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Synthesis and Structure of Vitamin B_{12}-Derivatives with a Modified Ribose-Unit

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Summary. The acylation at the 5′-OH group of the ribose-unit of vitamin B_{12} (cyanocobalamin) or of aquocobalamin with two conventional reagents gave mono-acylated B_{12}-derivatives with good to very high selectivity. The site of the modification was deduced from spectral data of the products and was further supported by the crystal structure data of three such modified B_{12}-derivatives. These three B_{12}-derivatives were found to crystallize in the space group $P2_12_12_1$, irrespective of the nature of the appendage. Acylation at 5′-OH has been used to protect (or block) this group in the context of functionalization of 2′-OH or elsewhere in the B_{12}-molecule. Attachment of the bifunctional succinyl-unit has allowed the preparation of further modified derivatives of vitamin B_{12} and binding of B_{12}-derivatives to biological carriers and other macromolecules. In aqueous solution, 5′-acylcobalamins turned out to be rather susceptible to hydrolytic loss of the acyl-functionality.

Keywords. Acylation; Cobalt-complex; Cofactor, Spectroscopy; X-ray structure determination.

Introduction

The ‘‘complete’’ corrinoids are essential cofactors in many spheres of life [1, 2] and are considered most complex [3] and fascinating [4] low molecular weight compounds. Chemical reactions of B_{12}-derivatives, which reflect their cofactor roles, typically take place directly in the coordination sphere of the corrin-bound cobalt-ion, and frequently occur with exquisite selectivity [5, 6]. Functional group modifications at the corrin ligand and nucleotide moieties of the multifunctional vitamin B_{12} (**1**) molecules are frequently less selective [7]. Obviously, the selective introduction of further functionality in such complex compounds has long been a challenge [3, 7]. However, the preparation of specific stable B_{12}-conjugates has become a target of special interest more recently, following considerable progress in studies on the take-up and transport of B_{12}-derivatives in whole organisms [8], including mammals and humans [9, 10]. The B_{12}-transport systems may tolerate the covalent attachment of a variety of new functional moieties, at the cobalt-centre and at the 5′-OH, in particular [11, 12]. Indeed, in a complementary sense, the natural ‘‘complete’’ corrinoids also may differ in the build-up of their nucleotide moiety [13–15]. The discovery of 176-norcobamides in de-chlorinating anaerobes [15] has induced us lately to prepare B_{12}-derivatives with a modified linker to the nucleotide function [16, 17]. On the other hand, the primary 5′-OH function at the ribose moiety of the nucleotide loop of vitamin B_{12} and other ‘‘complete’’ corrinoids may be directly considered as an anchor for covalent modification by acylation reactions: it is more accessible than the other hydroxyl group (the secondary 2′-OH), allowing for some

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selectivity [18]. Specifically refunctionalized B_{12}-derivatives are used as "Trojan" horses, exploiting the natural B_{12}-transport systems to carry cytotoxic loads into specific (*e.g.*, cancerous) tissues [11, 19], radioactive label into specific organs for treatment and diagnosis of tumours [20], or for other diagnostic purposes [21]. The covalent attachment of B_{12}-derivatives to carrier proteins using a linker at the 5′-OH has recently also become of interest, in the context of the production of a coenzyme B_{12}-specific antibody [22]. The complementary protection of the 5′-OH-group has also become useful, in order to achieve selective conjugation with (deoxy)nucleotides elsewhere at the nucleotide moiety [23, 24]. We report here on 5′-acetyl- and 5′-succinyl-derivatives of vitamin B_{12} (**1**) as well as of such a derivative of aquocobalamin (**2**), and on crystal structures of three such derivatives of **1**, modified at the ribose 5′-OH.

