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Synthesis and Characterization of a Cobalamin−Colchicine Conjugate as a Novel Tumor-Targeted Cytotoxin

Joshua D. Bagnato,[†] Alanna L. Eilers,[‡] Robert A. Horton,[†] and Charles B. Grissom*,[†]

Department of Chemistry, University of Utah, 315 S. 1400 E., Salt Lake City, Utah 84112-0850, and MantiCore Pharmaceuticals, Inc., 2401 Foothill Dr., Salt Lake City, Utah 84109-1405

grissomc@chemistry.utah.edu

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Colchicine was derivatized at C7 with *p*-alkoxyacetophenone and conjugated to cobalamin (vitamin B_{12}) through an acid-labile hydrazone linker. The cobalamin moiety leads to preferential uptake of the cobalamin−colchicine prodrug by cancer cells, whereupon the hydrazone linker undergoes hydrolysis in the lysosome to unmask colchicine, which acts as a potent cytotoxin by stabilizing microtubules and causing cell death. The bioconjugate is stable in cell culture media and at neutral pH but undergoes hydrolysis with a half-life of 138 min at pH 4.5. The colchicine−cobalamin bioconjugate exhibits nanomolar LC_{50} values against breast, brain, and melanoma cancer cell lines in culture. Attachment of colchicine to cobalamin is expected to increase the therapeutic index of the drug by limiting the side effects caused by the current nonselective administration of tubulin-targeted chemotherapeutic drugs.

A major challenge in cancer therapy is to selectively target cytotoxic agents to tumor cells. In an effort to decrease the undesirable side effects of small molecule anticancer agents, many targeting approaches have been examined. One of the most promising methods involves the covalent attachment of a potent cytotoxic warhead to a macromolecular carrier. Many carriers have been tested with varying success, including soluble polymers,[1−4] nanoparticles, proteins,[5] micelles, liposomes, folate, and antibodies.[6−8]

Vitamin B_{12} (hereafter referred to as cobalamin to describe the naturally occurring 5′-deoxyadenosylcobalamin, methylcobalamin, hydroxocobalamin, cyanocobalamin, or a synthetic derivative that includes the corrin ring and is recognized by B_{12} transport proteins and enzymes) shows promise as a drug delivery system to target the delivery of cytotoxic drugs to cancer cells.[9] Rapidly dividing cells require abnormally high quantities of cobalamin for the synthesis of thymidine to support DNA replication prior to cell division.[10] Cobalamin can be covalently modified to allow the attachment of a variety of drugs without disrupting the corrin ring pharmacophore that is recognized by B_{12} transport proteins.[9] Several such cobalamin bioconjugates have been prepared in our laboratory and tested in vitro and in vivo.[11−15]

Colchicine has been used since the 16th century for a broad range of diseases, including the treatment of gout

[†] University of Utah.

[‡] MantiCore Pharmaceuticals, Inc.

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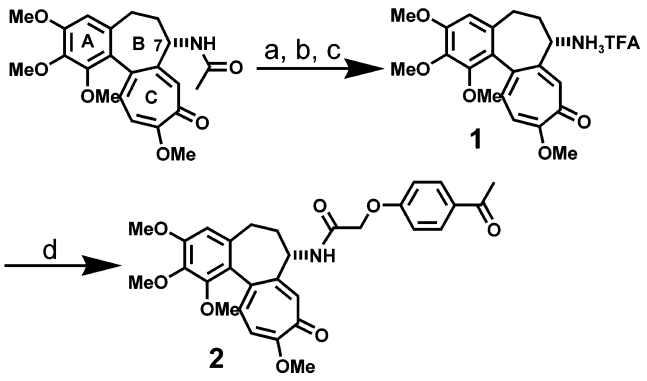
and acute gouty arthritis.[16] Colchicine is cytotoxic and has a mechanism of action similar to that of the taxanes by acting as a spindle poison. Colchicine prevents tubulin polymerization and blocks cell growth at metaphase in mitosis. As a result of the success of the taxanes in cancer therapy, colchicine research has experienced renewed interest because of its inherent water solubility and similar mechanism of action, as well as its commercial availability and low cost. Colchicine has not been useful for the treatment of cancer to date because of its overbearing systemic toxicity that produces unacceptable side effects when administered intravenously.[17−22] By using cobalamin to preferentially deliver colchicine to cancer cells, these side effects may be dramatically decreased.

