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Cyanocobalamin (vitamin B_{12}) conjugates with enhanced solubility

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Abstract—Cyanocobalamin (vitamin B_{12}) is an essential nutrient as well as a very useful carrier in drug delivery. Conjugates of vitamin B_{12} are investigated due to their wide range of therapeutic applications. We report the synthesis of six vitamin B_{12} conjugates, and the effect of conjugation on their solubilities and stabilities in various media. We reveal here that vitamin B_{12} can be released readily if a 2′-hydroxyl group is conjugated rather than the 5′-hydroxyl group, and the solubility (thus the equivalents of vitamin B_{12}) could be enhanced as much as 19-fold, by simple conjugates such as glycolates. Findings disclosed here provide insights into the reactivities of vitamin B_{12} conjugates, the design of future prodrugs and similar conjugated moieties using vitamin B_{12}.

1. Introduction

Vitamin B_{12} or cyanocobalamin (Chart 1), a member of the vitamin B complex, is important for the healthy survival of several organisms and human despite being produced by few bacterial species.[1] This important vitamin must be obtained from external sources in mammals including human because vitamin B_{12} is not synthesized in mammalian cells. This vitamin plays a key role in enzymatic processes in the mitochondria, cell nucleus, and cytoplasm.[2] It is necessary for the synthesis of red blood cells, for the maintenance of the nervous system, and the growth and development in children.[3] The uptake of cyanocobalamin in human is complex and requires at least three different transport proteins: intrinsic factor (IF), transcobalamin, and haptocorrin.[4] Deficiencies in absorption, transport and/or metabolism of vitamin B_{12} result in its intracellular deficiency leading to anemia, degeneration of nerve fibers, and irreversible neurological damage.[5] Vitamin B_{12} deficiency is common with aging and is linked to cognitive impairment.[6,7] Vitamin B_{12} is also essential at the site of enhanced proliferation, particularly in cancer cells or at the sites of bacterial infections.[8] Higher eukaryotes require vitamin B_{12} as an essential cofactor for the methylation of uracil prior to DNA synthesis and cell replication.[9,10] Thus, vitamin B_{12} was used for the targeted delivery of chemotherapeutic agents and radionuclides to cancer cells by conjugation of drug or radionuclide to cyanocobalamin. In the targeted delivery, vitamin B_{12} was used as a Trojan horse, thereby enabling receptor-mediated endocytosis of the cobalamin conjugate/transcobalamin complex.[11,12] Vitamin B_{12} has low toxicity thus excess intake of this vitamin may not be dangerous to the host.[13] Its uptake system exhibits enormous potential as an absorption enhancing mechanism for orally administered proteins, peptides, and immunogens, when such molecules are conjugated to vitamin B_{12}.[14] There are also attempts to conjugate vitamin B_{12} with drugs to utilize the efficient receptor-mediated endocytosis of vitamin B_{12} simultaneously achieving the co-absorption of the drug.[15] All above strategies require appropriate derivatization of vitamin B_{12}.

A number of synthetic handles are available on vitamin B_{12} for the introduction of ligand or receptor-binding molecules (Chart 1).[16] The conjugating groups at the cobalt center, the amide side chains (*b*, *c*, *d*, and *e* positions), as well as the 5′-hydroxyl (and to a lesser extent the 2′-hydroxyl or both the 5′- and 2′-hydroxyl groups) on the ribofuranoside are synthetically feasible positions to conjugate other molecules.[17–19] Due to the light sensitivity of the carbon–cobalt bond, and the low synthetic yields for the derivatization of the *b*, *c*, *d*, and *e* side-chain acids, the 5′-hydroxyl moiety of the ribofuranoside has been the most productive position for

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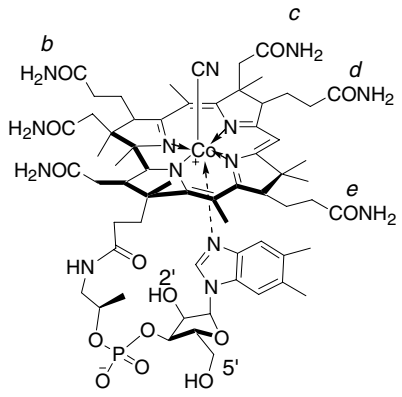


Chart 1. Structure of cyanocobalamin (vitamin B_{12}) and various positions for conjugation.

