 10^{-5} M procedure, complete reaction will result in the formation of 10^{-5} M HCl (Eq. (1)), whereas in the 10^{-2} M procedure, 10^{-2} M HCl will be produced. Hence, the solution pH appears to affect the position of equilibrium in Eq. (1). Confirming this, the addition of 1×10^{-2} M CF_3COOH in MeOH to a solution of $C_6H_{11}SCbl$ (10^{-5} M) in MeOH under nitrogen resulted in the rapid reformation of H_2OCbl^+ ($t_{1/2}$ ~ 1–2 min).

A subsequent synthesis was performed employing 1 equiv. HOCbl·HCl plus 10 equiv. $C_6H_{11}SH$ at room temperature; however, although $C_6H_{11}SCbl$ was completely formed, the product was unstable and a small amount of side-product, Cob(II)alamin (Co(II)Cbl, $\lambda_{max}=473$ nm [63]) was produced. Cob(II)alamin formation could be prevented by the use of a buffer (LiOAc) to control solution pH; however, as we found previously for C_6F_5SCbl [45], a L-SIMS (liquid secondary ion mass spectrometry) of the resulting $C_6H_{11}SCbl$ revealed about $30\pm10\%$ Li^+ salt contaminant, and this procedure was, therefore, also abandoned (see, however, the work of Randaccio et al. [64] with crystalline Li^+Cl^- adducts [2]).

[2] The formation of crystalline, pure Li^+X^- adducts of cobalamins is also possible, as demonstrated by Randaccio et al. [64] who prepared, and characterized by single-crystal X-ray diffraction, the crystalline Li^+Cl^- adducts ClCbl·2LiCl·$n$$H_2O$ and N_3Cbl·2LiCl·$n$$H_2O$.

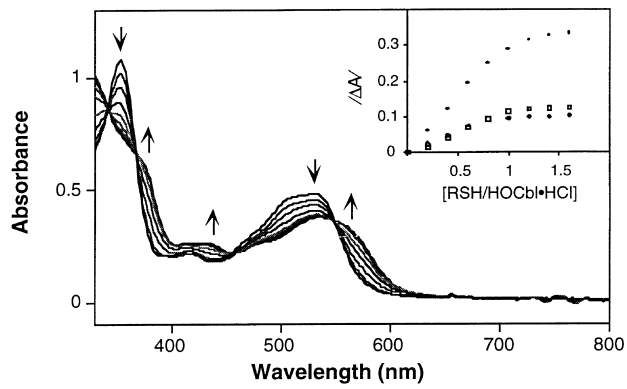


Fig. 2. Spectral changes during the titration of 5.40×10^{-5} M HOCbl·HCl with $C_6H_{11}SH$ (8.00×10^{-3} M) in MeOH at 25°C. Added [$C_6H_{11}SH$] = 0.000, 0.0108, 0.0216, 0.0324, 0.0432, 0.0540, 0.0648, 0.0756 and 0.0864 mM (in a molar ratio of [RSH/HOCbl·HCl] = 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6, respectively). The inset shows the absolute absorbance difference at each individual wavelength vs. [RSH/HOCbl·HCl] molar ratio (□: 560 nm, ◇: 532 nm, ○: 354 nm).

After the survey of numerous possible routes and conditions, crimson $C_6H_{11}SCbl$ was found to be best prepared, and in ~75% average yield (two separate syntheses gave yields of 83 and 64%), by the dropwise addition of 1 equiv. $C_6H_{11}SH$ in MeOH into 1 equiv. of HOCbl·HOAc in MeOH at room temperature in air-free, red light conditions:

$$\mathrm{HOCbl\cdot HOAc + C_6H_{11}SH \overset{\text{MeOH}}{\underset{\text{r.t.}}{\rightarrow}} C_6H_{11}SCbl + H_2O + HOAc} \quad (2)$$

This procedure, while similar to our previously reported synthesis of C_6F_5SCbl, incorporates three crucial differences important to the synthesis of $C_6H_{11}SCbl$:

(i) The mole ratio of thiol, RSH, to cobalamin was maintained as close to unity as possible (i.e., to avoid any possible contamination of the $C_6H_{11}SCbl$ with excess RSH or RSSR that might be formed during the synthesis, by-products which tend to co-precipitate with the product in the added-acetone isolation procedure). Also, since commercial cobalamins can contain up to 20% H_2O, the percentage H_2O present in the commercial HOCbl·HOAc starting material was determined by the standard procedure of conversion to $(CN)_2Cbl^-$ [65]; the resultant true amount of cobalamin present was then used to calculate the required 1.0 equiv. of RSH.

(ii) Room temperature (instead of −15°C used for C_6F_5SCbl) was employed so that the reaction proceeded rapidly to completion (at −15°C only $\sim35\pm10\%$ product was formed after 2 h, probably simply due to the reaction being too slow at −15°C).

(iii) The isolation procedure had to be changed slightly due to the greater solubility of $C_6H_{11}SCbl$ in MeOH; specifically, the $C_6H_{11}SCbl$ product was precipitated by the dropwise addition of the concentrated reaction solution into a large excess of cold acetone (−50°C), the opposite order of addition compared to that used in the isolation of C_6F_5SCbl [45].

Our recently developed ^{1}H NMR method [45,66], focusing on the well-separated and thus diagnostic aromatic chem-

ical shift region of cobalamin complexes, was used to determine the purity of the $C_6H_{11}SCbl$ product, $93 \pm 2\%$ (Fig. A, Section 5). The most readily identified, and thus most useful, ^1H NMR resonances from among the many overlapping ^1H NMR resonances observed for $C_6H_{11}SCbl$ are listed in Table 1, along with related chemical shifts of other thiolatocobalamins. The $7 \pm 2\%$ cobalamin impurity, with its diagnostic chemical shifts at $\delta = 7.25$, 6.88, 6.46, 6.24, and 5.96 ppm, is the same impurity found in C_6F_5SCbl ($6 \pm 2\%$ impurity in that case), an impurity we have determined is present initially, and at about the 11% level, in the commercial [3] HOCbl·HOAc reactant [45,67]. The $C_6H_{11}SCbl$ complex was also characterized by UV–visible spectroscopy and L-SIMS. As with C_6F_5SCbl [45], $C_6H_{11}SCbl$ could not be characterized by HPLC, since HPLC investigations failed to yield conditions where $C_6H_{11}SCbl$ is both stable (as it is in MeOH) and HPLC-separable from other possible cobalamin contaminants (e.g., pure MeOH as eluant did not provide the needed separation).

The UV–visible spectrum of a freshly prepared solution of $C_6H_{11}SCbl$ in MeOH (25°C) is given in Fig. 3, and exhibits absorption maxima at λ_{max} (ε, M^{-1} cm^{-1}) of 256 ($2.7(3) \times 10^4$), 280 ($2.3(1) \times 10^4$), 312 ($1.8(9) \times 10^4$), 338 ($1.9(8) \times 10^4$), 376 (shoulder, $1.3(7) \times 10^4$), 416 ($5.4(7) \times 10^3$) and 534 ($8.9(2) \times 10^3$) nm. A L-SIMS of $C_6H_{11}SCbl$ in a *m*-nitrobenzyl alcohol matrix exhibits the expected protonated parent peak at m/z 1444.9 ($[M+H]^+$ and a fragmentation peak at m/z 1329.8 corresponding to the $[(M+H)–C_6H_{11}S]^+$ ion (Fig. B, Section 5). As seen for C_6F_5SCbl [45], since the difference between the two peaks is 115.1 (i.e., 1444.9–1329.8 = 115.1) which corresponds to $C_6H_{11}S$ rather than $C_6H_{11}SH$, it can be concluded that the parent peak is due to the matrix-protonated species, $[C_6H_{11}SCbl + H]^+$, rather than due to the bound thiol complex, $[C_6H_{11}S(H)Cbl]^+$ (i.e., as the true species present). The isotropic distribution pattern of the parent peaks matches the expected computer-simulated spectrum (Fig. C, Section 5, Supplementary material). The isolated $C_6H_{11}SCbl$ gives a satisfactory C, H, N, S, and P elemental analysis.