Our previous work has focused on the delivery of the alkylating agent chlorambucil and the topoisomerase poison doxorubicin.[11−14] These DNA-targeted drugs must ultimately reach the nucleus to exert their cytotoxic effect. To further probe the versatility of cobalamin as a cancer drug delivery agent, we chose to deliver colchicine as the cytotoxic warhead because it has a cytosolic target that does not require transport across the nuclear membrane. A cytosolic target should be more readily delivered to its site of action as the drug does not have to both escape the lysosome and accumulate in the nucleus to achieve cell death. In addition, colchicine is a cytotoxin significantly more potent than those previously conjugated to cobalamin. The LC_{50} values range from low micromolar to nanomolar in all cancer cell lines tested. A major limitation to cobalamin delivery is that only a finite amount of cobalamin can be transported into a cell before all of the cellular receptors have been saturated. The potent toxin colchicine may result in greater efficacy because less of the drug is required to initiate cell death.

An acid-labile hydrazone has been used as the key linkage between cobalamin and colchicine. The use of an acid-labile hydrazone for the release of a drug at low pH has been discussed widely in the literature.[5−8,15,23−28] Gemtuzumab ozogamicin (Mylotarg) was the first antibody−cytotoxin bioconjugate that was approved by the FDA for the treatment of acute myeloid leukemia. Gemtuzumab ozogamicin contains a calicheamicin warhead that is linked to a monoclonal antibody via an acid-labile hydrazone bond.[8] A similar linker using 4-acetylphenoxyacetic acid was used as the electron-rich ketone in our work. Because a hydrazone is stable at neutral pH, this provides a stable bond until the bioconjugate undergoes endocytosis and localization in the low pH environment of the lysosome. Once the bioconjugate is inside of the lysosome, the hydrazone undergoes hydrolysis to release the active drug.

In previous experiments a cytotoxin was covalently bound to the β-face of cobalamin by direct attachment to the central cobalt atom core.[15] The resulting C−Co bond is a very low energy bond that can be cleaved by exposure to visible light. Cancer cells were incubated with these cobalamin bioconjugates and then exposed to light to unmask the cytotoxin inside of the cell. Evidence in our lab suggests that once the bioconjugate is taken into a cell, the endosome becomes a lysosome and the intravesicular pH drops. As an alternate method to light-triggered release of the drug, we have used a pH-sensitive linker that releases the drug in the acidic intralysosomal environment.

SCHEME 1. Synthesis of Colchicine Analog 2[a]



[a] (a) Boc_2O, DMAP, TEA in CH_3CN; (b) 2 M $NaOCH_3$ in CH_3OH; (c) TFA (85% yield, 3 steps); (d) APAA, BOP, NMM (84% yield).

Several analogues of colchicine have been prepared in the past.[23,29−31] Upon reviewing the literature it became evident that modifications of the B ring at the C7 acetate appeared to have the least effect on the overall toxicity of the resulting analogue. Modifications of the A ring and C ring tend to decrease toxicity of the analogue dramatically, and therefore modification at the C7 position of colchicine was selected for this study.

Results and Discussion

The C7 acetamido group of colchicine can be hydrolyzed under forcing acidic conditions;[32] however, this also results in isomerization of the C ring, and requires a tedious purification process. To avoid this isomerization, a three-step process based on a modification of a published procedure[33] was utilized to obtain the nucleophilic deacetylcolchicine (Scheme 1). The amide is first acylated