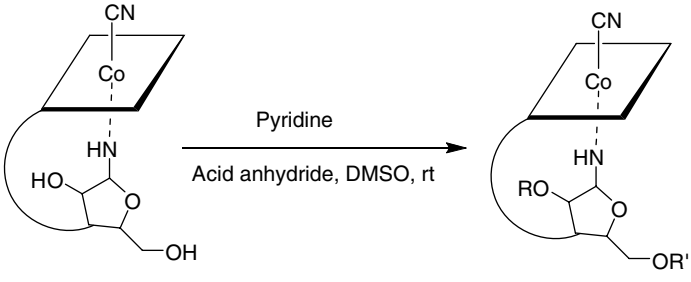
additional conjugation. Additionally, vitamin B_{12} with conjugated groups on the ribosyl moiety maintains high binding efficiency in general to transcobalamin II transporter protein, and exhibits improved stability to light.[20] In the process of preparing various vitamin B_{12} conjugates, previous studies have examined the reactivity of the 5′-hydroxy group toward acid anhydrides, succinic anhydride and glutaric anhydride.[21] In order to design a suitable conjugation strategy using vitamin B_{12}, an understanding of the chemistry and the effect of conjugation on the physicochemical properties is essential.

In an effort to improve the solubility properties of vitamin B_{12}, we were interested in developing novel vitamin B_{12} conjugates. Their stabilities in human serum and in buffer solutions were investigated to understand their corresponding abilities to deliver vitamin B_{12} in its native form after the hydrolysis of the conjugate. Such highly soluble vitamin B_{12} conjugates will be able to deliver larger quantities of vitamin B_{12} when necessary. Since these vitamin B_{12} derivatives have potential to be used as a Trojan horse in cancer cells and as carrier molecules for proteins, peptides, and immunogens, the solubility of these derivatives is of much interest.[22,23] Here, we report the synthesis of several ester conjugates of vitamin B_{12} using a generic and inexpensive method, their aqueous solubilities, and stabilities in biological media for these derivatives, in the context of designing a useful vitamin B_{12} conjugate.

2. Results and discussion

Four different conjugating groups were investigated carrying various functionalities to interrogate the solubility properties of vitamin B_{12} conjugates. These include phthalate, tartarate, succinate, and glycolate. Synthesis of these ribose-derivatized vitamin B_{12} analogs was accomplished using the corresponding anhydrides. While there are a number of options available to activate the 5′-hydroxyl group, such as 1,1′-carbonyldiimidazole (CDI), 1,1′-carbonyl-di-(1,2,4-triazole) (CDT), and di-(1-benzotriazolyl) carbonate (DBTC), we found that anhydrous pyridine achieved this task efficiently and is also a cheaper reagent (Scheme 1). Thus, vitamin B_{12} was activated with anhydrous pyridine in DMSO and an acid anhydride was added to this mixture. When vitamin B_{12} was conjugated with succinic anhydride, the reaction was complete in about 16 h yielding compound **1**. For the preparation of compounds **2** and **3**, it required a longer time for the completion of the reaction, up to 40 h. When vitamin B_{12} was reacted with diglycolic anhydride, compounds **4**, **5**, and **6** were generated in a single reaction in the ratio of 3:2:8 after 48 h and these compounds were easily separable. All synthesized compounds were evaluated for their solubility and stability in their amorphous forms under various conditions.

Compounds **1–6** were evaluated for their apparent solubility in their free amorphous form, in their corresponding ammonium salt form, and sodium salt form in PBS buffer, and in distilled water (Fig. 1 and Table 1). The three conjugating groups, that is, succinate, tartarate, and glycolate carry multiple polar functional groups and are expected to enhance the solubility. The fourth conjugating group, phthalate, carries aromatic as well as hydrophilic moieties which could influence solubility as well as the hydrolysis rates to generate the parent vitamin B_{12}. These compounds exhibited enhanced solubility compared to vitamin B_{12}, as anticipated. There are



Vitamin B_{12}

1, R = H, R' = $COCH_2CH_2COOH$
2, R = H, R' = CO-(o-COOH)Ph
3, R = H, R' = COCH(OAc)CH(OAc)COOH
4, R = H, R' = $COCH_2OCH_2COOH$
5, R = $COCH_2OCH_2COOH$, R' = H
6, R = $COCH_2OCH_2COOH$, R' = $COCH_2OCH_2COOH$

Scheme 1.