2.1.2. Glutathionylcobalamin (GluSCbl)

This naturally occurring [49], and thus important, RSCbl has been prepared before in aqueous solution by two methods [27,48,49]. The relatively high stability of GluSCbl allowed it to be the first isolable RSCbl, although it was isolated in only one of the two earlier studies [48], and has never been isolated before in analytically pure form. The most recent, best previous procedure employs column chromatography to obtain GluSCbl with a purity greater than 98% cobalamin (by HPLC) but in an unreported, presumably low yield. The resultant GluSCbl is, however, impure as a solid due to its contamination with the NaOAc elutant employed in the column chromatography. (Note that the previous synthesis of GluSCbl was adequate for the reported 600 MHz NMR studies; i.e., the previous work had no need to improve the synthesis to yield solid GluSCbl in an analytically pure form.)

Table 1
Aromatic ^1H NMR chemical shifts of the B2, B4, B7, C10 and R1 signals of thiolatocobalamins at room temperature

RSCbl	δ (ppm)
$C_6H_{11}SCbl$ [a]	7.17, 6.99, 6.42, 6.21(d), 6.03
GluSCbl [a]	7.17, 7.01, 6.46, 6.20(d), 6.06
GluSCbl [b]	7.20, 6.95, 6.40, 6.29(d), 6.10
(Literature [c]	7.25, 6.97, 6.42, 6.33(d), 6.12)
C_6F_5SCbl [a,d]	7.17, 6.78, 6.42, 6.19(d), 6.04

[a] In CD_3OD; internally referenced to TMS (0 ppm).
[b] From this work; 0.05 M MES buffer in D_2O, pH 6.5 ± 0.1; internally referenced to TSP (0 ppm).
[c] From Ref. [48], D_2O (TSP), pH 6.5.
[d] From Ref. [45].

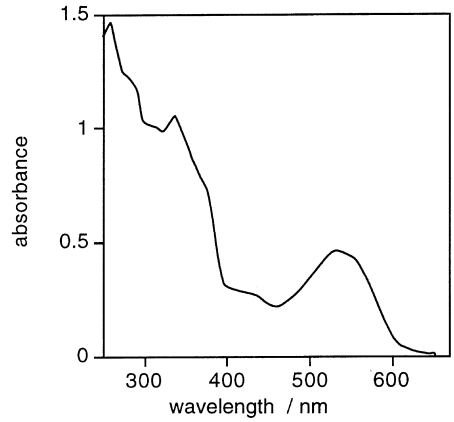


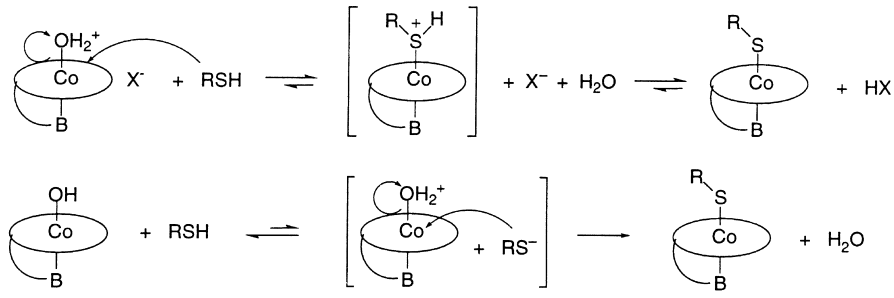
Fig. 3. UV–visible spectrum of $C_6H_{11}SCbl$ in aerobic MeOH at 25°C, directly (~15 min) after solution preparation.

In a synthesis that we worked out chronologically before we discovered the differences between using the $X = Cl^-$ versus OAc^- forms of HOCbl·HX, GluSCbl was synthesized by the addition of HOCbl·HCl to a solution of excess GluSH in MeOH:

$$\text{HOCbl·HCl} + \text{excess GluSH} \underset{\text{r.t.}}{\overset{\text{MeOH}}{\rightleftharpoons}} \text{GluSCbl} + H_2O + \text{HCl} + \text{excess GluSH} \quad (3)$$

The reaction was left for 2 h to proceed to completion, due to the limited solubility of GluSH in MeOH. The need for an

[3] When queried, Sigma replied that their current batch of HOCbl·HOAc (which was used for these experiments) was about 10 years old. Previous studies claim that HOCbl·HX stored even in the refrigerator for several years can contain up to 15% of a species initially named 'B_{12} prime' (i.e., B_{12}'), but which has subsequently been shown by Marzilli et al. [67], in the case of cyanocobalamin, to consist of monocarboxylic cyanocobalamin derivatives. It seems quite likely, then, that such monocarboxylic impurities are also the culprit(s) in HOCbl·HX. Unfortunately, however, the monocarboxylic acids of HOCbl·HX have, to our knowledge, never been isolated and characterized, and thus positive identification of the impurity(ies) in the commercial cobalamins, and their removal, remain to be accomplished. (We also see this impurity at about 5% levels in Sigma's HOCbl·HCl, and at about 15% levels in HOCbl prepared via an amberlite IRA-400 column in the basic form [45]; the increased approx. 15% impurity in the latter case is consistent with hydrolysis of one or more amide side chains in the cobalamin to a monocarboxylic acid(s).)



Scheme 1. Proposed mechanism for the formation of thiolatocobalamins from either HOCbl·HX or HOCbl.

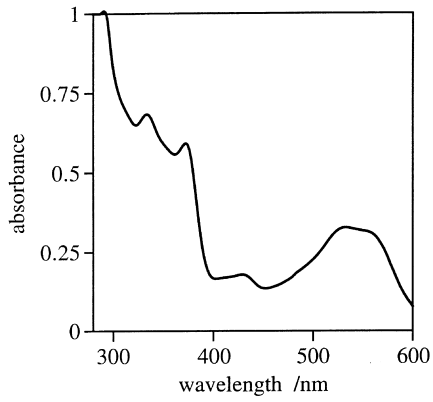


Fig. 4. UV–visible spectrum of GluSCbl in aerobic H_2O at 25°C, directly (~15 min) after solution preparation.

excess of GluSH can now be rationalized by the fact that the HOCbl·HCl salt produces HCl and, therefore, requires more GluSH to drive the reaction to completion (i.e., to the right in Eq. (3)). Excess GluSH was removed by column chromatography using Bio-Gel P2, according to the literature procedure [48], only using distilled water as the elutant to avoid salt (i.e., NaOAc) contamination of the product.

The purity of the resultant purple GluSCbl is high (~98% by ^{1}H NMR, see Fig. D in Section 5). Unlike other RSCbls, GluSCbl is relatively stable under the aqueous solution conditions required for the separation of GluSCbl from its possible H_2OCbl^+ contaminants. The ^{1}H NMR chemical shifts of the aromatic region for GluSCbl in both CD_3OD and D_2O (pH 6.5) are given in Table 1, and the latter values are in good agreement with literature values (see Table 1, entries 3 and 4) [4]. The UV–visible spectrum of GluSCbl in H_2O is given in Fig. 4 and exhibits absorption maxima at $\lambda_{max} = 252$ ($2.3(9)\times10^4$), 288 ($2.7(2)\times10^4$), 334 ($1.7(8)\times10^4$), 372 ($1.5(4)\times10^4$), 428 ($4.9(1)\times10^3$) and 534 ($8.7(3)\times10^3$) nm. A L-SIMS of GluSCbl exhibits a protonated parent peak at m/z 1635.8 ($[M+H^+]$) and a fragmentation peak at 1329.5 corresponding to $[(M+H)–C_6H_{11}S]^+$ (Fig. E, Section 5). The computer-simulated isotopic distribution pattern of the protonated parent peak (Fig. F, Section 5) is in excellent agreement with the experimentally observed pattern. The isolated GluSCbl also gives a satisfactory C, H, N and S elemental analysis.