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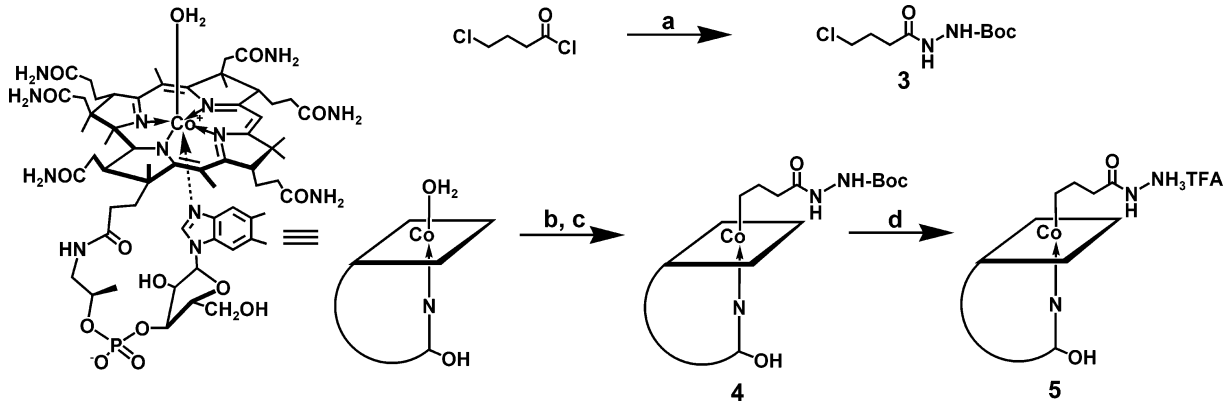
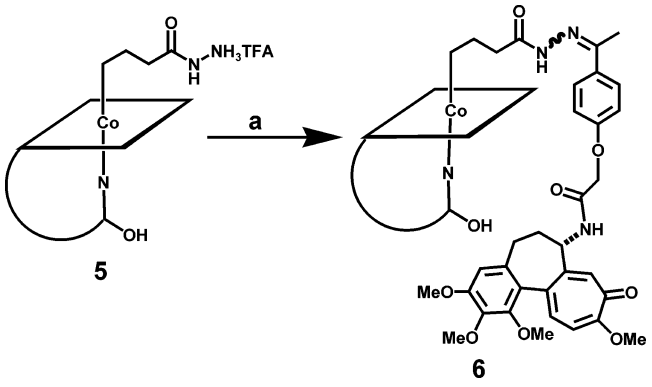
SCHEME 2. Synthesis of Cobalamin Hydrazide 5[a]



[a] (a) *tert*-Butyl-carbazate in CH_2Cl_2 (61% yield); (b) Zn, NH_4Cl in H_2O; (c) hydrazide **3** in CH_3OH; (d) TFA (95% yield, 3 steps).

SCHEME 3. Synthesis of Cobalamin Bioconjugate 6[a]



[a] (a) Colchicine analog **2** in CH_3OH (40% yield).

with Boc-anhydride using DMAP to form an *N*-acetylcarbamate, followed by selective methanolysis of the acetate and subsequent deprotection with TFA to yield the desired compound **1**. With the primary amine in hand, acylation with acetylphenoxyacetic acid (APAA) and Castro's reagent (BOP) as activating agent proceeded smoothly to yield compound **2** in 84% yield.

The efficient synthesis of a hydrazide moiety on cobalamin may be useful as a general starting material for the attachment of a number of chemotherapeutic agents. To that end, 4-chlorobutyric acid chloride was reacted with *tert*-butyl carbazate to give protected hydrazide **4** (Scheme 2). The primary alkyl chloride was combined with hydroxocobalamin under reducing conditions to yield a protected version of the desired hydrazide. Deprotection occurs smoothly, and this synthetic approach is scaleable to the production of multigram quantities to give compound **5** in high yields with no purification. Preparation of the colchicine−cobalamin bioconjugate occurs rapidly in CH_3OH at 25 °C by mixing colchicine analogue **2** with the prepared cobalamin hydrazide **5** to yield bioconjugate **6** (Scheme 3).

As a control, a light- and pH-stabile colchicine−cobalamin bioconjugate was prepared as depicted in Scheme 4. Compound **9** does not have a labile hydrazone and was expected not to be cytotoxic if colchicine must be released from cobalamin to induce cell death. *N*-deacetylcolchicine **1** was acylated with glutaric anhydride, followed by activation of the resulting carboxylic acid with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) and *N*-hydroxysuccinimide (NHS) to generate **7**. Compound **7** was added to the 5′-modified cobalamin **8**, as prepared previously,[34] to generate stabile bioconjugate **9**.

NMR Characterization of 6 in DMSO-d_6. The structure of **6** (Figure 1) was determined using ^1H, ^{13}C, DEPT, DQ-COSY, NOESY, gHSQC, and gHMBC multidimensional NMR experiments in DMSO-d_6.

Evidence of two isomers is observed in the NMR spectrum. The two compounds result from isomerization about the C−N bond commonly seen in other acylhydrazones.[35] By careful analysis of the data, the structure of the two major isomers was assigned (Table 1). Because no HMBC correlations to the terminal side chain amide carbons are observed, the amide side chain protons were assigned by reference to a previous structure.[34]

To distinguish the two isoforms from one another, a prime designation (′) is used to denote the assignment of the second set of resonances. The majority of the side chain methylene carbons on cobalamin had two distinct HSQC correlations, so each proton was denoted as either a or b. In the case of the colchicine portion, if chirality was present, each proton was denoted as either α or β corresponding to the relative position of the proton as drawn in Figure 1.