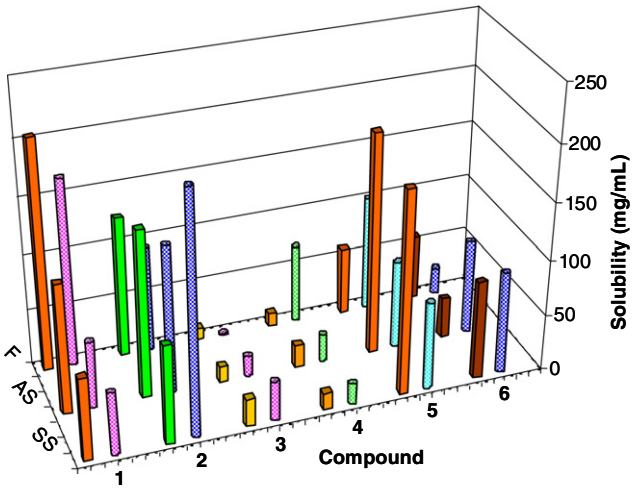


Figure 1. The solubility of compounds **1–6**, their ammonium and sodium salt in PBS buffer and water (F, free form; AS, ammonium salt; SS, sodium salt; the square solid columns show the solubility in PBS buffer; the round semi-transparent columns illustrate the solubility profiles in water).

Table 1. Solubilities of Vitamin B_{12} and its conjugated derivatives (mg/mL)

Compound	Salt form	Solubility (mg/mL)	
		in PBS	in water
Vitamin B_{12}	F	10.7	10.2
1	F	205.0	166.7
	AS	115.0	60.0
	SS	73.3	55.0
2	F	125.0	94.3
	AS	148.8	131.6
	SS	86.9	213.3
3	F	11.3	1.0
	AS	13.9	18.3
	SS	25.0	35.0
4	F	11.4	70.0
	AS	19.4	23.8
	SS	15.4	18.4
5	F	60.0	102.5
	AS	196.0	78.2
	SS	180.0	77.1
6	F	58.3	23.8
	AS	35.7	84.3
	SS	87.5	90.9

also unexpected solubility profiles especially in their salt forms (vide infra). Vitamin B_{12} exhibited a solubility of 10.2 and 10.7 mg/mL, in distilled water and PBS buffer, respectively. In PBS, the free forms of compounds **1** and **2** have a solubility of 205.0 and 125.0 mg/mL, respectively, which is a remarkable improvement of over 12-fold when compared to that of vitamin B_{12}. In water, the free forms of compounds **1**, **2**, and **5** were soluble at 166.7, 94.3, and 102.5 mg/mL indicating that the presence of polar groups improved the solubilities 16- and 10-fold, respectively (in comparison to that of vitamin B_{12} at 10.2 mg/mL), even in their native free forms. Ammonium and sodium salts of compounds **2** and **6** exhibited enhanced solubilities in the range of 8- to 20-fold, and the sodium salt of **2** showed the highest solubility at 213.3 mg/mL (Fig. 1). These are significant enhancements over the solubility of vitamin B_{12} (vide supra), although we note here that the equilibrium solubilities might be slightly lower than the apparent solubilities. It is interesting to note that the solubility of diacetyl tartarate analog **3** is almost equivalent to that of the parent compound, vitamin B_{12}. Overall, vitamin B_{12} conjugates with succinate, phthalate, and 2′-glycolate exhibited the highest improvements in their solubilities, up to 19- to 20-fold in water and PBS.

While it is common to anticipate enhanced solubility for salt forms, we observed some surprising results. For example, the solubility for compound **1** in its free form is higher by at least 2-fold than its ammonium or sodium salt form. Interestingly for compounds **3** and **4**, the free acid and the salt forms are exhibiting poor improvements in solubility in comparison to that for vitamin B_{12}. Additionally, the free form of compound **5** in water has higher solubility than in PBS (almost 70% higher). These exceptions to the general rule indicate the influence of the molecular interactions that are modulated due to the changes in the salt forms of the compounds and the solution environment (example water vs PBS) on the solubility. The 5′-glycolate derivative **4** and 3′-glycolate **5** exhibit higher solubilities in their respective free forms than the diglycolate derivative **6** in its free form. Due to the proximity of the glycolic acid substitutions at 3′- and 5′-positions, there is a strong possibility of intramolecular hydrogen bonding in compound **6**. This may be facilitated by water molecules integrally hydrogen bonded with the carboxyl groups of the glycolate moieties at 3′- and 5′-positions of compound **6**. At this stage, it is impractical to provide any additional rational explanation at the molecular level for these interesting observations, and certainly additional structural studies are necessary.

In order to have higher quantities of vitamin B_{12} released from these highly soluble conjugates, the rates of hydrolysis of the above compounds should be reasonably faster in aqueous and/or biological media such as serum. We investigated compounds **1–6** for their ability to release vitamin B_{12} in distilled water (at 37 °C as well as at room temperature), in the presence of porcine liver esterase (PLE) and human serum at 37 °C (Fig. 2 and Table 2).