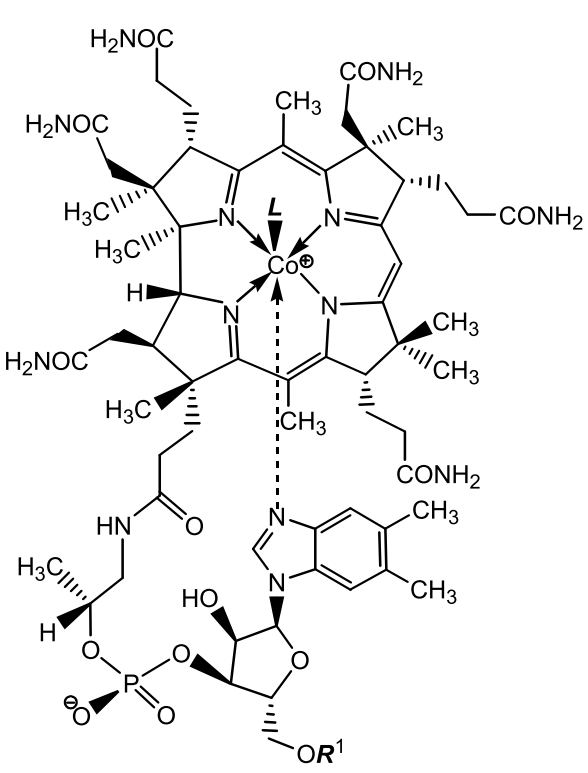
Results and Discussion

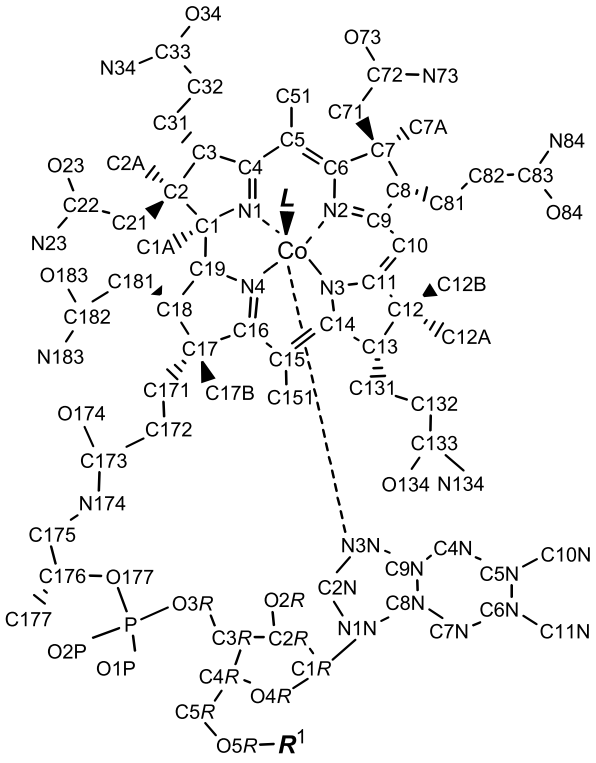
Aquocobalamin chloride (**2**), a derivative of vitamin B_{12} (**1**) in which a water molecule is bound at the cobalt-centre (see Fig. 1), underwent nearly quantitative mono-succinylation of the 5′-OH group under standard conditions for acylation, allowing for the direct preparation of crystalline Co_β-chloro-O5R-succinylcobalamin (**4a**, 97% yield). The high selectivity of this acylation reaction at the 5′-OH rather than the 2′-OH of the ribose unit appeared to be due to two main contributing factors: the primary *vs.* secondary nature of the two ribose hydroxyl groups, as well as the specifically encumbered environment of 2′-OH in the aquocobalamin cation [25]. Mono-succinylation of vitamin B_{12} (**1**) has been observed earlier to be selective and was assumed to give Co_β-cyano-O5R-succinylcobalamin (**4c**) on the basis of the primary *vs.* the secondary nature of the 5′-OH and 2′-OH groups of the ribose [18, 26]. In aqueous solution, the succinyl-derivative **4c** was found to readily revert to the unfunctionalized cobalamin **1** [18, 27]. Rapid work-up and/or direct precipitation and crystallization were developed here in order to achieve good yields of pure products (such as **4a** and **4c**). The corresponding B_{12}-conjugates, Co_β-cyano-O5R-[3-(methoxycarbonyl)propionyl]cobalamin (**5**) and the amide Co_β-cyano-O5R-[3-(benzylcarboxamido)propionyl]cobalamin (**6**) were also made and tested here qualitatively, in order to explore the influence of the second carboxylic acid function on the stability of the acylated B_{12}-derivatives.

The structures of **4a**, **4b**, and of the cyano analogue **4c**, were (first) delineated by their full spectroscopic characterization. The attachment of a succinyl unit at O5R induced shifts (of about 0.4, 2.2, and −3.1 ppm, respectively) of the signals of H-(C5R), C5R, and C4R in the NMR-spectra of **4c** (see Table 2), when compared to the corresponding spectra of vitamin B_{12} (**1**) [28]. Our spectroscopic data thus confirmed the earlier tentative structural assignments for **4c** [18, 26]. The identities of **4a**/**4b** could be confirmed by further analysis of the crystal structure of **4a**. The chlorocobalamin **4a** crystallized in the space group $P2_12_12_1$ and showed similar structural properties for the Co(III)-cobalamin moiety as observed earlier for chlorocobalamin (**7**) itself (see Table 1 and *e.g.* Refs. [29, 30]). Analogous to the situation with **7**, the B_{12}-derivative **4a** was also indicated by its spectra to be hydrolyzed in aqueous solution to ionic Co_β-aquo-O5′-succinylcobalamin chloride (**4b**). In the crystal, the succinyl appendage of **4a** was present in a synclinal conformation with rather short contacts between the carbonyl oxygen atoms (O52R and O55R) of 335(2) pm. It also exhibited a pair of intramolecular H-bonds between the succinyl-carboxylic acid function and the carboxamide group of the d-propionamide side chain in one of the two major conformations of this disordered side chain (see Fig. 2).

The related synthesis of Co_β-cyano-O5R-acetyl-cobalamin (**3**) has been outlined recently [23] and resulted in crystalline samples, whose crystal structures were also analyzed here: acyl-group attachments in **3** and in **4a** occur with a similar acyl-group conformation, indicating inherent electronic properties and local effects of crystal packing to be relevant, rather than the specific requirements of (any observed) intra-molecular H-bonding in **4a** (see Fig. 3). Along this line, both the acyl- and the succinyl-groups were noted to be lost readily under neutral and slightly basic conditions [18, 23].

The refunctionalization of the succinate **4c** to the methyl ester **5** was achieved readily in methanol and at 0°C with carbodiimide chemistry (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (*EDC* hydrochloride) and *N*-hydroxybenztriazole). The crystalline di-ester **5** could be isolated in 77% yield by direct precipitation from the reaction mixture and crystallization from aqueous acetone. In the





1: R^1 = H; *L* = CN

2: R^1 = H, *L* = $H_2O^+(Cl^-)$

3: R^1 = O=C(CH$_3$)–CH$_3$, *L* = CN

4a–4c: R^1 = O=C(CH$_3$)–CH_2–CH_2–CO_2H { **4a**: *L* = Cl; **4b**: *L* = $H_2O^+(Cl^-)$; **4c**: *L* = CN }

5: R^1 = O=C(CH$_3$)–CH_2–CH_2–CO_2CH_3, *L* = CN

6: R^1 = O=C(CH$_3$)–CH_2–CH_2–CO–NH–CH_2–C_6H_5, *L* = CN

7: R^1 = H; *L* = Cl

$\boldsymbol{R^1}$ = O52*R*=C51*R*–C52*R*

$\boldsymbol{R^1}$ = O52*R*=C51*R*–C52*R*–C53*R*–C54*R*(X)=O55*R*

Fig. 1. Left. Structural formulae of B_{12}-derivatives: vitamin B_{12} (**1**), aquocobalamin chloride (**2**), Co_β-cyano-O5R-acetyl-cobalamin (**3**), Co_β-chloro-O5R-succinylcobalamin (**4a**), Co_β-aquo-O5R-succinylcobalamin chloride (**4b**), Co_β-cyano-O5R-succinylcobalamin (**4c**), Co_β-cyano-O5R-[3-(methoxycarbonyl)propionyl]cobalamin (**5**), Co_β-cyano-O5R-[3-(benzylcarboxamido)propionyl]cobalamin (**6**), chlorocobalamin (**7**). Right. Atom numbering used here [36]

crystal, the cyano-Co(III)-corrin **5** exhibited similar structural properties as the acetylcobalamin **3**, including the linker acyl-function at the 5′-OH. However, the methyl ester terminus of **5** was oriented in a synclinal conformation again (see structure of **4a**), resulting in a rather short intramolecular contact between the carbonyl oxygen atoms (O52R and O55R) of 377 pm. In this structure, the terminal ester function was rather disordered and not oriented by specific intramolecular H-bonding interactions, as found for the mono-succinate **4a** (see Figs. 2 and 4).