A number of NMR assignments of cobalamin have been published previously.[34,36−40] This discussion will focus on the assignment of the β ligand of the bioconjugate. The NMR resonances from carbon atoms that are α and β to cobalt show severe line broadening because of the electric quadrapole moment of cobalt.[40] Knowing this, there are two carbons that have a rather broad carbon resonance

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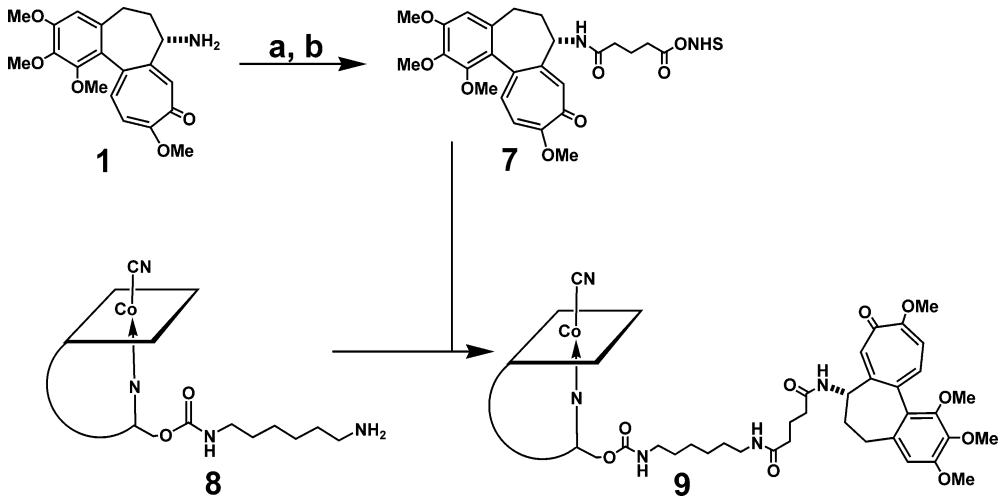
SCHEME 4. Synthesis of Stable Bioconjugate 9[a]



[a] (a) Glutaric anhydride in DMSO; (b) EDCI, NHS in DMSO (34% yield, 3 steps).