The hydrolysis of compounds **1** and **2** was ca. 1% in water, and for compound **3** it was less than 10% in 4 days implying that these compounds are stable prodrugs and do not release vitamin B_{12} readily in water at 25 °C as well as 37 °C (Table 2, Fig. 2, Panels A and B). Compounds **4**, **5**, and **6** were hydrolyzed in the range of 20–48% in water at 37 °C, and in the range of 4–34% at 25 °C, in water over 24 h (Table 2). Thus, overall hydrolysis and the release of vitamin B_{12} from the ester conjugates **1–6** was not significant (i.e., less than 50% over 24 h period), either at 25 °C or 37 °C in water.

The stability of compounds **1–6** in the presence of porcine liver esterase (PLE) was determined to understand the susceptibilities of these esters to nonspecific esterases

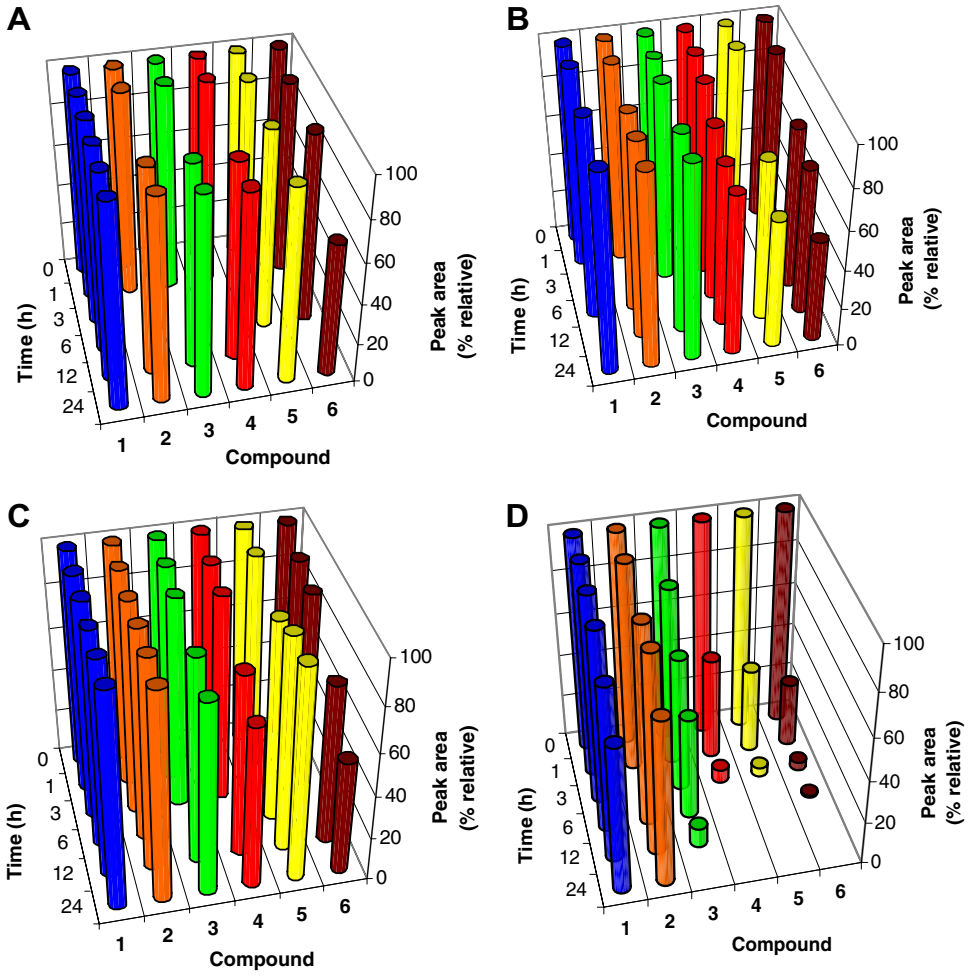


Figure 2. Stability profiles for compounds **1–6** in distilled water over a period of 24 h (A) at room temperature, (B) at 37 °C, (C) in PBS buffer with PLE enzyme at 37 °C, and (D) in human plasma at 37 °C.

Table 2. Stabilities (expressed as % decomposition) of compounds **1-6** in distilled water, in the presence of porcine liver esterase (PLE), and in human serum after 24 h

Compound	Water at 37 °C (%)	Water at rt (%)	PLE in PBS at 37 °C (%)	Serum at 37 °C (%)
1	0.46	0.00	1.33	31.32
2	3.57	0.57	3.88	22.76
3	2.80	2.76	11.98	90.99 (12 h)
4	20.00	4.28	25.38	93.71 (3 h)
5	35.30	5.10	12.86	95.70 (3 h)
6	48.63	34.14	47.44	99.04 (6 h)

and possibly proteases (Table 2, Fig. 2C). Thus, the above compounds were incubated in the presence of porcine liver esterase (PLE) at 37 °C, which has a wide range of substrate specificity. Compounds **4** and **6**, both of which are derivatized with a glycolyl moiety at 5′-position, exhibited 25% and 47% hydrolysis leading to the release of the parent vitamin B_{12}. All other compounds, including the 2′-derivatized vitamin B_{12}, did not exhibit reasonable rates of release of the parent compound in the presence of PLE. Thus, certain esterases may not be activating agents at least for the above synthesized conjugates.