The related condensation of **4c** with benzyl amine to the amide **6** was similarly achieved in a nearly quantitative way at 0°C using the water soluble carbodiimide *EDC* (hydrochloride) in aqueous solution. Amide **6** was precipitated from the reaction mixture

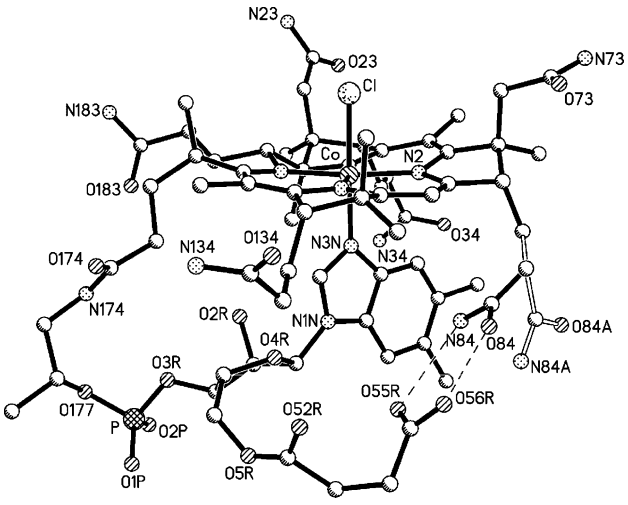


Fig. 2. Molecular structure (ball and stick model) of Co_{β}-chloro-O5R-succinylcobalamin (**4a**) according to X-ray analysis; the d-propionamide side chain is disordered; one conformation exhibits a pair of H-bonds to the carboxylic acid function of the succinyl residue

Table 1. Crystallographic data for **3**, **4a**, and **5**. Bond lengths/Å and fold angles/°

	3 (L = CN)	**4a** (L = Cl)	**5** (L = CN)
Co-N1	1.878 (6)	1.878 (5)	1.888 (6)
Co-N2	1.903 (6)	1.910 (6)	1.906 (6)
Co-N3	1.910 (6)	1.913 (5)	1.918 (6)
Co-N4	1.894 (6)	1.890 (6)	1.882 (6)
Co-N3N	2.049 (7)	1.984 (6)	2.036 (7)
Co-L	1.869 (11)	2.262 (2)	1.868 (11)
fold angle	15.2 (2)	16.8 (2)	17.7 (2)

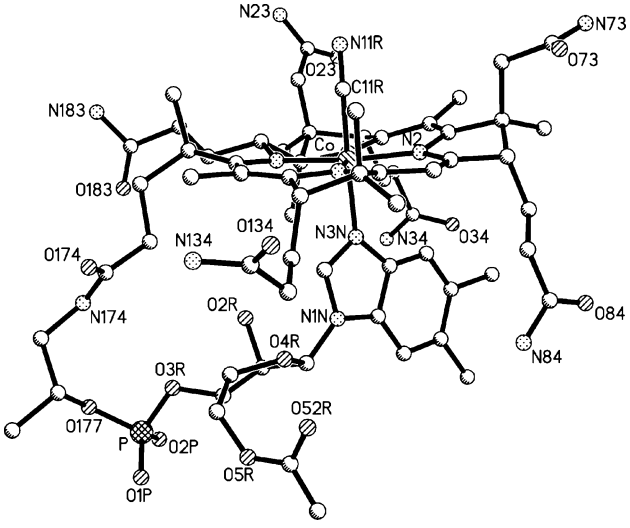


Fig. 3. Molecular structure (ball and stick model) of Co_{β}-cyano-O5R-acetylcobalamin (**3**) according to X-ray analysis

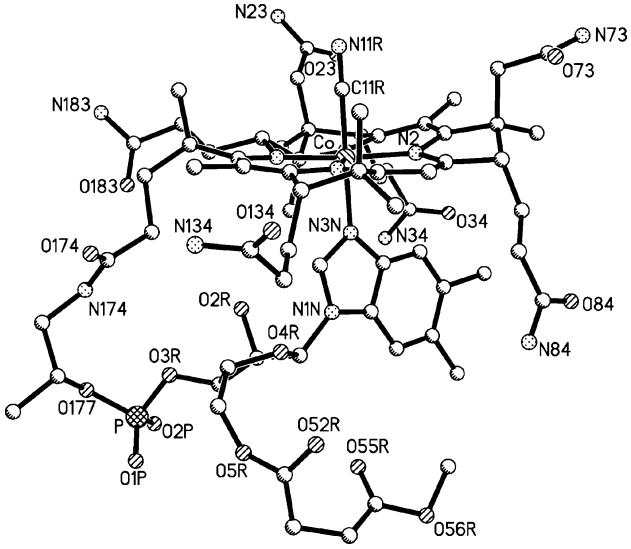


Fig. 4. Molecular structure (ball and stick model) of Co_{β}-cyano-O5R-[3-(methoxycarbonyl)propionyl]cobalamin (**5**) according to X-ray analysis; the methyl ester function is disordered in the crystal and not well defined

by addition of acetone, repeated washing of the precipitate with acetone, and drying under high vacuum at room temperature. The amide **6** was obtained as a red powder in about 92% yield and in spectroscopically pure form. Crystallization of **6** was not attempted due to its ready decomposition in neutral aqueous solution at room temperature: An ^{1}H NMR-spectrum of **6** (at 500 MHz) in perdeuteromethanol displayed the signals of the benzyl- and phenyl-hydrogen atoms (AB-system at 4.3 ppm and multiplet at 7.3 ppm, respectively). Storage of a solution of **6** in un-buffered D_2O for 3 days led to nearly complete decomposition with formation of vitamin B_{12} (**1**) and *N*-benzylsuccinimide, as deduced from the 500 MHz ^{1}H NMR spectrum of the reaction solution. Such a spectrum indeed exhibited the complete signal pattern of the spectrum of **1** and two new singlets near 2.7 ppm (*ca.* 4H) and 4.6 ppm (*ca.* 2H) of *N*-benzylsuccinimide.