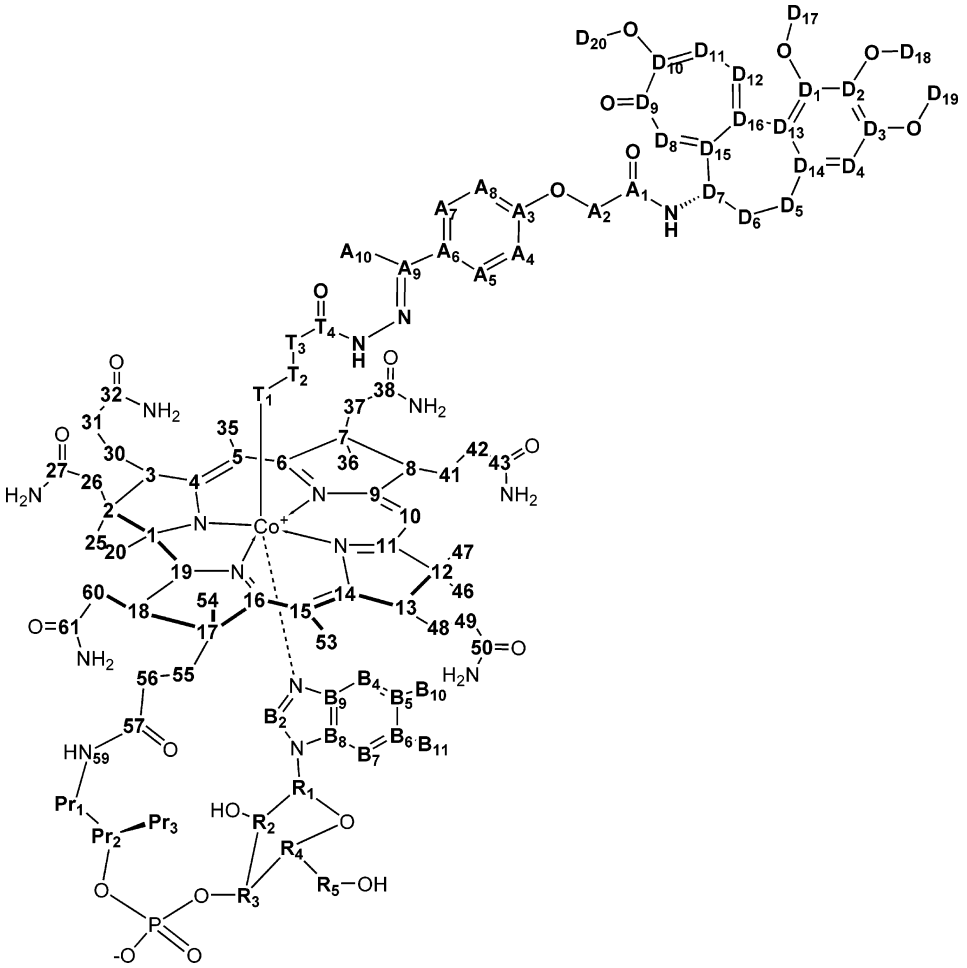


FIGURE 1. Numbering scheme of cobalamin bioconjugate **6**.

at 25.72 and 26.45 ppm. The HSQC correlations to these carbons are shifted downfield due to deshielding. The carbon at 25.72 ppm assigned to the α carbon (relative to cobalt, T1) has HSQC correlations to protons at 0.46 and 1.32 ppm, and the carbon assigned to the β position (T2) at 26.45 has protons associated at 0.39 and −0.12 ppm. These values are supported by their similarity to the published values for ^{13}C enriched ethylcobalamin, in which the α carbon is reported to be at 24.4 ppm and the associated protons are at 0.566 and 0.847 ppm. The β carbon is unidentified because of quadrapolar broadening, but the protons are assigned at −0.42 and 0.04 ppm.[40] The protons on the γ carbon (T3a and T3b) are then assigned on the basis of COSY correlations to these

TABLE 1. NMR Assignments for Bioconjugate 6 in DMSO-d_6

freq ppm	DEPT	carbon assign	gHSQC	gHMBC[a]	DQ-COSY	NOESY
177.95		D9		D11, D8		
175.38		C16		C53, C54, C55		
175.31		C4		C35		
174.00		*C50*[b]				
173.81		*C32*				
173.42		*C9*				
172.89		*C43*				
172.79		C11		C46, C47		
171.43		*C57*/*C61*				
171.33		*C27*				
170.64		*C38*				
168.29		T4		T4NH′		
167.15		A1		A2, D7NH		
167.02		A1′		A2′, D7NH		
163.62		C6		C35, C36, C37		
163.57		D10		D8, D11, D12, D20		
162.65		C14		C53		
158.58		A3		A2, A5/A7		
158.36		A3′		A2′, A5′/A7′		
152.96		D3		D4, D19		
150.43		D1		D4, D17		
150.29		D16		D7, D8, D11		
146.39		A9		A5/A7, A10, T4NH		
142.42	CH	B2	7.24	R1		
140.74		D2		D4, D18		
138.57		B9		B2, B7		
135.09		B9′		B2, B7		
134.49	CH	D12	7.03	D8, D11	D11	
134.49	CH	D11	7.11	D12	D12	D20
134.17		D14		D4, D5α, D6β		
131.23		A6		A4/A8, A10		
131.10		A6′		A4/A8, A10		
130.98		B5		B11		
130.80		B6		B10		
130.57	CH	D8	7.22			D7NH
129.59		B8		B2		
127.65	CH	A5/A7	7.65		A4/A8	A4/A8, A10
127.15	CH	A5′/A7′	7.51		A4′/A8′	A10′
125.36		D13		D5α, D5β, D4, D8, D12		
118.33	CH	B4	6.43			C20, C30b, C31a, C31b
118.33	CH	B4′	6.49			C30b, C31a, C31b, C41a
114.32	CH	A4′/A8′	6.93		A5′/A7′	A2/A2′, A5′/A7′, D17
112.18	CH	A4/A8	7.02		A5/A7	A2/A2′, A5/A7
112.18		D15		D8, D12		
110.47	CH	B7	7.21	B11		R1, R3, R4, B11
107.78	CH	D4	6.78	D5α, D5β		D5β, D19
104.60		C5		C35		
104.39		C5′		C35		
103.78		C15		C53		
103.66		C15′		C53		
93.82	CH	C10	6.12			C8, C37a, C46, C47
93.45	CH	C10′	6.12			C8′, C37a, C46, C47
85.06		C1		C20		
84.94	CH	R1	6.15		R2	B7, B11, C56b, N59, R2, R3, R4, R5
82.03	CH	R4	3.95		R3, R5	B7, C48a, N59, Pr2, R1, R3
74.13	CH	R3	4.50		R2, R4	R1, R2, R4, R5
74.13	CH	C19	4.06		C18	C18, C26a, C26b, C54, N63a, T1a, T2a
70.41	CH	Pr2	4.14	Pr3	Pr1a, Pr3	N59, Pr3, R4, R5
69.74	CH	R2	4.06		R1, R3, R7	C56b, R1, R3, R5
66.70	CH_2	A2	4.58			A4/A8, D7NH
66.59	CH_2	A2′	4.58			A4′/A8′, D7NH
61.60	CH_2	R5	3.53		R4, R8	B11, C53, Pr2, R1, R2, R3
60.80	CH_3	D17	3.52			D18, D20
60.67	CH_3	D18	3.78			D17
57.81		C17		C54		
56.04	CH_3	D20	3.87			A4′/A8′, C26b, D11
55.84	CH_3	D19	3.83			D4
54.85	CH	C3	4.23	C25	C30b	C25, C26a, C26b, C35
54.55	CH	C3′	4.35	C25	C30b	C25, C26′, C30′, C35′
53.63	CH	C8	3.76	C10, C36	C41a, C41b	C10, C35, C37a, C41a, C41b
53.63	CH	C8′	3.66	C10,C36	C41a,C41b	C10
52.24	CH	C13	3.16	C46, C47	C48a	C53
52.24	CH	C13′	3.08	C46′, C47	C48a	C46′, C47, C48a, C48b, C53