A further synthesis of GluSCbl was attempted from HOCbl and in MeOH (Eq. (4)) analogous to the conditions that successfully yielded C_6F_5SCbl [45] (albeit in the case of C_6F_5SCbl, only 1.0 equiv. of C_6F_5SH is required):

$$HOCbl + 2GluSH \underset{\text{r.t.}}{\overset{\text{MeOH}}{\rightleftharpoons}} GluSCbl + H_2O + GluSH \quad (4)$$

Interestingly, no observable (<5%) reaction occurred. One possible explanation is that, in MeOH, glutathione will exist mainly as GluSH (pK_a 9.05 in aqueous solution [68]), whereas in the latter case the stronger acid, C_6F_5SH (pK_a 2.68 in aqueous solution [69]), will protonate HOCbl to yield the H_2OCbl^+ plus the $C_6F_5S^-$ conjugate-base nucleophile required for H_2O displacement reaction, Scheme 1.

Based on the syntheses of $C_6H_{11}SCbl$ and, previously, C_6F_5SCbl [45], which were developed, chronologically, after the above GluSCbl synthesis, it became of interest to see whether the need for excess GluSH and, then, for a column chromatography step to remove the excess GluSH, could be avoided by the use of HOCbl·HOAc. A reaction using HOCbl·HOAc and 2.0 equiv. of GluSH in MeOH at 25°C, followed by the use of UV–visible spectroscopy showed no reaction after 24 h. In another experiment, HOCbl·HOAc was reacted with 20 equiv. of GluSH in MeOH at 25°C. Incomplete reaction (~50%) was observed even after 6 h. In short, the otherwise preferred solvent (MeOH) fails as a suitable solvent for the synthesis of GluSCbl *from HOCbl·HOAc*.

We have also reinvestigated the puzzling report in the literature of 'break-point' between 1 and 2 equiv. in a UV–visible titration of [GluSH] and [HOCbl·HCl] [27] in water. Specifically, we titrated GluSH with HOCbl·HCl in aqueous solution, and at pH 5.5, while monitoring the reaction by UV–visible spectroscopy allowing complete return to equilibrium between each point (30 to 60 min, Figs. G and H, Section 5). The data allowed us to determine a formation

[4] A complete ^{1}H 600 MHz and associated ^{13}C NMR study of GluSCbl is available elsewhere for the interested reader based on the work of Brown et al. [48]. The study reports the two-dimensional COSY, HOHAHA, NOSEY, HMBC and HMQC spectra that are now well known to be required to observe, and to assign, individual resonances that otherwise overlap in the one-dimensional NMR spectra of cobalamin complexes. The simplified approach emphasized herein is the use of the ^{1}H NMR of the aromatic region of cobalamin complexes to verify their identity and purity [66]. This approach is used deliberately since it is most useful to the average researcher wishing simply to verify the identity and purity of a given RSCbl complex.

constant, $K_f = (1.1 \pm 0.3) \times 10^5\ M^{-1}$ at 25°C, for the equilibrium $H_2OCbl^+ + GluSH \rightleftharpoons GluSCbl + H_2O + H^+$ (pH 5.5) (data of Fig. H, and equation used to obtain this K_f value are available, see Section 5). Our K_f value is in general agreement with the results of Adler et al. [27], who found that excess GluSH is required for complete formation of GluSCbl in water. However, our equilibrium measurement indicates that no true 'break-point' between 1 and 2 equiv. of GluSH to HOCbl·HCl is present for this equilibrium process and in pH 5.5 solution (see the data in Fig. H).

2.1.3. Cysteinylcobalamin (CysSCbl): the design and development of syntheses in water, not MeOH

Despite its description in solution several times previously, no one has been able to isolate cysteinylcobalamin [27,28,34–38]. In our hands, CysSCbl has proved to be the most challenging of the RSCbls investigated to synthesize cleanly, to isolate, and to characterize as far as its metastability would allow.

Before proceeding with the synthesis that was eventually worked out for CysSCbl, it is worth taking a moment to summarize the non-cysteinyl RSCbl syntheses that we have derived to date, along with their specific conditions and isolation procedures, so that one can understand the problems that had to be overcome, and the revised reaction variables that resulted, in the synthesis of CysSCbl to follow. In the case of our previous work, C_6F_5SCbl [45], the best synthesis employed: HOCbl·HOAc (not the HCl salt), 1 equiv. C_6F_5SH, −15°C, MeOH solvent (which allowed the −15°C reaction temperature), dropwise addition of C_6F_5SH, a relatively short, 2–5 min reaction time even at the −15°C reaction temperature, then precipitation and isolation by the addition of −50°C acetone. In the case of $C_6H_{11}SCbl$, the synthesis was similar: HOCbl·HOAc, 1 equiv. of freshly distilled $C_6H_{11}SH$ added dropwise, MeOH as solvent, but a 25°C reaction temperature (since the reaction was too slow at −15°C), and the use again of the acetone precipitation and then an isolation procedure, but now using the inverse order of addition (dripping the $C_6H_{11}SCbl$ into −50°C acetone) in order to isolate the product due to the solubilizing effect of the $C_6H_{11}S$– group. In the case of GluSCbl, the synthesis required the use of HOCbl·HCl, a large 20-fold excess of GluSH, and a relatively long reaction time of 2 h at 25°C in MeOH. Purification and isolation required column chromatography, followed by the slow addition of 30 ml of acetone to each, approx. 5 ml fraction off the column. Subsequent cooling in a −22°C freezer caused precipitation of the desired GluSCbl.

Returning now to the synthesis of CysSCbl, the analogous synthesis that is obvious from the above syntheses was tried, namely HOCbl·HOAc plus 1 equiv. of CysSH in MeOH and at 0°C, but this synthesis failed even after 12 h. The reason is simple: the insolubility of CysSH in MeOH (see Section 4, which includes controls ruling out kinetic contributions to the (therefore thermodynamic) insolubility of CysSH in MeOH).

The failure of the above, otherwise preferred, HOCbl·HOAc and cold MeOH synthesis meant that a return to water as solvent was the next preferred solvent choice; this, in turn, meant that the amounts of CysSH, the pH, reaction temperature, optimum time and isolation procedure were all open to 'redevelopment' as a result of the insolubility of CysSH in MeOH. As it has turned out, carefully determined, somewhat longer reaction times, and a small excess of CysSH, yielded the best synthesis of the metastable CysSCbl.

Crucial information for the design of the CysSCbl synthesis which follows is from Nome and Fendler's report [34]: in 0.1 M pH 5.5 buffer, of a $\Delta H^{\ddagger}_1$(apparent) = 14.9 (±0.3) kcal/mol and $\Delta S^{\ddagger}_1$(apparent) = −3.6 (±2.0) e.u., so that a k_1(apparent) = 1.1 $M^{-1}\ s^{-1}$ at 0°C can be estimated for the synthesis at 0°C employed below. Also of value here are: k_1(apparent) = 11.7 $M^{-1}\ s^{-1}$ at 25°C; estimate of the k_{-1}(apparent) = $5.2 \times 10^{-5}\ s^{-1}$ (at 25°C and in 0.1 M pH 5.5 buffer), a number which, if reliable, indicates that once $CysS(H)Cbl^+$ is formed the back-reaction is slow ($t_{1/2}$ = 222 min); and the formation constant, K_1 (by Nome and Fendler's notation [34]) that can, therefore, be estimated as the relatively large value $K_1 = 2.2 \times 10^5\ M^{-1}$ for the reaction $H_2OCbl^+ + CysSH$ to give $CysS(H)Cbl^+ + H_2O$. There is confusion about whether one should ever write $RS(H)Cbl^+$ versus RSCbl as the true product under pH 5.5 conditions of Nome and Fendler, as the pK_a (equals the published pK^{II} [34]) for the equilibrium $RS(H)Cbl^+$ to $RSCbl + H^+$ is reported as an unreasonable pK_a = −10.9. (Even if real, this value would have been leveled by the phosphodiester conjugate acid pK_a = 0.22 [70] or, after that, by the axial base with its pK_a < 5.6, the pK_a value of the free 1-α-D-ribofuranosyl-5,6-dimethybenzimidazole (α-ribozole) [71].) Additional physical constants relevant here are the pK_a values of $CysS(H)Cbl^+$ versus that of $CysSCbl(H)^+$ (the protonated 5,6-dimethyl benzimidazole form), pK_a values which ideally one would have prior to designing any synthesis of CysSCbl. Unfortunately, these pK_a values are unknown; however, working estimates can be constructed as follows: one can estimate $0.5 \leq pK_a \ll 8.5$ for $CysS(H)Cbl^+$ (based by analogy to p$K_a(H_2O)$ = 15.7 versus p$K_a(H_2OCoCbl^+)$ = 7.8 [72–75]), and one can estimate $\sim 3.7 \leq pK_a < 5.6$ for the protonated axial base form $CysSCbl(H)^+$ (based on the upper limit of the free α-ribozole pK_a = 5.6 [71] and a crude estimate of the lower limit based on the protonated 5,6-dimethylbenzimidazole in $AdoCbl(H)^+$ pK_a = 3.7 [76]) [5].