Conclusions

Acylation of vitamin B_{12} (**1**) and of aquocobalamin (**2**) occurs selectively at the primary 5′-OH of the ribose unit, as expected. As a result of the high selectivity, 5OR-acyl-derivatives of **1** and **2** are available readily. They frequently crystallize well (in the space group $P2_12_12_1$, as typically found for crystalline B_{12}-derivatives [31], so that their direct isolation

and crystallization can be achieved. The investigated type of refunctionalization of the ''complete'' corrinoids at O5R affects neither the structural characteristics of the cobamide moiety (such as bonding around the cobalt-centre) nor their organometallic reactivity. However, rapid isolation is important as the 5OR-acyl-derivatives readily loose their acyl substituents, in aqueous solution (even at neutral *pH*) [27]. Alternative isolation methods rather than the earlier used phenol extraction procedure [18] are thus in demand. Direct crystallization of B_{12}-derivatives frequently is achieved [32, 33] and may be crucial for the preparative success. The frequently used succinyl-linker is prone to hydrolytic removal and may be of limited use for reliable and stable attachment of B_{12} to, *e.g.*, fluorescent labels, large macromolecules and solid surfaces.

Experimental

Vitamin B_{12} (cyanocobalamin, **1**), Hoffmann-La Roche; aquocobalamin chloride (**2**), Rhone-Poulenc, water purified using Epure, Barnstead Co.; acetone, *Me*OH, acetic anhydride (Ac_2O), aq. HCl solution, all Fluka puriss. p.a., *DMSO*, Fluka, puriss., absolute, over molecular sieve ($H_2O \leq 0.01\%$); pyridine, Fluka, puriss., absolute, over molecular sieve ($H_2O \leq 0.005\%$); *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (*EDC* hydrochloride), Fluka BioChemika, >99%; *Me*CN, 4-(dimethylamino)pyridine (*DMAP*), 1-hydroxybenzotriazole (HO*BT*), 3-(trimethylsilyl)propanesulfonate (*TSP*), all Fluka purum; reversed phase chromatography materials were purchased from Merck.

Spectroscopy. UV/Vis spectra: Hitachi U-3000 apparatus. CD spectra: Jasco J-715 spectropolarimeter. NMR spectra: Varian Unity 500 plus spectrometer; ^1H NMR in D_2O (δ(HDO) = 4.76 ppm), ^{13}C NMR in D_2O (external standard $\delta(TSP) = 0$ ppm), apparent coupling constants J/Hz; all spectra were recorded at 26°C; ^1H- and ^{13}C-signals were assigned based on HSQC and ROESY-spectra (except for where noted otherwise). FAB-MS spectra (m/z (rel. int.)): (+)-ions; Finnigan MAT 95S, with nitrobenzyl alcohol (*NOBA*) matrix and Cs^+ bombardment.

Crystallography. Crystal preparation. Crystals (all red prisms, belonging to the orthorhombic system, space group $P2_12_12_1$ (No. 19)) were fused in a glass-capillary with mother liquor.

X-Ray Measurement and Structure Determination

Data collection was performed at 293 K on a Nonius Kappa CCD equipped with graphite-monochromatized Mo-K_α-radiation ($\lambda = 71.073$ pm) and a nominal crystal to area detector distance of 36 mm. Intensities were integrated using DENZO and scaled with SCALEPACK [34]. The structures were solved with direct methods SHELXS86 and refined against F^2 SHELXL-97 [35] (see Tables 3–5 for more details). Most of the non-hydrogen atoms were refined with anisotropic displacement parameters. Positions of hydrogen atoms were calculated except for those at solvent, the disordered carboxamide group of **4a** and succinyl units of **4a** and **5**, which were omitted. The thermal ellipsoids of the carbon and oxygen atoms of the succinyl units are 2–4 times higher than for the other atoms of the B_{12}-molecule, showing a higher mobility at this part of the crystal lattice with the possibilities of different conformational arrangement. Their positions were not stable in a free refinement and bond restraints and partial fixed temperature factors must be used to refine the major conformation. The carbon atoms C51R–C54R of **4a** and carbon and oxygen atoms C53R–O56R were refined with isotropic displacement parameters. The carboxamide group N84-C83-O84 of **4a** is disordered; one (of the two) main conformation forms hydrogen bonds to the acid group of the succinyl unit (bond distances [pm] of N84 $\cdots$ O55R: 299(2) and O84 $\cdots$ O56R: 233(3)). The latter calculated distance is short, due to an inexact location of O56R, which shows two times higher thermal ellipsoids than the atoms N84, O84, and O55R. The water molecules O1–O5 have two hydrogen bonds to the B_{12}-molecules and are nearly at the same position in all three crystal structures. They have normal temperature factors and were refined anisotropically. All other solvent molecules had higher temperature factors and were refined isotropically.

Supporting information available: the crystallographic data for **3**, **4a**, and **5** was deposited at the Cambridge Crystallographic Data Centre with the Nos. CCDC-649866, CCDC-649867, and CCDC-649868, resp. The coordinates can be obtained, on request, from the Director, Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, U.K. (fax: +44-1223-336033, e-mail: deposit@ccdc.cam.ac.uk, or http://www.ccdc.cam.ac.uk).