Following these, we considered human serum to measure the stability and to understand the rates of hydrolysis of the conjugates over 24 h (Fig. 2D). Although PLE hydrolysis may be a slight indication, it was of interest to see if human serum will make a difference as opposed to a model enzyme such as PLE. Compounds **3–6**, that is, the diacetyl tartarate analog, and the glycolate derivatives released vitamin B_{12}, but not the succinate and the phthalate derivatives, **1** and **2**, respectively. The products of hydrolysis are cyanocobalamin as identified by the HPLC analyses and the mass spectral analyses. These interesting findings when viewed with those in distilled water and in the presence of PLE indicate that serum enzymes perhaps may be involved in the hydrolysis of these ester conjugates. Compounds **1** and **2**, however, did not show any significant hydrolysis in either media (less than 4%), thus compounds **3–6** must be activated by specific esterases/proteases in the human serum. These results show that the specificity and hydrolytic rates of vitamin B_{12} conjugates might differ substantially based on the conjugating group.

Enhanced solubilities of compound **5** in its ammonium and sodium salt forms by 16-fold in PBS (in comparison

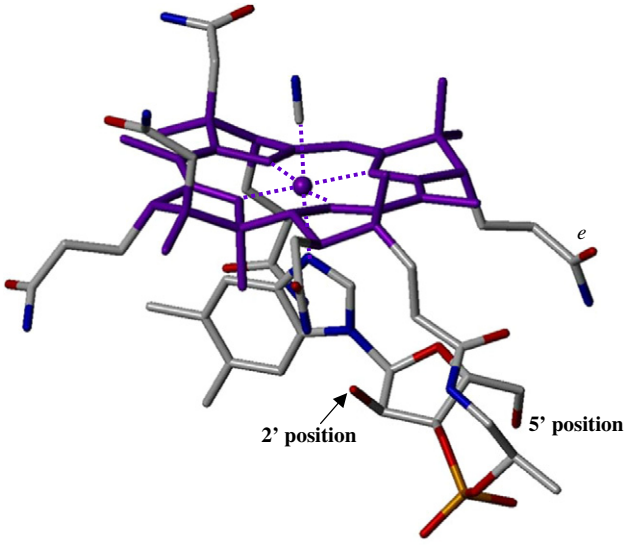


Figure 3. Three-dimensional structure of vitamin B_{12} (obtained from protein databank, code 1DDY).

to vitamin B_{12}), and the conversion of more than 95% of **5** into vitamin B_{12} in human serum at 37 °C indicate that the 2′-glycolate derivative is the best among the synthesized derivatives to provide enhanced concentrations of vitamin B_{12}. Although one would anticipate that the 5′-derivative **4** might be hydrolyzed at comparable or better rate than the corresponding 2′-derivative **5**, the exposed 2′-hydroxyl group to the solution acts counter-intuitively leading to the observations presented here (Fig. 3). We analyzed the structure of vitamin B_{12} and investigated the location of 5′-hydroxyl group versus 2′-hydroxyl moiety (Fig. 3). The *e*-ethylamide side chain and the phosphate moiety are in the vicinity of 5′-hydroxyl moiety and the conjugating group at this position will create further crowding thus affecting the approach of an enzyme or other nucleophilic reagents such as water to hydrolyze the conjugated vitamin B_{12}. Here, the chemical reactivity (i.e., primary vs secondary hydroxyl moieties) is overshadowed due to the steric interactions and accessibility to the reaction center, which is the ester at 5′- versus 2′-position, respectively. Thus, vitamin B_{12} due to its complex three-dimensional structure should be treated with care to design prodrugs and/or conjugated strategies.

3. Experimental

3.1. General

All anhydrous reactions were performed under nitrogen atmosphere. All reactions were conducted in dark. The workup of the chemical reactions, HPLC analyses, solubility studies, and stability studies were conducted in a dark room and the equipment/glassware were covered with aluminum foil. A water bath was used as a source of heat during stability studies. All solvents and reagents were obtained from commercial sources and were used as received except where noted. Vitamin B_{12} and human serum were generously provided by Transition Therapeutics (Toronto, Ontario, Canada). The porcine liver esterase was purchased from Sigma Chemicals (specific activity 20 U/mg). Ion exchange chromatography was conducted with 2% cross-linked Dowex (200–400 mesh, acetate form, prepared by refluxing the Dowex 1-chloride in saturated sodium acetate for 2 h and then washed with appropriate distilled water). PBS buffer (1 M) is at pH 7.4. Solvents for HPLC were filtered through a 0.2 μm membrane filter prior to use. ^{1}H NMR spectra were recorded at 400 MHz and the chemical shifts were reported in δ ppm using tetramethylsilane as a standard.[24] The high-resolution mass spectra were acquired on a QStar mass spectrometer (ESI) at the Department of Chemistry Mass Spectrometry facility, University of Toronto.