In summary, the following, carefully chosen conditions were picked for the synthesis of CysSCbl in water: ~1.1

[5] Note that while Ado^- (with its estimated, conjugate acid Ado–H pK_a in the 45–50 range) is likely to be a much stronger σ donor than $CysS^-$ (with its conjugate acid pK_a = 8.5), there is the interesting, potentially strong, π-donor properties of a $CysS^-$ with is two lone pairs on sulfur, a feature which alkyl ligands such as Ado^- lack completely. The effects of these different σ and π effects of a RS^- ligand make the lower limit estimate of the $CysSCbl(H)^+$ pK_a ~ 3.7 quite uncertain, but a subject worthy of future investigation.

equiv. CysSH, 0°C, pH = 5.5, and 30 min reaction time (for 0°C and the concentrations used, vide infra).

2.1.4. Cysteinylcobalamin (CysSCbl)

The actual synthesis and isolation was accomplished by the dropwise addition of 1.15 equiv. CysSH [6] in aqueous MES buffer (0.05 M, pH 5.5 ± 0.2, MES = 2-[*N*-morpholino]ethanesulfonic acid, Na^+ salt) to a solution of 1 equiv. HOCbl·HCl in the same buffer at 0°C, followed by 30 min reaction time at 0°C (Eq. (5)). Fig. 5 gives the UV–visible spectrum of the CysSCbl product in anaerobic pH 5.5 water just before its isolation; the spectrum indicates complete (> 95%) formation of the desired CysSCbl in solution. The observed λ_{max} values of 332, 372, 426 and 534 nm in pH 5.5 MES buffer are in good agreement with literature values obtained in the presence of excess CysSH [34] (335, 374, 430, 532 (560) nm).

Precipitation of the desired, dark crimson CysSCbl was accomplished by the dropwise addition of cold (0°C) acetone; the resultant yield was 65%. The isolated CysSCbl exhibited a satisfactory elemental analysis (C, H, N, and S). The metastable product was stored in a refrigerator at 4°C under argon with minimal exposure to light.

$$\text{HOCbl·HCl} + \text{CysSH} \xrightarrow[0°C]{\text{aq. MES buffer}} \text{CysSCbl} + H_2O + \text{HCl} \qquad (5)$$

Regarding the choice of buffer, the literature reports that Co(II)Cbl will be produced by attempting the synthesis of CysSCbl from CysSH and HOCbl·HCl if the solution pH ≥ 7 [34–38]; pH 5.5 was chosen because of the large reported apparent formation constant for CysSCbl at this particular pH [34], $K_{app} = 2.3 \times 10^5\ M^{-1}$, pH 5.50, 25°C. Our observation that CysSCbl can be made by reacting 1.15 equiv. of thiol with HOCbl·HCl at pH 5.5 is, therefore, in agreement with the extensive equilibrium measurements of this system reported by Nome and Fendler [34]; our results confirm Nome and Fendler's finding that only slighly more than 1 equiv. of cysteine is required, not the 9 equiv. implied elsewhere [27].

As expected, characterization of the resultant metastable CysSCbl proved to be difficult, but we were able to characterize CysSCbl by L-SIMS, elemental analysis, UV–visible spectroscopy, and ^1H NMR (using a pD 5.5 D_2O buffer system). L-SIMS of CysSCbl in the *m*-nitrobenzyl alcohol matrix exhibits the expected protonated parent ion peak at m/z 1449.6 ($[M+H]^+$) and a fragmentation peak at m/z 1329.6 corresponding to $[(M+H)–CysS]^+$ (Fig. J, Section 5). The isotopic distribution pattern of the protonated parent peak is in excellent agreement with the computer-simulated protonated parent peak (Fig. K, Section 5).

[6] As detailed in Section 4, an initial control testing the solubility of a 100% excess of CysSH (i.e., 2.0 equiv. total) in the same amount of acetone/water (buffer) used to precipitate the CysSCbl product (adding the acetone first, then the buffer) revealed that the excess CysSH is insoluble under the conditions used to precipitate the CysSCbl product. This solubility control experiment predicts that the use of 2.0 equiv. of CysSH in the synthesis in an attempt to improve the yield or shorten the reaction time yields CysSCbl product contaminated with CysSH (~0.5 equiv., as confirmed by elemental analysis; see Section 4). Hence, our best, final synthesis employs 1.15 equiv. of CysSH and 30 min at 0°C to give a 65% yield of CysSCbl.

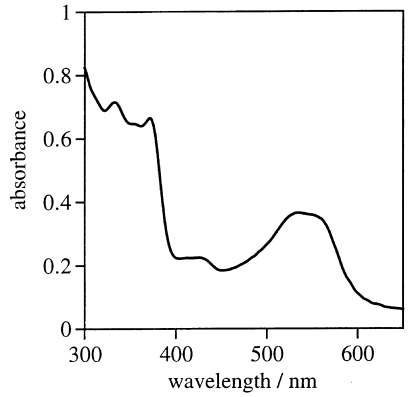


Fig. 5. UV–visible spectrum of the CysSCbl product in anaerobic pH 5.5 water just before its isolation.

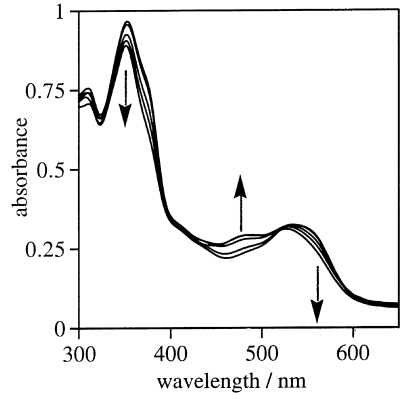


Fig. 6. UV–visible spectra of a solution of $C_6H_{11}SCbl$ in anaerobic water, pH 7.2, at 25°C as a function of time: $t = 0$, 1, 5, 10, 17 h.

However, once isolated, CysSCbl dissolved in anaerobic pH 7.2 water, or anaerobic dry MeOH, decomposes to Co(II)Cbl before a UV–visible spectrum of the product could be taken (about 1 min after solution preparation, and even at 15 ± 5°C) as indicated by the appearance of a peak at 313 nm and a shoulder at 476 nm (see Fig. L, Section 5). In anaerobic pH 5.5 MES buffer, isolated CysSCbl is more stable, but still decomposes to Co(II)Cbl in less than 1 h. Other solvents were tested, without success, for their ability to stabilize CysSCbl in solution (specifically acetone, acetonitrile, propylene carbonate, and dimethylformamide). Hence, it is clear that the Co–S bond in CysSCbl is metastable, especially at pH values above 5.5, and even at about 15°C.

The greater stability towards the Co–SR homolysis of CysSCbl in pH 5.5 versus pH 7.2 buffers is worth noting. We suspect that this greater stability at the lower pH is due to the formation of some fraction of the protonated axial benzimidazole base, and thus base-off and more stable, CysSCbl(H)$^+$ form. Studies are in progress [51] to support or refute this hypothesis.