Co_β-cyano-O5R-acetylcobalamin (**3**, $C_{65}H_{90}CoN_{14}O_{15}P$)

In 30 cm^3 dry *DMSO* 500 mg vitamin B_{12} (**1**, 0.369 mmol) were dissolved and the solution was degassed four times by freezing and thawing. *DMAP* (4.5 mg, 36.9 μmol), triethyl amine (514 mm^3, 373 mg, 3.69 mmol, filtered over Alox (bas.)), and acetic anhydride (697 mm^3, 753 mg, 7.38 mmol) were added and the solution was stirred for 17.5 h under an argon atmosphere. Addition of about 50 cm^3 acetone to the reaction mixture gave a red precipitate, which was washed with acetone several times. The acetone washings were filtered through a *Büchner* funnel and the retained red solid was combined with the first precipitate. The crude solid was purified by column chromatography on RP-18 (119.0 g) with an acetonitrile/water eluent (gradient from 8 to 17% acetonitrile in steps of 1%, 200 cm^3 of each). The fraction containing the product (Co_β-cyano-O5R-acetylcobalamin, **3**) was collected and 188.7 mg (36.6%) **3** were obtained as dark red needles by crystallization from water/acetone. $R_f = 0.55$ (RP-18, *Me*CN/H_2O 3:7); ^1H NMR and ^{13}C NMR data (D_2O): see Table 2; UV/Vis (H_2O, $c = 4.72 * 10^{-4}$ *M*): λ_{max} (log ε) = 548.5 (3.91), 360.0 (4.42), 321.5 (3.86), 277.0 (4.16) nm; CD (H_2O, $c = 4.72 * 10^{-4}$ *M*): $\lambda_{max/min}$ ([θ]) = 544.5 (−5100), 486.5 (−12700), 431.5 (46600), 362.5 (−58600), 326.0 (−13300), 308.5 (−8400), 276.0 (16900), 250.5 (−39500)

Table 2. List of assigned signals in the 125 MHz ^{13}C and 500 MHz ^{1}H NMR spectra of Co_{β}-cyano-O5′-acetylcobalamin (**3**) and of Co_{β}-cyano-5′-succinylcobalamin (**4c**) (in D_2O)

3			**4c**			**3**			**4c**		
	$\delta(^{13}C)$	$\delta(^{1}H)$		$\delta(^{13}C)$	$\delta(^{1}H)$**		$\delta(^{13}C)$	$\delta(^{1}H)$		$\delta(^{13}C)$	$\delta(^{1}H)$**
C1	87.8		C1	87.5		C133	180.2				
C1A	21.9	0.47	C1A	21.7	0.37	C14	168.7				
C2	49.9		C2	49.4		C15	106.8		C15	106.5	
C2A	19.4	1.41	C2A	19.4	1.30	C151	17.8	2.57	C151	17.7	2.43
C21	45.4	2.41	C21	45.2	2.31	C16	181.6				
C22	178.5					C17	61.8		C17	60.1	
C3	58.9	4.19	C3	58.5	4.09	C17B	18.4	1.39	C17B	18.3	1.29
C31	28.6	2.03	C31	28.3	1.89	C171	34.6*	1.84/	C171	34.2*	1.74/
C32	37.5	2.50/	C32	37.4	2.40/			2.63*			2.51*
		2.56			2.46	C172	34.8*	2.11/	C172	34.2*	2.03/
C33	180.5							2.54*			2.45*
C4	182.7					C173	177.2				
C5	110.2		C5	109.8		C175	47.7	3.01/	C175	47.5	2.93/
C51	18.0	2.54	C51	17.9	2.46			3.60			3.49
C6	168.0					C176	75.6	4.31	C176	75.5	4.22
C7	54.2		C7	53.6		C177	21.6	1.24	C177	21.1	1.13
C7A	21.6	1.86	C7A	21.1	1.75	C18	41.6	2.76	C18	41.3	2.66
C71	45.5	2.18/	C71	45.2	2.08/	C181	34.1	2.68/	C181	34.0	2.58/
		2.57			2.47			2.75			2.66
C72	177.7					C182	178.3				
C8	58.3	3.42	C8	58.2	3.30	C19	77.5	4.10	C19	77.1	4.00
C81	28.5	0.99/	C81	28.1	0.89/	C1R	89.6	6.37	C1R	89.6	6.28
		1.99			1.89	C2R	71.3	4.31	C2R	70.9	4.22
C82	34.1	1.00/	C82	33.6	0.89/	C3R	76.1	4.84	C3R	75.6	4.76
		1.82			1.70	C4R	81.9	4.25	C4R	81.8	4.17
C83	179.6					C5R	65.7	4.21/	C5R	65.5	4.11/
C9	176.2							4.56			4.48
C10	97.5	6.08	C10	97.4	5.97	C52R	23.1	2.18	C52R*	34.3	2.42
C11	179.5					C51R	176.7		C53R*	32.8	2.60
C12	50.8		C12	50.3		C2N	144.3	7.11	C2N	144.1	7.01
C12A	21.8	1.44	C12A	21.7	1.34	C4N	119.1	6.50	C4N	119.2	6.39
C12B	33.9	1.19	C12B	33.5	1.08	C5N	135.8		C5N	137.8	
C13	56.3	3.34	C13	56.1	3.23	C6N	137.9		C6N	135.5	
C131	30.3	1.94/	C131	30.3	1.84/	C7N	114.0	7.26	C7N	113.9	7.20
		2.03			1.94	C8N	132.6		C8N	132.4	
C132	37.2	2.66	C132	36.7	2.55	C9N	139.3		C9N	138.9	
						C10N	22.5	2.26	C10N	22.2	2.15
						C11N	22.0	2.26	C11N	21.7	2.15

* Tentative individual assignment, ** $\delta(\text{HDO}) = 4.68$ ppm

nm (10 deg cm^2 mol^{-1}); MS (FAB): m/z (%) = 1400.5 (20), 1399.6 (53), 1398.6 (81), 1397.6 (100, $[M+H]^+$), 1371.9 (20, $[M+H\text{-}CN]^+$), 1369.8 (27); X-ray: Crystals of **3** were grown from water/acetone at room temperature under protection from light. Crystal data of **3** are summarized in Tables 1 and 3; see Fig. 3 for a view of the structure of **3**.