Table 1 (Continued)

freq ppm	DEPT	carbon assign	gHSQC	gHMBC[a]	DQ-COSY	NOESY
51.24	CH	D7	4.41	D6α, D5*β*, D8, D12	D6α	D6*β*, D7NH
49.11		C7		C36		
48.93		C7′		C36		
46.34		C12		C10, C46		
46.23		C12′		C10, C46′		
45.63		C2		C25		
45.24	CH2	Pr1	2.83a	Pr3	N59, Pr1b, Pr2	N59, Pr1b, Pr2, Pr3
			3.44b	Pr3	N59, Pr1a	N59, Pr1a, Pr3
42.57	CH2	C26	2.05a	C25		C18, C19, C25, C30a, N29a, N29b, N34a
			2.22b	C25		C3, C19
42.33	CH2	C26′	2.16	C25′		B4, B4′, C3, C3′, C31, N34b
41.95	CH_2	C37	1.71a	C36	C37b	C8, C10, C37b, T1b
			2.41b	C36	C37a	C37a, C41a
38.62	CH	C18	2.74	C54, C55a	C19, C60b	C19, C20, C25, C26b, C56a, C56b, N63b
35.46	CH_2	D6	2.01α	D5α, D5*β*, D7	D7	D5*β*, D7, D7NH
			2.26*β*	D5α, D5*β*, D7	D5α	D5α, D7, D7NH
33.54	CH_2	T3	1.85a		T2b	T3b, T4NH
			2.24b		T2a	T3a
32.47	CH_2	C31	1.73a	N34	C30a, C31b	B4, B4′
			2.20b	N34	C30a, C31a	C25, C26′
32.20	CH_2	C60	2.11a			
			2.47b		C18	C56a, T1a
31.78	CH_2	C49	1.77		C48a, C48b	C47
31.56	CH_2	C42	2.31a		C41a	C41b
			2.34b		C41a	C41b
31.34	CH_3	C46	1.01	C47		C10, C47
31.34	CH_3	C46′	0.97	C47		C10, C13′, C47, T1a
29.88	CH_2	C56	1.96a		C56b	C18, C54, C56b, C60, N59, N63a, N63b
			2.46b		C56a	C18, C54, R1, R2
29.14	CH_2	D5	2.61α	D4, D7	D5*β*, D6*β*	D6α
			2.23*β*	D4, D7	D5α	D4
28.96	CH_2	C55	1.18a	C54		
			1.23b	C54		
26.73	CH_2	C48	1.83a		C13, C13′	C13′, C47, N52b
			1.96b			C13′, C47, N52a, R4
26.45	CH_2	C41	1.07a		C8, C8′	B4′, C8, C8′, C37b, C41b
			1.77b		C8, C8′, C42a, C42b	C41a, C42
26.45	CH	T2	0.39a		T1a, T2b, T3a	C19
			−0.12b		T1a, T1b, T2a,T3a	C19
25.72	CH_2	C30	1.75		C3, C3′	C26a
			1.81		C31a, C31b	C3, C3′
25.72	CH_2	T1	0.46a		T1b, T2a, T2b	C19, C46, C46′, C60, T1b
			1.32b		T1a, T2a, T2b	T1a, C37a
21.49	CH_3	C20	0.45			B4′, C18, C25′
21.27	CH_3	C20′	0.45			
20.76	CH_3	C47	1.38	C46		C10, C13, C46, C48a, C48b, C49
20.67	CH_3	C47′	1.38	C46′		C10, C13′, C46′, C48a, C48b, C49
20.12	CH_3	Pr3	1.06		Pr2	N59, Pr1a, Pr1b, Pr2
19.90	CH_3	B10	2.27			
19.79	CH_3	B11	2.19	B7		B7, R1, R5
18.99	CH_3	C36	1.68			N40a, N40b
18.81	CH_3	C36′	1.68			
17.78	CH_3	C25	1.28			C3, C18, C20, C26a, C31a, N29a, N29b
17.61	CH_3	C25′	1.23			C3′, C18, C20, C26a, C31a, N29a, N29b
16.60	CH_3	C54	1.25			C19, C19, C53, C56a, C56b, N63b
15.79	CH_3	C35	2.41			C3, C3′, C8, N40a, N40b
15.57	CH_3	C35′	2.41			C3, C3′, C8, N40a, N40b
15.46	CH_3	C53	2.25			C13, C13′, C54, N52a, R5
13.84	CH_3	A10′	2.08			A5′/A7′, T4NH′
13.20	CH_3	A10	2.06			A5/A7, T4NH