2.2. Preliminary evidence for clean RS–Co homolysis to yield RS˙ and ˙Co(II)Cbl radical pairs

Fig. 6 gives the UV–visible spectra of a solution of $C_6H_{11}SCbl$ in anaerobic water, pH 7.2, as a function of time at 25°C. The results show isosbestic points at 400 and 520 nm, with the formation of Co(II)Cbl (and, by mass and charge balance, initially RS˙) with a $t_{1/2}$ of about 6.3 ± 1.8 h:

$$C_6H_{11}SCbl \rightleftharpoons Co(II)Cbl\cdot + C_6H_{11}S\cdot \rightarrow 1/2\ C_6H_{11}S\text{–}SC_6H_{11} \quad (6)$$

Analogous clean formation of Co(II) has been seen in preliminary Co–SR thermolysis experiments for the other RS–Cbls that we have prepared and under the proper solvent, pH and anaerobic conditions (for example, for CysSCbl; see Fig. M, Section 5) [27] but in some cases (e.g., $RS^- = C_6F_5S^-$ [45,51]) the products are more complicated than just the expected RS–SR disulfide (Eq. (6)) or likely involve precedented follow-up reactions of radicals such as CysS˙ and GluS˙ [77,78]. Hence, further studies of Co–SR homolysis products, kinetic and Co–SR BDEs are under their own, separate investigation [51].

2.3. Literature reports of protein–S–Cbl species in B_{12}-dependent enzymic reactions

Protein–SCbl complexes [7] have been detected recently by Stubbe and co-workers [52,60] in RTPR, side-products formed from the catalytically active protein–S˙ and ˙Co(II)Cbl intermediates undergoing degradative side-reactions. The protein–SCbl side-products were characterized by comparison to a sample of GluSCbl prepared as part of the present work and provided to the Stubbe group as a UV–visible and ES-mass spectroscopy model of the observed, protein–S–Cbl complexes, a good example of the synergism between biochemical and bioinorganic chemical-precedent studies. Of further interest here is that, despite CysSCbl being unstable under neutral aqueous conditions in solution, the protein–CysS–Cbl fragment reported by Stubbe and co-workers is relatively stable at pH 5–6; hence, there is an important difference in stability between the protein–CysSCbl and simple CysS–Cbl in solution, the origins of which remain to be elucidated. One possibility is that the RS–Co bond in protein–S–Cbl complexes is stabilized via protein radical-cage effects [79,80]; other effects are under investigation as well [8].

The present syntheses of isolable RSCbl complexes, from HOCbl·HX and RSH precursors, that is, RSH + HOCbl·HX → RS–Cbl + H_2O + HX, may be biologically relevant in another way besides their connection to the protein–S–Cbl products seen in the Stubbe laboratories or the production of RS˙ and ˙Co(II)Cbl shown in Eq. (6). Specifically, a number of literature reports describe unusually strong, 'irreversible' inhibition and apparent tight binding when using HOCbl·HX as an AdoCbl cofactor analog in thiol-containing B_{12}-dependent apoenzymes. The work presented herein, showing that RSCbl complexes are rapidly formed in protic solvents from RSH and H_2OCbl^+, reveals the possibility that the sulfhydryl side-chains in B_{12}-dependent enzymes react with added 'inhibitor' [9] HOCbl·HX to form protein–S–Cbl complexes rather than the previously assumed, active-site-bound HOCbl·HX species [10]. Certainly, the now chemically precedented and thus plausible formation of protein–S–Cbl complexes is an issue that needs to be rethought and experimentally re-examined with the proteins themselves. Our work reveals that it is not automatically valid to assume that HOCbl·HX will go solely to, or even reach at all, the active site in B_{12}-dependent enzymes that also contain cysteine residues.

3. Summary

Isolable, pure $C_6H_{11}SCbl$, GluSCbl and isolable CysSCbl have been prepared in 75, 55 and 65% yield, respectively. This is the first time that $C_6H_{11}SCbl$ has been described, the

[7] Stubbe and co-workers [52,60] have found that the inactivation of RTPR by the substrate analog 2′-deoxy-2′-2′-difluorocytidine is accompanied by the formation of protein–S–Cbl species involving, initially, predominantly the active site Cys419, i.e., they find that a Co–S bond is formed at some stage during the inactivation process. In a second paper studying another inhibition reaction of RTPR [61], Stubbe and co-workers identified a range of protein–S–Cbls involving the various cysteine side chains of the enzyme, the most predominant probably being the outwardly situated C-terminal Cys736. It was concluded, however, that in this case the protein–S–Cbls are formed via subsequent solution chemistry rather than being part of the intimate mechanism of enzymic inhibition by the 2′-deoxy-2′-2′-difluorocytidine substrate analog.

[8] In recent work we have obtained kinetic evidence for the destabilizing effect on the RS–Co bond of the adjacent $–NH_3^+$ group of cysteine in CysSCbl [81].

[9] Specifically, glycerol dehydratase [53], diol dehydratase [53–55], and ethanolamine dehydratase [56,57] report the use of HOCbl·HX as an 'inhibitor'. For example, for diol dehydratase it was found that, unlike methylcobalamin, adenosylcobalamin or cyanocobalamin, the binding of H_2OCbl^+ was not dependent on substrate or potassium ion concentration [54], i.e., it was different for some unexplained reason. For ethanolamine deaminase, 1 mol of enzyme became inactive after the addition of 2–3 mol H_2OCbl^+, suggesting, it was thought, that the enzyme may contain 2–3 active sites [57]. However, the enzyme was also capable of strongly binding another approx. 4 mol of H_2OCbl^+, and the H_2OCbl^+ remained bound (covalently), to the enzyme even after extensive dialysis [57]. In subsequent circular dichroism studies of the interaction between H_2OCbl^+ and ethanolamine deaminase [56], it was found that relatively large spectral changes (which were not seen for adenosylcobalamin) were complete upon the addition of 2 mol of H_2OCbl^+ to the enzyme [56]; these spectral changes were interpreted to suggest that the enzyme has two 'active sites' [56].

[10] Also worth mentioning here is the possibility of H_2OCbl^+ binding to other amino acid side-chains of the protein. Indeed, for bovine serum albumin [57] it has been shown that 16 of its 17 imidazole N atoms of its histidine side-chains titrate with an expected $pK_a \sim 6.9$, and that 16 mol of H_2OCbl^+ bind with bovine serum albumin [57]. The authors suggest that this type of non-active site binding could account for the nonspecific, excessive binding of H_2OCbl^+ to methyltransferase and ethanolamine deaminase.

first characterization of pure isolated GluSCbl, and also the first report where CysSCbl has been obtained as an isolable solid. Seven variables which influence the choice of the best reaction conditions and the ultimate success of RSCbl syntheses have been discovered and are as follows (with the generally preferred conditions in parentheses):

(i) The choice of the HOCbl·HX precursor ($X = OAc^-$ preferred over Cl^- for syntheses of $C_6H_{11}SCbl$).

(ii) The solvent (MeOH is best, unless the RSH is insoluble in MeOH, in part since MeOH allows the use of lower temperatures).

(iii) The amount of RSH (1.0 equiv. or a small excess if clean RSCbl formation can be achieved with only 1.0 equiv. of RSH; this minimizes the precipitation of excess RSH or RS–SR [11] with the desired RSCbl).

(iv) The reaction temperature (-15 to 0°C if possible to stabilize the RSCbl product; the offsetting variable here is the longer time for formation of RSCbls at lower temperatures; for more stable RSCbls such as GluSCbl, 25°C can be used).

(v) The reaction time (is variable as expected for different RSH or for MeOH versus H_2O solvent).