Co_{β}-chloro-O5R-succinylcobalamin
(**4a**, $C_{66}H_{92}ClCoN_{13}O_{17}P$)
An aqueous solution of 288.8 mg Co_{β}-aquocobalamin chloride (**2**) (209 μmol) was lyophilized over night. The red residue was re-dissolved in 10 cm^3 absolute *DMSO*, the solution was degassed (by freezing and thawing four times) and the reaction vessel filled with Ar. With protection from air, pyridine (16.8 mm^3, 16.5 mg, 209 μmol), *DMAP* (2.55 mg, 20.8 μmol), and succinic anhydride (418 mg, 4.17 mmol) were added. The reaction mixture was stirred for four days at room temperature and in the dark. Addition of about 50 cm^3 acetone produced a dark red precipitate, which was separated from the nearly colourless solution. The red residue was washed with acetone and dried. It was then dissolved in about 5 cm^3 water and 18.7 mm^3 10 *M* aqueous HCl were added. Acetone was

Table 3. Crystal data, data collection, and structure refinement for **3**

Molecular formula	$C_{65}H_{90}CoN_{14}O_{15}P \times 16H_2O$
Formula weight	1685.67
Unit cell dimensions	$a/\text{pm} = 1597.72(7)$ $\alpha = 90°$ $b/\text{pm} = 2226.97(6)$ $\beta = 90°$ $c/\text{pm} = 2614.90(9)$ $\gamma = 90°$
Z	4
Density (calculated)/ mg m^{-3}	1.203
Crystal size/mm^3	$0.35 \times 0.12 \times 0.10$
Goodness-of-fit on F^2	1.058
Final R indices [$I > 2\sigma(I)$]	$R1 = 0.0774$, $wR2 = 0.1980$
R indices (all data)	$R1 = 0.1016$, $wR2 = 0.2153$
Refinement details	5 (of 16) water molecules were refined anisotropically, Solvent H-atoms were omitted

also added until a slight turbidity was noticed. Upon storage of the mixture in a refrigerator, the dark red product crystallized out (to give, after drying 285.9 mg pure **4a**). From the mother liquor an additional batch of 36.6 mg **4a** was obtained as a precipitate (yield total: 295.5 mg **4a** (202 μmol, 97%). The crystalline product was subjected to the spectral analysis, as reported below:

^{1}H NMR (D_2O): $\delta = 0.422$ (s, 3H, C1A), 0.8–1.0 (m, 2H, C81/C82), 1.15 (d, 3H, $J = 6.2$ Hz, C177), 1.25/1.29 (s, 6H, C12B, C171B), 1.38/1.39 (s, 6H, C12A, C2A), 1.7–1.9 (m), superimposed by 1.83 (s, C7A), total of 4H, 1.9–2.2 (m), superimposed by 2.12/2.18 (2s, C10N, C11N), total of 11H, 2.33 (dd, 2H, C21), 2.4–2.75 (m), superimposed by 2.53/2.60 (2s, C51, C151), total of 20H, 2.84 (dd, 1H, $J_1 = 14.4$, $J_2 = 9.4$ Hz, C175), 2.91 (m, 1H, C18), 3.36 (m, 1H, C8), 3.47 (m, 1H, C13), 3.52 (m, 1H, $J_1 = 14.5$ Hz, C175), 4.08–4.19 (m, 6H, C3, C19, C176, C2R, C4R, C5R), 4.54 (m, 1H, C5R), 4.71 (m, 1H, C3R), 6.12 (d, 1H, $J = 4.6$ Hz, C1R), 6.18 (s, 1H, C10), 6.36 (s, 1H, C4N), 6.43 (s, 1H, C2N), 7.07 (s, 1H, C7N) ppm; ^{13}C NMR (D_2O): $\delta = 18.0$, 18.2, 19.3, 20.3, 21.7, 21.8, 22.2, 22.5, 22.5, 22.8 (10q), 29.0, 30.4, 31.6, 31.7, 33.9, 34.0 (6t), 34.8 (q), 35.0, 35.2, 37.1, 37.5 (4t), 42.3 (d), 46.0, 48.0, 48.3 (3t), 50.3, 50.9, 53.6 (3s), 56.6, 59.7, 60.1 (3d), 61.7 (s), 65.3 (t), 71.2, 75.5, 75.8, 77.7, 82.3 (5d), 87.7 (s), 90.1, 97.6 (2d), 106.9, 110.7 (2s), 114.5, 118.2 (2d), 128.0, 131.8, 136.6, 138.7 (4s), 144.0 (d), 166.8, 168.6, 171.5 (3s), 177.1, 177.4, 178.2, 178.5, 179.5, 180.1, 180.3, 181.6, 184.0, 184.1 (12s) ppm; IR (KBr): $\bar{\nu} = 3422$vs, 3200s, 2971w, 2934w, 1734w, 1665vs, 1574m, 1499m, 1478w, 1405m, 1375w, 1350w, 1314w, 1219m, 1175m, 1146m, 1084m, 1071w, 1001w cm^{-1}; UV/Vis (H_2O, $c = 3.91 * 10^{-4}$ *M*): λ_{max} (log ε) = 524 (3.90), 498 (3.88), 411 (3.57), 390 (3.57), 351 (4.38), 274 (4.29) nm; CD (H_2O, $c = 3.91 * 10^{-4}$ *M*): $\lambda_{max/min}$ ([θ]) = 550 (−2.2), 516 (+3.5), 422 (−12.4), 388 (+2.3), 471 (−2.3), 349 (+15.8), 315 (−5.3), 301 (−2.8), 276 (+14.2), 247 (−9.2) nm (10 deg cm^2 mol^{-1}); MS (FAB): m/z (%) = 1483.8 (9), 1482.4 (12, $[M + H]^+$), 1467.7 (28), 1466.7 (48), 1464.7 (59), 1465.7 (100, $[M + H\text{-}H_2O]^+$), 1444.8 (10), 1431.8 (36), 1430.7 (71), 1429.8 (91), 1428.5 (47, $[M\text{-}H_2O\text{-}Cl]^+$), 1427.5 (50); TLC ($CH_3OH$:$H_2O$ = 9:1): R_f = *ca.* 0.0; X-ray: Crystals of **4a** were grown by dissolving from water/acetone in a refrigerator. Crystal data of **4a** are summarized in Tables 1 and 4; see Fig. 2 for the molecular structure.