[a] This is the proton correlation. [b] Italics indicate an assignment that is made by reference to previous structures because of a lack of HMBC correlations.

β protons, and the corresponding *γ* carbon (T3) is assigned from HSQC correlations. The hydrazide proton is determined on the basis of a NOESY correlation observed between T3a and a proton at 9.94 ppm (assigned T4NH′) that is consistent with a hydrazide proton. The two isomers of the hydrazone are shown in Figure 2 with the corresponding correlations depicted to clarify the assignments made. The T4NH′ proton also has a weak HMBC correlation to T4, further confirming this assignment. This hydrazide proton at T4NH′ also has a NOESY correlation to the A10′ methyl that serves as a bridge to correlate the alkyl chain (T1−T4) to the acetophenone portion of **2**.

As expected from two isomers, there are two distinct A10 carbons (13.84 and 13.20 ppm) and corresponding *δ* ^{1}H of 2.08 and 2.06 ppm. A very strong NOESY correla-

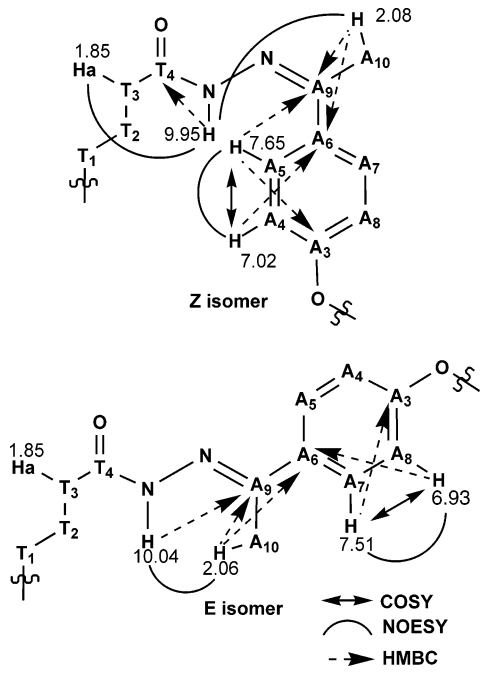


FIGURE 2. Two isomers of the hydrazone of bioconjugate **6** and 2D NMR correlations of each isomer. The proton resonances are listed for key protons.

tion between a proton at 10.04 ppm (assigned T4NH) and 2.06 ppm indicates that this is the *E* isomer. The *Z* isomer has a much weaker NOESY correlation between T4NH′ (9.95 ppm) and A10′ (2.08 ppm). Interestingly, the *E* isomer shows no NOESY correlation between the T4NH and the T3a proton, but there is a weak HMBC correlation to A9. The remainder of the acetophenone proton (A3−A10) is assigned via HMBC and COSY correlations.