(vi) The pH in the case of syntheses in H_2O or RSH of unusual pK_a values (e.g., the solution pH should be about 2–3 pH units below the pK_a of the particular RSH so that the [RS(H)]–$CoCbl^+$ intermediate is deprotonated, but yet no appreciable RS^- is present, since RS^- reduces Co(III)Cbls to Co(II)Cbls; also, a pH value too acidic will decompose the RSCbl product). The unknowns here are the exact pK_a values of the [RS(H)]–$CoCbl^+$ intermediate and of the protonated-axial-base form, $RSCbl(H)^+$, as well as the extent to which this base-off form stabilizes, we hypothesize, the Co–SR bond towards homolysis or heterolysis. This latter effect may be quite significant as the first crystal structure of a RSCbl (in progress, [81]) shows a rather short, Co–N(axial-benzimidazole base) bond distance, a rather large corrin upward fold angle (corrin butterfly conformation), and thus presumably a quite significant labilizing effect on the Co–SR bond [81].

(vii) Finally, the order of addition, and temperature, of the added-acetone isolation procedure (the preferred method here depends upon the solubilities of the RSCbl, of any excess RSH, and of any possible RS–SR by-product; these are best pre-determined in simple solubility control experiments with authentic compounds).

The net result of the importance of the above seven variables in the otherwise seemingly (deceptively) simple, one-step '$H_2OCbl^+ + RSH \rightarrow RSCbl + H^+$' reaction is to make the synthesis of isolable, pure RSCbls much more challenging than first meets the eye.

[11] The catalytic oxidation of $2RSH + O_2 \rightarrow RSSR + H_2O_2$ is known [29,31] and is one precedented route for formation of the RSSR if air is present at any time during the synthesis, along with cobalamin catalyst. The reaction $2RSH \rightarrow RSSR + H_2$ is also known, at least for cobaloxime B_{12} model complexes [32], and related reactions exist even for simple $CoCl_2$ in CH_3CN [33].

Presently we are investigating the structural, protonated axial-base and other factors affecting the stability of RSCbls in kinetic studies of Co–SR homolysis and heterolysis. In particular we are investigating whether GluSCbl and related analogs really are unusually stable or, as it now appears, whether CysSCbl (and related complexes) are especially unstable due to their adjacent $-NH_3^+$ group [81]. Studies on the bond homolysis of Co–SR complexes to produce clean ·Co(II)Cbl plus RS· radical pairs are under investigation, along with any RS–Cbl bond dissociation energies that prove measurable. Chemical model studies on the H· abstraction step from RSCbl and other RS· precursors are also under investigation.

4. Experimental

Hydroxocobalamin hydrochloride ($HOCbl \cdot HCl \cdot nH_2O$, stated purity by manufacturer's TLC method ≥98%; ~95% purity by ^1H NMR [66]), the acetate salt of hydroxocobalamin ($HOCbl \cdot HOAc \cdot nH_2O$, stated purity by manufacturer's TLC method ≥97%; ~89% purity by ^1H NMR [66]), L-cysteine (CysSH, ≥98%), glutathione (GluSH, ≥98%) and 2-[*N*-morpholino]ethanesulfonic acid (MES buffer, K^+ salt, ≥99%) were obtained from Sigma. Cyclohexanethiol ($C_6H_{11}SH$, 97%) was purchased from Aldrich and was distilled before use at about 43°C (vapor temperature) under partial vacuum (water aspirator), then stored under argon. Distilled water was further purified by filtration through a Barnsted Nano-pure system. Methanol was refluxed over $Mg(OMe)_2$ and always freshly distilled prior to use. Ellman's reagent was obtained from Sigma and used as received.

UV–visible absorption spectra (±1 nm) were recorded on a Hewlett-Packard model 8452A UV–visible diode array spectrophotometer equipped with a built-in thermoelectric Pelter cell block temperature controller operating at 25.0±0.1°C unless stated otherwise. Air-sensitive samples were prepared in Schlenk cuvettes [82] using standard Schlenk techniques [83].

^1H NMR spectra were recorded on either a Varian Mercury-300 or Inova-300 spectrometer operating at room temperature and were referenced internally to 0 ppm with either TMS (CD_3OD) or TSP (D_2O). Anaerobic RSCbl solutions for NMR measurements were prepared in a Vacuum Atmospheres glovebox (N_2, ≤2 ppm O_2).

L-SIMS was performed on a VG AutoSpec double focusing mass spectrometer operating in the positive detection mode. Samples were dissolved in *m*-nitrobenzyl alcohol matrices. Simulations were obtained with the Micromass program OpusV3.5X.

All experimental errors are reported as the standard deviation of the mean value.

4.1. UV–visible spectroscopy titrations

The concentrations of HOCbl·HCl, $C_6H_{11}SCbl$ and GluSH were determined according to literature methods [65,84,85]. Specifically, the HOCbl·HCl concentration was obtained by converting HOCbl·HCl to $(NC)_2Cbl^-$ [65]; 0.1 M KCN, pH 10, $\varepsilon_{367} = 3.04 \times 10^4\ M^{-1}\ cm^{-1}$. A control experiment with HOCbl·HCl, predried under vacuum over P_2O_5 at 80°C for 12 h, showed 97(±5)% cobalamin. The concentrations of $C_6H_{11}SH$ and GluSH were determined using Ellman's reagent [79,80]; the RSH purity agreed within experimental error with the purity given by the supplier.

Titration experiments were performed at room temperature by UV–visible spectroscopy on low concentration MeOH solutions of HOCbl·HCl titrated with thiol solutions added through a microsyringe and by monitoring, for $C_6H_{11}SCbl$ for example, the disappearance of HOCbl·HCl at the α and γ bands (532 and 354 nm, respectively) and the appearance of $C_6H_{11}SCbl$ at 544 nm. Amounts and other details are given in the figure captions.

4.2. Synthesis of $C_6H_{11}SCbl$

All syntheses were performed, as a general precaution, under conditions of red light only in an argon atmosphere using standard Schlenk techniques [77,78] even though the light sensitivity of RSCbls is lower than their RCbl analogs. The percentage of Cbl present in commercial HOCbl·HOAc·nH$_2$O was determined to be 80±1% by the standard method of conversion of the HOCbl·HOAc·nH$_2$O to $(NC)_2Cbl^-$ [65]. Three separate determinations were performed, giving %Cbl = 80, 78 and 81%, respectively. HOCbl·HOAc was allowed to react for more than 1 h in 0.1 M KCN/0.1 M phosphate buffer, pH 10.3±0.1. Freshly distilled MeOH was degassed by three freeze/pump/thaw cycles (<0.01 Torr).

HOCbl·HOAc (54.5 mg, 3.14×10^{-5} mol, after allowing for 80% HOCbl·HOAc content) was dissolved in ~2 ml MeOH in a 50 ml side-armed Schlenk flask containing a stir bar. A solution of freshly distilled $C_6H_{11}SH$ (3.85 μl, 3.15×10^{-5} mol) was added dropwise to the cobalamin solution via a 5 ml syringe with stirring at room temperature. Additional MeOH (~1 ml) was used to complete the transfer of the remaining $C_6H_{11}SH$ into the flask containing the cobalamin solution, and the resultant solution was stirred at 25°C for about 2–5 min. A 15 μl aliquot of the solution was withdrawn for dilution in ~3 ml MeOH (i.e., to $\sim 5 \times 10^{-5}$ M) to confirm that the desired RSCbl formation reaction occurred by UV–visible spectroscopy (λ_{max} = 312, 338, 376 (s), 416 and 534 nm in MeOH, 25°C). The solution was concentrated to ~0.3 ml under vacuum at room temperature and dripped into a flask containing ~80 ml cold (−50°C, ethanol/dry ice bath), degassed acetone (degassed by bubbling argon through it for 1 h) via a 1 ml syringe to yield a crimson precipitate. The solid $C_6H_{11}SCbl$ product was removed by filtration via a Schlenk apparatus, washed with three ~10 ml portions of cold (−50°C) degassed acetone, and dried under vacuum overnight (0.01 Torr, room temperature).