3.2. Synthesis

All reactions were performed using anhydrous DMSO as the solvent. Reactions were monitored using thin-layer chromatography with 2-propanol/NH_4OH(28%)/water (7:1:2) as the eluting system. The ^{1}H NMR spectra are included in the supplementary data.

3.2.1. Cyanocobalamin succinate (1). Prepared according to the reported procedure.[21] Yield: 83.6%. Structure was confirmed using ^{1}H NMR and mass spectral analyses.

3.2.2. Cyanocobalamin phthalate (2). Cyanocobalamin (60 mg, 0.045 mmol) was dissolved in DMSO (13 mL) containing phthalic anhydride (3.33 g, 22.5 mmol) and pyridine (2.2 mL). After 40 h at room temperature, excess phthalic anhydride was neutralized by the addition of water (150 mL). The pH of the reaction mixture was adjusted to 6 using 10% KOH. Then potassium cyanide (0.01 M final concentration) was added and the pH of the solution was readjusted to 6 using 3 N HCl solution. After 1 h, cyanocobalamin components were desalted and were extracted from DMSO by phenol extraction (3× 15 mL). The combined phenol layers were washed with water (3× 10 mL), following which diethyl ether (50 mL) and hexanes (10 mL) were added to the phenol layer. The cobalamin components went to aqueous layer and the phenol layer was further extracted with water (3× 10 mL). The combined aqueous layers were washed with ether (2× 10 mL) to remove traces of phenol and then washed with hexanes (2× 10 mL) to remove traces of ether. The water was removed by freeze-drying and the residue was applied to a Dowex column (25× 2.5 cm^2). The column was first eluted with water to remove traces of unreacted cyanocobalamin until the water is colorless and then eluted with 1% acetic acid until the eluent was colorless. This fraction was concentrated under 35 °C and the crude product (65 mg, purity 87%) was obtained by freeze-drying. This crude was purified by HPLC to obtain compound **2** (54.7 mg, 81% yield) as a dark red solid. ^{1}H NMR (CD_3OD) δ ppm 0.45 (s, 3H), 0.82–0.98 (m, 1H), 1.02–1.18 (m, 1H), 1.17 (s, 3H), 1.24 (d, J = 6.15 Hz, 3H), 1.29 (m, 2H), 1.37 (s, 3H), 1.38 (s, 3H), 1.43 (s, 3H), 1.65–1.75 (m, 1H), 1.80–1.92 (m, 5H), 1.95–2.10 (m, 4H), 2.11–2.21 (m, 1H), 2.24 (s, 3H), 2.27 (s, 3H), 2.32–2.52 (m, 6H), 2.51–2.69 (m, 11H), 2.82–2.94 (m, 2H), 3.18–3.27

(m, 1H), 3.59–3.69 (m, 2H), 4.13 (d, J = 11.65 Hz, 1H), 4.23 (br s, 1H), 4.32–4.41 (m, 2H), 4.45 (m, 2H), 4.98 (m, 1H), 6.01 (s, 1H), 6.30 (d, J = 1.64 Hz, 1H), 6.56 (s, 1H), 6.74–6.77 (m, 1H), 7.12–7.16 (m, 2H), 7.22 (s, 1H), 7.57–7.64 (m, 1H), 7.72–7.78 (m, 1H), 7.81–7.86 (m, 1H); HRMS calcd for $C_{71}H_{94}N_{14}O_{17}PCoH_2$: 752.2990 (*m*/*z*, charge +2). Found: 752.3020.