Co$_\beta$-cyano-O5R-succinylcobalamin (**4c**, $C_{67}H_{92}CoN_{14}O_{17}P$) In 10 cm^3 *DMSO* 502 mg (370 μmol) lyophilized cyanocobalamin (**1**) were dissolved and the solution was degassed by three cycles of freezing (liq. N_2), evacuation under high vacuum and thawing at room temperature. Under an argon atmosphere 741 mg (20 equiv, 7.41 mmol) succinyl anhydride, 9.05 mg (0.741 mmol) *DMAP*, and 29.8 mm^3 (29.3 mg, 370 μmol, 1 equiv) pyridine were added to the red, stirred solution. The reaction mixture was stirred at room temperature for 3 days. Then the raw product (**4c**) was directly precipitated by addition of about 50 cm^3 acetone. The mother liquor was removed and the precipitate was first washed with acetone. Raw **4c** was dissolved in about 5 cm^3 water and acetone was added, until the solution started to become turbid. Crystalline **4c** separated out overnight and was dried under high vacuum. Yield: 394 mg crystalline **4c** (271 μmol, 73%).

Table 4. Crystal data, data collection, and structure refinement for **4a**

Molecular formula	$C_{66}H_{92}ClCoN_{13}O_{17}P \times 11H_2O \times 2$ acetone
Formula weight	1779.21
Unit cell dimensions	$a/\text{pm} = 1595.15(4)$ $\alpha = 90°$ $b/\text{pm} = 2225.57(9)$ $\beta = 90°$ $c/\text{pm} = 2626.90(9)$ $\gamma = 90°$
Z	4
Density (calculated)/mg m^{-3}	1.267
Crystal size/mm^3	$0.4 \times 0.1 \times 0.1$
Goodness-of-fit on F^2	1.090
Final R indices [$I > 2\sigma(I)$]	$R1 = 0.0726$, $wR2 = 0.1782$
R indices (all data)	$R1 = 0.0987$, $wR2 = 0.1934$
Refinement details	5 (of 11) water molecules were refined anisotropically. H-atoms of solvent, disordered amide, and succinyl groups were omitted. The succinyl unit has high mobility: it was refined with bond restraints and isotropic parameters of the C-atoms. The d-amide group is 1:1 disordered. Occupancy disorder of the three acetone molecules of 0.5, 0.75, and 0.75

^{1}H NMR (D_2O, δ(HDO) = 4.68 ppm): $\delta = 0.37$ (s, 3H, C1A), 0.89 (m, 2H), 1.08 (s, 3H, C12B), 1.13 (d, 3H, $J = 5.9$ Hz, C177), 1.29–1.34 (3s, 9H, C2A, C12A, C17B), 1.61–20.4 (m) superimposed by 1.75 (s, C7A), total 10H, 1.71–2.31 (m) superimposed by 2.15 (s, C10N, C11N), total 10H, 2.40–2.66 (m), superimposed by 2.43/2.46 (2s, C51, C151), total 18H, 2.93 (dd, 1H, $J_1 = 12.7$, $J_2 = 8.8$ Hz, C175), 3.23 (d, 1H, $J = 10.3$ Hz, C13), 3.30 (m, 1H, C8), 3.49 (dm, 1H, $J = 14$ Hz, C175), 4.00 (m, 1H, C19), 4.09–4.22 (m, 5H), 4.48 (m, 1H, C5R), 4.76 (m, 1H, C3R), 5.97 (s, 1H, C10), 6.28 (d, 1H, $J < 1$ Hz, C1R), 6.39 (s, 1H, C4N), 7.01 (s, 1H, C2N), 7.07 (s, 1H, C7N) ppm; ^{13}C NMR (D_2O): $\delta = 17.7$, 17.9, 18.3, 19.4, 21.1, 21.6, 21.72, 22.2 (10q), 28.1, 28.3, 30.3, 32.8 (4t), 33.48 (q), 33.5, 33.7, 34.2, 34.3, 36.4, 37.4 (7t), 41.3 (d), 45.2, 47.5 (3t), 49.4, 50.3, 53.6 (3s), 56.1, 58.2, 58.5 (3d), 60.1 (s), 65.5 (t), 70.9, 75.5, 75.6, 77.1, 81.8 (5d), 87.5 (s), 89.6, 97.4 (2d), 106.5, 110.0 (2s), 113.9, 119.2 (2d), 132.4, 135.6, 137.8, 138.9 (4s), 144.1 (d), 167.8, 168.4, 176.3, 177.1, 177.6, 178.2, 179.1, 179.4, 181.3, 182.4, 183.2 (14q) ppm; IR (KBr): $\bar{\nu} = 3387$vs, 3200s, 2970m, 2936m, 2135w, 1726m, 1669vs, 1572s, 1545m, 1499s, 1478m, 1449m, 1403s, 1366m, 1350m, 1314m, 1219s, 1157s, 1146s, 1111m, 1082s, 1073sh, 1028sh, 1001m cm^{-1}; UV/Vis (H_2O, $c = 5.29 * 10^{-4}$ *M*): λ_{max} (log ε) = 549 (3.89), 519 (3.84), 480sh (3.65), 407 (3.55), 361 (4.40), 323 (3.88), 305 (3.95), 279 (4.16) nm; CD (H_2O, $c = 5.29 * 10^{-4}$ *M*): $\lambda_{max/min}$ ([θ]) = 549 (−1.2), 486 (−3.4), 433(+12.6), 388 (+1.0), 362 (−16.1), 327 (−3.6), 309 (−2.2), 291 (+1.8), 277 (+4.5), 250 (−10.3) nm (10 deg cm^2 mol^{-1}); MS (FAB): m/z (%) = 1458.7 (9), 1457.8 (30), 1456.7 (100, [M + 1]$^+$), 1455.7 (86), 1454.7 (10), 1430.6 (15), 1429.6 (33, [M-CN + 1]$^+$), 1428.6 (26), 1427.6 (20); TLC (CH_3OH:H_2O = 10:1): $R_f = 0.56$.