Two independent syntheses gave yields of 83 and 64%, respectively. ^{1}H NMR aromatic signals (in CD_3OD, referenced to TMS, room temperature): δ 7.17, 6.99, 6.42, 6.21 (d) and 6.03 ppm; 93±4% cobalamin purity by integration (see Fig. A, Section 5). L-SIMS (in *m*-nitrobenzyl alcohol matrix) calc. molecular mass for $C_{68}H_{100}O_{14}N_{13}PSCo$ (i.e., $[M+H]^+$) = 1444.6; found: *m/e* 1444.9 ($[M+H]^+$) and 1329.8 ($[(M+H)–C_6H_{11}S]^+$) (Figs. B and C, Section 5). Elemental analysis on a sample predried at 25°C under vacuum, which showed ~88(±5)% cobalamin by the dicyano method, then dried further at Galbraith Laboratories for 8 h under vacuum at 80°C: $C_6H_{11}SCbl \cdot 5H_2O$ (i.e., ~6(±5)% H_2O based on the $\cdot 5H_2O$ formulation) (found): C, 53.22 (52.95); H, 7.16 (7.06); N, 11.86 (11.71); S, 2.02 (1.77); P, 2.09 (1.81). UV–visible λ_{max} (ε, $M^{-1}\ cm^{-1}$, in MeOH): 256 ($2.7(3) \times 10^4$), 280 ($2.3(1) \times 10^4$), 312 ($1.8(9) \times 10^4$), 338 ($1.9(8) \times 10^4$), 376 (shoulder, $1.3(7) \times 10^4$), 416 ($5.4(7) \times 10^3$) and 534 ($8.9(2) \times 10^3$) nm. Solid $C_6H_{11}SCbl$ can be handled in air; recommended storage is in a refrigerator (or freezer, for longer term storage) and under argon. In anaerobic aqueous pH 7.2 (±0.1) potassium phosphate buffer (0.05 M), decomposition of $C_6H_{11}SCbl$ (10^{-5} M) to Co(II)Cbl occurs with $t_{1/2} \sim 6.3 \pm 1.8$ h ($3t_{1/2}$ data collected), whereas in pH 4.0 potassium hydrogenphthalate buffer, $C_6H_{11}SCbl$ is hydrolyzed to H_2OCbl^+ with $t_{1/2}$ = 45 min. $C_6H_{11}SCbl$ appears to undergo very slight decomposition ($\Delta A_{max} \sim 0.03$ in the 300–800 nm region over 12 h) in anaerobic MeOH, as observed by UV–visible spectroscopy, although no change in the aromatic region of the ^{1}H NMR is observed over a period of 2 days.

4.3. Synthesis of GluSCbl

4.3.1. From HOCbl·HCl in MeOH

GluSH (182 mg, 6.0×10^{-4} mol) was dissolved in ~17 ml MeOH in a 100 ml side-armed Schlenk flask containing a stir bar. HOCbl·HCl (40 mg, 2.3×10^{-5} mol, corrected for %Cbl) was added all at once and the resulting mixture left stirring for 2 h under argon. (Note that the order of addition of the reagents is important, the most reproducible results obtained with the indicated order of addition [12]). A ~0.1 ml aliquot of the solution was withdrawn for dilution in about 3 ml MeOH (i.e., to about 5×10^{-5} M) to confirm that the reaction was complete by UV–visible spectroscopy (λ_{max} = 252, 288, 334, 372 and 532 nm in H_2O, 25°C). Since GluSCbl is stable in the presence of air, most of the excess GluSH was removed by filtration through celite (water aspirator). To remove any residual GluSH, the filtrate was run

[12] If the opposite order of addition is used, i.e., HOCbl·HCl is added to the MeOH before the GluSH, in two of six such experiments no discernible reaction was observed, perhaps due to the decreased solubility of GluSH in the presence of predissolved HOCbl·HCl.

through a 3×9 cm Bio-Gel P2 column that was equilibrated with distilled (and Nano-pure filtered) water for 24 h prior to packing the column [48]. The column was eluted with distilled water at a flow rate of ~1 ml/min. Fractions were taken every 5 min and 30 ml of acetone was added to each. The resulting mixtures were cooled to −22°C overnight and a fine purple precipitate was formed in the first three red fractions. The precipitate from the first two fractions was collected separately by centrifugation, the supernatant from each fraction was removed with a pipette, and the separate product from fractions 1 and 2 was washed with cold acetone and then dried under vacuum (it was also possible to collect the precipitate by filtration, but its tendency to stick to the filter reduced the yield). The isolated product from each the first two red fractions was tested by the $(NC)_2Cbl^-$ method [64]; this revealed the first of the red fractions to be ~97% pure. ^{1}H NMR confirmed this by showing a purity of ~98% by ^{1}H NMR, vide infra. The second fraction proved to be only ~70% pure. The yield of the first fraction was 8 mg (25%).

A higher yield of GluSCbl was obtained by repeating the above synthesis exactly, but changing the column chromatography procedure to optimize it somewhat as follows: the filtrate was run through the same type and size column with a flow rate of ~1 ml/min, but fractions were taken every 30 s starting with the first red fraction ~2 min after the product solution was poured onto the column and the water eluant was immediately started. Each resulting fraction was reduced to about 0.1 ml in volume via rotary evaporation, and then 15 ml of acetone was added to each fraction. The fractions were cooled to −10°C overnight in a freezer causing precipitation in fractions #3–10 and #16–22. The material in fractions #3–10 was isolated by the same centrifugation method as cited above. (Fractions #16–22 were also collected, but were only about 70% pure by the $(NC)_2Cbl^-$ method, and hence were discarded.) Fractions #3–10 were combined for an improved yield of 55%.

Solid GluSCbl can be handled under air; storage in a refrigerator (or freezer, for longer term storage) and under argon is recommended. ^{1}H NMR aromatic signals: (i) in room temperature CD_3OD (referenced to TMS) δ 7.17, 7.01, 6.46, 6.20 (d) and 6.06 ppm; ~98% cobalamin purity by ^{1}H NMR; (ii) in D_2O, pH 6.50±0.05 (0.04 M MES buffer, referenced to TSP) δ 7.20, 6.95, 6.40, 6.29 (d) and 6.10 ppm; (iii) in D_2O (see Fig. D, Section 5, referenced to TSP) δ 7.19, 6.95, 6.39, 6.27 (d) and 6.10 ppm. (A 600 MHz NMR investigation of GluSCbl is also available elsewhere [48].) UV–visible λ_{max} (in H_2O): 252 ($2.3(9)\times10^4$), 288 ($2.7(2)\times10^4$), 334 ($1.7(8)\times10^4$), 372 ($1.5(4)\times10^4$), 428 ($4.9(1)\times10^3$) and 534 ($8.7(3)\times10^3$) nm are in agreement with the GluSCbl λ_{max} reported in the literature at 291, 333, 375, 428 and 535 nm [86,87]. L-SIMS (in *m*-nitrobenzyl alcohol matrix) calc. molecular mass for $C_{72}H_{105}O_{20}N_{16}PSCo$ (i.e., $[M+H]^+$) = 1635.6; found: *m/e* 1635.8 ($[M+H]^+$) and 1329.5 ($[(M+H)–GluS]^+$) (Figs. E and F, Section 5). Elemental analysis on a sample predried under vacuum over P_2O_5 at 80°C for 12 h (and which showed ~97(±5)% cobalamin by the dicyano method after drying), and which was then also dried at Galbraith Laboratories for 8 h under vacuum at 80°C, prior to the actual analysis (done in air): $GluSCbl\cdot7H_2O$ (i.e., ~7(±5)% H_2O based on the $\cdot11H_2O$ formulation), (found): C, 49.09 (49.31); H, 6.75 (6.90); N, 12.72 (11.38); S, 1.82 (2.59).

4.3.2. Attempted GluSCbl syntheses from HOCbl·HOAc in MeOH

GluSH (16 mg, 5.2×10^{-5} mol) was dissolved in ~15 ml MeOH in a 100 ml side-armed Schlenk flask containing a stir bar. HOCbl·HOAc (45.7 mg, 2.6×10^{-5} mol, corrected for %Cbl) was added all at once and the resulting mixture left stirring for 24 h under argon at room temperature with no visible reaction, monitored by UV–visible spectroscopy. Another experiment, using 23 equiv. of GluSH (182 mg, 6.0×10^{-4} mol) showed incomplete ~50% reaction after 6 h under identical conditions.