3.2.3. Cyanocobalamin diacetyl-L-tartarate (3). Cyanocobalamin (60 mg, 0.045 mmol) was dissolved in DMSO (20 mL) containing diacetyl-L-tartaric anhydride (4.8 g, 22.5 mmol) and pyridine (2.2 mL). After stirring for 40 h at room temperature, the reaction was worked up as described for the synthesis of **2**. The residue was purified on a Dowex column (25× 2.5 cm^2). The column was first eluted with water until the water was colorless to remove trace unreacted cyanocobalamin. Then the column was eluted with 2% acetic acid in water until the eluted acid was colorless. This fraction (about 150 mL) was concentrated under 35 °C and the crude product was freeze-dried (104 mg). The crude product was purified by HPLC to yield compound **3** (48.3 mg, 68% yield) as a red solid. ^{1}H NMR (CD_3OD) δ ppm 0.48 (s, 3H), 1.16 (m, 2H), 1.18 (s, 3H), 1.24 (d, J = 2.2 Hz, 3H), 1.37 (s, 3H), 1.39 (s, 3H), 1.43 (s, 3H), 1.70–1.80 (m, 2H), 1.87 (s, 6H), 2.10–2.15 (m, 2H), 2.15 (s, 3H), 2.28 (s, 5H), 2.32–2.60 (m, 15H), 2.65 (s, 10H), 2.82–2.98 (m, 2H), 3.23–3.27 (m, 1H), 3.60–3.70 (m, 2H), 4.14 (d, J = 11.21 Hz, 1H), 4.17 (s, 1H), 4.27 (br d, 1H), 4.33 (br s, 1H), 4.36 (br s, 1H), 4.51 (br d, 1H), 5.85 (dd, J = 2.64, 10.77 Hz, 2H), 6.06 (s, 1H), 6.24 (d, J = 2.86 Hz, 1H), 6.56 (s, 1H), 7.12 (s, 1H), 7.32 (s, 1H), 7.75 (m, 1H, NH); HRMS calcd for $C_{71}H_{96}N_{14}O_{2s1}PCoH_2$: 786.3044 (*m*/*z*, charge +2). Found: 786.3067.

3.2.4. Cyanocobalamin diglycolates (4, 5, 6). Cyanocobalamin (180 mg, 0.135 mmol) was dissolved in DMSO (39 mL) containing diglycolic anhydride (7.8 g, 67.5 mmol) and pyridine (6.6 mL). After 48 h at room temperature, the reaction was worked up as described for the synthesis of **2**. The residue was purified on a Dowex column (30× 4 cm^2). First, the column was eluted with water to remove trace unreacted cyanocobalamin until the water was colorless and then with 1% acetic acid (100 mL) to obtain compound **4**, followed by elution with 2% acetic acid to obtain compound **5** (100 mL). Finally, elution with 5% of acetic acid in water (150 mL) yielded compound **6**. All three fractions were concentrated under 35 °C and the concentrate was further purified by HPLC to obtain compounds **4**, **5**, and **6** (38.2, 23.7, and 96.6 mg, respectively).

Compound **4**, yield 19%; ^{1}H NMR (CD_3OD) δ ppm 0.46 (s, 3H), 0.84–0.96 (m, 1H), 1.06–1.14 (m, 1H), 1.18 (s, 3H), 1.24 (d, J = 6.15 Hz, 3H), 1.36 (s, 3H), 1.38 (s, 3H), 1.45 (s, 3H), 1.64–1.75 (m, 1H), 1.80–1.92 (m, 6H), 1.96–2.10 (m, 5H), 2.11–2.21 (m, 1H), 2.27 (s, 3H), 2.32 (s, 3H), 2.34–2.40 (m, 2H), 2.40–2.69 (m, 16H), 2.84–2.94 (m, 2H), 3.25–3.28 (m, 1H), 3.59–3.69 (m, 2H), 4.13 (d, J = 11.64 Hz, 1H), 4.19 (m, 1H), 4.21 (s, 2H), 4.24–4.28 (m, 1H), 4.31–4.32 (m, 2H), 4.33 (s, 2H) 4.48–4.52 (m, 1H), 6.03 (s, 1H), 6.27 (d, J = 2.64 Hz, 1H), 6.56 (s, 1H), 7.14 (s, 1H), 7.28 (s, 1H), 7.76–7.82 (m, 1H); HRMS calcd for $C_{67}H_{91}N_{14}O_{18}PCo$: 1469.5710 (*m*/*z*, charge −1). Found: 1469.5775.

Compound **5**, yield 12%; ^{1}HNMR (CD_3OD) δ ppm 0.55 (s, 3H), 0.92–1.02 (m, 1H), 1.10–1.15 (m, 1H), 1.16 (s, 3H), 1.24 (d, J = 6.15 Hz, 3H), 1.31–1.40 (m, 1H), 1.42 (s, 3H), 1.46 (s, 3H), 1.48 (s, 3H), 1.62–1.70 (m, 1H), 1.85 (s, 3H), 1.86–2.05 (m, 6H), 2.10–2.22 (m, 2H), 2.25 (s, 3H), 2.26 (s, 3H), 2.37 (s, 2H), 2.40–2.56 (m, 6H), 2.57 (s, 3H), 2.59–2.64 (m, 4H), 2.65 (s, 3H), 2.67–2.80 (m, 4H), 2.84–2.94 (m, 1H), 3.04–3.14 (m, 1H), 3.22–3.26 (m, 1H), 3.50–3.65 (m, 3H), 3.74–3.82 (m, 1H), 3.92–4.02 (m, 2H), 4.08–4.20 (m, 2H), 4.28–4.38 (m, 1H), 4.55–4.60 (m, 1H), 4.76–4.80 (m, 2H), 6.09 (s, 1H), 6.12 m, 1H), 6.35 (d, J = 2.66 Hz, 1H), 6.49 (s, 1H), 7.12 (s, 1H), 7.15 (s, 1H); HRMS calcd for $C_{67}H_{91}N_{14}O_{18}PCo$: 1469.5710 (*m*/*z*, charge −1). Found: 1469.5791.