Co$_\beta$-cyano-O5R-[3-(methoxycarbonyl)propionyl]cobalamin (**5**, $C_{68}H_{94}CoN_{14}O_{17}P$)

In 1.0 cm^3 methanol HO*BT* (2.8 mg, 20.7 μmol) was dissolved and the solution was cooled with an ice bath. Co$_\beta$-cyano-O5R-succinylcobalamin (**4c**, 25.0 mg, 17.2 μmol) and *EDC* * HCl (7.1 mg, 37.0 μmol) were added to this solution, which was then stirred at 0°C for 2 h. Stirring was continued at r.t. for one day. The reaction mixture was evaporated to dryness, the residue was re-dissolved in water and precipitated by the addition of acetone. The solid residue was re-crystallized from water/acetone to obtain 19.5 mg (77.2%) **5** as red crystals. ^{1}H NMR (D_2O): $\delta = 0.44$ (s, 3H), 1.01 (m, 2H), 1.18 (s, 3H), 1.23 (d, 3H, $J = 5.5$ Hz), 1.38 (s, 3H), 1.39 (s, 3H), 1.42 (s, 3H), 1.79–2.28 (m) superimposed by 1.85 (s), 2.25 (s), total of 18H, 2.36–2.80 (m), superimposed by 2.52 (s), 2.55 (s), total of 22H, 3.00 (dd, 1H, $J = 13.9$, 8.0 Hz), 3.32 (d, 1H, $J = 10.5$ Hz), 3.41 (m, 1H), 3.53 (s, 3H), 3.58 (d, 1H, $J = 13.3$ Hz), 4.08 (d, 1H, $J = 9.8$ Hz), 4.16–4.32 (m, 5H), 4.61 (d, 1H, $J = 11.3$ Hz), 4.84 (m, 1H), 6.05 (s, 1H), 6.31 (s, broad, 1H), 6.49 (s, 1H), 7.09 (s, 1H), 7.28 (s, 1H) ppm; UV/Vis (H_2O/D_2O, $c = ca.$ 32 μ*M*): λ_{max} (ε_{rel}) = 548.0 (0.31), 517 (0.27), 408 (0.12), 359.5 (1.00), 322 (0.27), 305 (0.32), 277.0 (0.54) nm; MS (FAB pos., *NOBA*): m/z (%) = 1492.0 (19, [M-H + Na]$^+$), 1471.8 (30), 1470.9 (75), 1469.9 (100, [M + H]$^+$), 1444.8 (19), 1443.84 (24, [M + H-CN]$^+$), 1441.55 (18); X-ray: Crystals of **5** were grown from water/acetone at room temperature under protection from light. Crystal data of **5** are summarized in Tables 1 and 5, for the crystal structure see Fig. 4.

Co$_\beta$-cyano-O5R-[3-(benzylcarboxamido)propionyl]-cobalamin (**6**, $C_{74}H_{99}CoN_{15}O_{16}P$)

To a solution of 1.9 mm^3 benzyl amine (1.8 mg, 17.2 μmol) and 2.6 mg HO*BT* (17.2 μmol) in 1 cm^3 H_2O, which was cooled to 0°C with an external ice-water bath, 25.0 mg (17.2 μmol) cyano-O5′-succinylcobalamin (**4c**) and 6.60 mg *EDC* (34.4 μmol) were added and the resulting solution was stirred for 75 min with cooling at 0°C. The external cooling bath was removed and the reaction mixture warmed to about 24°C. Analytical TLC indicated nearly complete conversion and the amide **6** was precipitated by addition of about 20 cm^3 acetone. The red precipitate was washed twice with acetone and was dried under high vacuum, to give 23.8 mg (15.4 μmol, 92%) of **6** as a red powder.

Table 5. Crystal data, data collection, and structure refinement for **5**

Molecular formula	$C_{68}H_{94}CoN_{14}O_{17}P \times 11H_2O$
Formula weight	1667.65
Unit cell dimensions	a/pm = 1592.67(4) $\alpha = 90°$ b/pm = 2255.9(1) $\beta = 90°$ c/pm = 2561.7(1) $\gamma = 90°$
Z	4
Density (calculated)/mg m^{-3}	1.203
Crystal size/mm^3	$0.4 \times 0.1 \times 0.1$
Goodness-of-fit on F^2	1.071
Final R indices [$I > 2\sigma(I)$]	$R1 = 0.0767$, $wR2 = 0.1950$
R indices (all data)	$R1 = 0.1071$, $wR2 = 0.2166$
Refinement details	5 (of 11) water molecules were refined anisotropically. H-atoms of solvent and succinyl ester group were omitted. The (terminal) ester group had high mobility and was refined with bond restraints and fixed temperature factors for C53R and C54R. C53R, C54R, O55R, and O56R were refined isotropically

^{1}H NMR (CD_3OD): $\delta = 0.49$ (s, 3H, C1A); 1.06–1.30 (m), superimposed by 1.20 (s, C12B), 1.25 (d, $J = 6.2$, C177), total 7H, 1.36–1.51 (m), superimposed by 1.38/1.40/1.47 (3s) total 10H, 1.68–2.22 (m), superimposed by 1.90 (s, C7A), total 13H, 2.2 (3H)); 2.24–2.80 (m), superimposed by 2.27 (s), 2.29 (s), total 31H, 3.26–3.37 (m) superimposed by m (CHD_2OD), total *ca.* 10H, 10H, 3.60–3.71 (m, 2H), 4.12–4.42 (m, 7H), 4.52 (d, 1H, $J = 8.8$ Hz, C5R), 4.59 (m, 1H), 4.77 (m, 1H, $J_1 = 9.0$ Hz, C5R), 6.06 (s, 1H, C10), 6.31 (d, 1H, $J = 2.6$ Hz, C1R), 6.59 (s, 1H, C4N), 7.17 (s, 1H, C2N), 7.20–7.37 (m, 5H) ppm; IR (KBr): $\bar{\nu} = 3420$vs, 3229s, 2969w, 2133vw, 1669vs, 1572m, 1499m, 1404m, 1215m, 1146m, 1084m, 1003w, 617w cm^{-1}; UV/Vis (CH_3OH, $c = 5.40 * 10^{-4}\,M$): λ_{max} (log ε) = 549 (8500), 518 (7700), 485sh (4500), 402sh (3400), 361 (24600), 323 (8400), 305 (9400), 279 (14800) nm; MS (FAB): m/z (%) = 1548.4 (19), 1547.4 (17), 1546.3 (100), 1545.4 (86, $[M + 1]^+$), 1520.3 (25), 1519.4 (19, $[M\text{-}CN + 1]^+$), 1518.2 (6), 1517.3 (22), 1356.3 (12); TLC (CH_3OH:H_2O = 9:1): $R_f = 0.42$.

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