4.3.3. Attempted synthesis of GluSCbl from HOCbl in MeOH

HOCbl was obtained as previously described from HOCbl·HX and an IRA-400 Amberlite column in the basic form [88]. A 2 ml solution of HOCbl (120 mg, 8.8×10^{-5} mol) in MeOH was added to a 50 ml suspension of a glutathione (120 mg, 1.8×10^{-4} mol) solution in MeOH (50 ml) in a 100 ml side-armed Schlenk flask under argon. The solution was stirred for 3 days and checked at 12 h intervals by UV–visible spectroscopy; no observable reaction occurred (<5%). However, when 17 ml of water was added to the reaction solution, giving an overall 75% MeOH aqueous solution, ≤50% GluSCbl was produced.

4.4. Synthesis of CysSCbl

The percentage of cobalamin present in commercial $HOCbl\cdot HCl\cdot nH_2O$ was determined to be 81±5% by conversion of HOCbl·HCl to $(NC)_2Cbl^-$ [65] (the HOCbl·HCl was allowed to react for more than 1 h in 0.1 M KCN/0.1 M phosphate buffer, pH 10.3±0.1). Three separate determinations were performed, giving %Cbl = 78, 82 and 83% (±5%), respectively.

4.4.1. Attempted synthesis from HOCbl·HOAc in MeOH

MeOH was degassed by three freeze/pump/thaw cycles. MeOH (4.0 ml) was added to an argon-filled Schlenk flask containing CysSH (4.0 mg 3.23×10^{-5} mol, corrected for 98% purity). The solution was heated to 60°C for 12 h. No visible dissolution of the CysSH and no detectable CysSCbl formation by UV–visible spectroscopy were observed. A series of control experiments confirmed that the insolubility of CysSH in MeOH is of thermodynamic, and not kinetic, origin (specifically controls, showing that heating plus ultrasonication of CysSH suspended in MeOH did not solubilize

it; also, controls showing that adding MeOH to CysSH in water caused precipitation of the CysSH).

4.4.2. Attempted synthesis from HOCbl·HCl in pH 5.5 MES buffer and with 2.0 equiv. of CysSH

Aqueous MES buffer (0.05 M, pH 5.5 ± 0.2, adjusted by the dropwise addition of ~5 M $HClO_4$) was degassed by three freeze/pump/thaw cycles, and refilled with argon. HOCbl·HCl (61.5 mg, 3.60×10^{-5} mol, corrected for 81% Cbl) was dissolved in 2 ml MES buffer in a 50 ml side-armed Schlenk flask under argon containing a stir bar, and the solution cooled to 0°C via an ice bath. A solution of CysSH (9.0 mg, 7.20×10^{-5} mol, 2.0 equiv., corrected for 2% impurity) dissolved in 0.5 ml MES buffer under argon was added dropwise to the cobalamin solution via a 5 ml syringe with stirring. Additional MES buffer (~1 ml) was used to complete the transfer of remaining CysSH into the flask containing the cobalamin solution. The resultant solution was stirred for 30 min at 0°C under argon. The product was precipitated under argon by the slow addition of 30 ml cold (0°C) degassed acetone (degassed by bubbling argon through it for 1 h) via a 10 ml syringe to yield a dark crimson precipitate. Control experiments demonstrated that both the MES buffer plus excess CysSH remain in solution under the (added-acetone) conditions used to precipitate the product. Yield: 45 mg (85%). L-SIMS (in *m*-nitrobenzyl alcohol matrix) confirmed the calc. molecular mass for $C_{65}H_{95}O_{16}N_{14}PSCo$ (i.e., $[M+H]^+ = 1449.6$; found: *m/e* 1449.6 ($[M+H]^+$) and 1329.8 ($[(M+H)–CysS]^+$). However, a sample dried under vacuum showed only ~80(±5)% cobalamin by the dicyano method, and elemental analysis on this sample, further dried at Galbraith Laboratories (8 h under vacuum at 80°C) showed it to be contaminated with about 0.5 equiv. CysSH: CysSCbl·$6H_2O$·0.5CysSH (i.e., ~89(±5)% CysSCbl), calc. (found): C, 49.38 (48.51); H, 6.79 (6.83); N, 12.55(12.94); S, 2.97 (2.91)%.

4.4.3. Synthesis from HOCbl·HCl in pH 5.5 MES buffer and with 1.15 equiv. of CysSH

The above synthesis in Section 4.4.2 was repeated exactly except that only 1.15 equiv. (5.3 mg) of CysSH were used. Yield: 35 mg (65%). Solid CysSCbl can be handled in air; storage in a refrigerator (or freezer, for longer term storage) and under argon over a drying agent such as P_2O_5 are recommended. L-SIMS (in *m*-nitrobenzyl alcohol matrix) confirmed the calc. molecular mass for $C_{65}H_{95}O_{16}N_{14}PSCo$ (i.e., $[M+H]^+ = 1449.6$; found: *m/e* 1449.8 ($[M+H]^+$) and 1329.6 ($[(M+H)–CysS]^+$) (Figs. J and K, Section 5). A sample dried under vacuum showed ~90 (±5)% cobalamin by the dicyano method. Elemental analysis on this sample, further dried at Galbraith Laboratories (8 h under vacuum at 80°C), provided a satisfactory analysis: CysSCbl·$7H_2O$ (~11(±5)% H_2O based on the ·$7H_2O$ formation), calc. (found): C, 47.91 (48.03); H, 7.05 (6.69); N, 11.31 (12.03); S, 1.97 (2.66)%. Repeat analysis on a non-dried sample from the same synthesis, CysSCbl·$18H_2O$, calc. (found): C, 44.01 (44.26); H, 7.39 (6.95); N, 11.06 (10.54)%. ^{1}H NMR (see Fig. I, Section 5, referenced to TSP) aromatic region, on a sample in pD 5.5 D_2O (0.01 M MES buffer in D_2O, pH measured at pH 5.1 corresponding to pD 5.5 [89]) and taken within 5 min of sample preparation: δ 7.24, 6.98, 6.45, 6.33 (d) and 6.13 ppm. There are also peaks visible at δ 7.21, 6.55, 6.50, 6.35 (shoulder), and 6.28 (d) corresponding to H_2OCbl^+ [66] (Fig. I, Section 5). CysSCbl proved too unstable for HPLC in water, acetonitrile, acetone, or methanol.

5. Supplementary material

Supporting information is available from the senior author, R.G. Finke: ^{1}H NMR spectrum (aromatic region) of $C_6H_{11}SCbl$ in anaerobic CD_3OD at room temperture (Fig. A); L-SIMS spectra of $C_6H_{11}SCbl$ (Figs. B–C); ^{1}H NMR spectrum (aromatic region) of GluSCbl in aerobic D_2O at r.t. (Fig. D); L-SIMS spectra of GluSCbl (Figs. E–F); UV–visible titration spectra for HOCbl·HCl + GluSH (Fig. G); derivation of the equation used to fit the GluSCbl titration data and to give the $K_f = (1.1 \pm 0.3) \times 10^5$ M^{-1} cited in the main text; plot of absorbance versus total glutathione concentration for the GluSCbl titration data at 350 nm (Fig. H) and the equivalent results at three other wavelengths; ^{1}H NMR spectrum (aromatic region) of CysSCbl in aerobic D_2O buffered to pD 5.5 at room temperture (Fig. I); L-SIMS spectra of CysSCbl (Figs. J–K); UV–visible spectum of isolated CysSCbl in pH 7.2 H_2O taken 1 min after solution preparation (Fig. L); UV–visible spectrum of CysSCbl in anaerobic MeOH at 25°C at longer reaction times of $t = 1$, 6, 11, 16, 21, and 26 min (Fig. M).

Acknowledgements

Financial support was provided by the National Institutes of Health Grant DK26214. N.E.B. wishes to thank the Australian National University for postponing the starting date of her Rita Cornforth Fellowship, which in turn allowed her to undertake a postdoctoral year at Colorado State University.

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