Compound **6**, yield 45%; ^{1}H NMR (CD_3OD) δ ppm 0.49 (s, 3H), 0.93–1.03 (m, 1H), 1.16 (s, 3H), 1.17–1.21 (m, 1H), 1.24 (d, J = 6.14 Hz, 3H), 1.43 (s, 3H), 1.44 (s, 3H), 1.46 (s, 3H), 1.62–1.73 (m, 1H), 1.86 (s, 3H), 1.87–1.94 (m, 4H), 2.02–2.22 (m, 5H), 2.26 (s, 6H), 2.37 (s, 2H), 2.38–2.50 (m, 3H), 2.52 (s, 1H), 2.54–2.63 (m, 6H), 2.64–2.68 (m, 2H), 2.69 (s, 3H), 2.70–2.76 (m, 1H), 2.85–2.89 (m, 1H), 3.24–3.27 (m, 1H), 3.54 (br d, 1H), 3.61–3.65 (m, 1H), 3.68 (d, 1H), 4.00 (d, 1H), 4.14–4.17 (m, 1H), 4.19 (s, 1H), 4.20–4.24 (m, 2H), 4.24 (s, 2H), 4.29 (d, 1H), 4.33 (d, 1H), 4.39 (s, 2H), 4.56 (d, 1H), 4.82 (m, 1H), 4.96–5.02 (m, 1H), 6.08 (s, 1H), 6.14 (m, 1H), 6.36 (d, J = 2.66 Hz, 1H), 6.49 (s, 1H), 7.16 (s, 1H), 7.18 (s, 1H). HRMS calcd for $C_{71}H_{96}N_{14}O_{22}PCoH_2$: 794.3019 (*m*/*z*, charge +2). Found: 794.3018.

3.3. HPLC analysis

All HPLC analyses were conducted on a Waters Delta600 HPLC system equipped with a Waters 996 Photodiode Array Detector (wavelength was set at 360 nm). HPLC analysis for the monitoring of reactions was conducted at a flow rate of 1 mL/min on a Symmetry® C_{18} 3.5 μm 4.6× 75 mm^2 column using a gradient solvent system. Solvent A was 1% HOAc in water. Solvent B was CH_3CN. Gradient elution initially started at 100% A and in 50 min, eluting system was linearly adjusted to 1:1 ratio of the solvents A and B.

Separation of the reaction mixtures was conducted at a flow rate of 3.7 mL/min on a Altantis® dC_{18}, 10 μm, 10× 250 mm^2 column using the isocratic mode (85% A and 15% B). HPLC analysis for the stability studies was conducted at a flow rate of 1 mL/min on a Symmetry® C_{18} 3.5 μm (4.6× 75 mm^2) column using a gradient solvent system (100% A at t = 0 min and then the gradient was increased to 30% B over the next 20 min).

3.4. Solubility

Cyanocobalamin derivatives as well as their ammonium salts and sodium salts were evaluated for their solubility

in water and PBS buffer. It should be noted that the solubilities were saturated concentrations of the corresponding solutions using the amorphous powders and are not corrected for true or equilibrium solubilities. Corresponding ammonium and sodium salts were made by dissolving 5 μmol of each derivative in 0.5 mL of 0.01 N sodium hydroxide or ammonium hydroxide solution at 0 °C. Distilled water was used for solubility tests.

3.5. Stability studies

All cyanocobalamin derivatives were evaluated in human serum, porcine liver esterase/PBS, and in water. The analysis of the conjugated compounds and the amount of vitamin B_{12} released were determined using HPLC. The PDA detector was set at 360 nm. The concentration for each compound was 1 mg/mL. The concentration of PLE in PBS was 1 mg/mL.[25] The data were recorded at 0 (i.e., immediately after the addition of the compound to the appropriate solution), 1, 3, 6, 12, and 24 h.

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Supplementary data

^{1}H NMR spectra for compounds **1–6** are presented in the supplementary material.

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