Carbon A2 has two resonances assigned to carbons 66.70 ppm (A2) and 66.59 ppm (A2′), yet HMBC correlations for each carbon have protons at 4.78 ppm. NOESY correlations between A2/A2′, A4/A8, and A4′/A8′ as well as a NOESY correlation to a proton at 8.95 (assigned D7NH) aid in the assignment of this portion of the molecule. An HMBC correlation between A2/A2′ and A3 as well as to A1 confirms these assignments. The D7NH proton is then correlated to the D7 proton via a NOESY interaction. Using a combination of COSY and NOESY correlations, the relative stereochemistry of the prochiral D6 and D5 protons are assigned on the basis of the Karplus relationship, and the corresponding carbons are assigned from the HSQC correlations. The stereochemistry of these protons is assigned on the basis of a NOESY correlation between the D4 proton (6.78 ppm) and the 2.61 ppm resonance (assigned D5α). As a result of the geometry of the ring system, only the α-proton is sufficiently close to show a NOESY correlation based on computer modeling. The rest of the colchicine portion of the molecule is assigned using HMBC and HSQC correlations. To distinguish D1−D3, which are in nearly equivalent environments, first a NOESY correlation between D4 and a large singlet peak at 3.83 ppm in the ^{1}H spectra (assigned D19) is identified. Because there are four large singlet absorption peaks in the ^{1}H spectra that correspond to the four methoxy groups of colchicine, these are used to distinguish the corresponding quaternary carbons, as they each show one HMBC correlation. An HMBC correlation from the D19 proton to a quaternary carbon at 152.96 is then used to assign D3. Two strong HMBC correlations from the D4 proton to two quaternary carbons at 150.29 and 140.74 ppm are used to identify D13 and D2. An HMBC correlation from one of the methoxy singlets at 3.78 ppm to the same carbon at 140.74 ppm confirmed this carbon as D2, and by default the carbon at 150.29 ppm is D13. An HMBC correlation to D13 from a proton at 7.03 ppm, which has an HSQC correlation to a carbon at 134.46 ppm is then assigned D12. This D12 proton also has an HMBC correlation to a carbon at 163.57 ppm that is assigned D10. An HMBC correlation from 3.87 ppm to this carbon is used to identify the proton at D20, and HSQC identifies the D20 carbon at 56.04 ppm. The only remaining methoxy proton at 3.52 ppm is then assigned to D17 by default. This proton shows an HMBC correlation to the resonance at 150.43 ppm that is assigned D10.

TABLE 2. Calculated Half-Life of Bioconjugate 6 in Various Buffers

buffer	half-life
pH 4.5 NaOAc	138 min
pH 5.5 succinic acid buffer	13 h
pH 7.0 $(H_3N)_2HPO_4$ buffer	9.4 d
MEM buffer (10% FBS)	>7 d[a]
Dulbecco-MEM buffer (10% FBS)	>7 d[a]

[a] Monitoring stopped after 10 days as a result of bacterial growth.

The rest of the core ring structure and side chains of cobalamin was assigned as reported previously.[34] One interesting observation of note was that there are several NOESY correlations from T1a to unique sites on the corrin ring. NOESY correlations between T1a to C60b as well as T1a to C19 and even T1a to C46 suggest that the Co−C bond does not freely rotate on axis and the upper ligand has constrained movement placing the T1a C−H bond pointing toward the southern portion of the molecule between the *e* and *f* side chains. There may be additional NOESY correlations to T1b as well as T2a and T2b to sites on the corrin ring, but they are indistinguishable because of the large number of protons in the region between 1 and 2 ppm.

Serum Stability Studies. Bioconjugate **6** was tested for stability in a variety of buffers, and the results were fitted to first-order decay kinetics (data not shown) to calculate the half-lives (Table 2). As expected, the hydrazone undergoes clean hydrolysis with a short half-life at pH 4.5 of under 2.5 h. The half-life increases to 13 h at pH 5.5 and to over 9 days at pH 7.0. The bioconjugate was further tested for stability in the same cell media used in the cell viability assays in the presence of 10% heat-inactivated fetal bovine serum (FBS), and bioconjugate **6** had a half-life of >7 days in both media.

Cell Viability Results. The data and results that follow are divided into two groups in which either the CellTiter-Glo Luminescent Cell Viability Assay (Luc. Assay) or the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT Assay) was used to quantify cell viability. The cell viability versus concentration was plotted on a log scale, and the LC_{50} values were estimated from the graph and tabulated in Table 3.

The dose response curve for **2** and **6** using either cell viability assay was nearly identical to that of colchicine

TABLE 3. LC_{50} Values for Cancer Cell Lines Treated with Colchicine, Colchicine Analogues 2, and Two Colchicine−Cobalamin Bioconjugates 6 and 9

	Luc. assay			MTT assay		
	SK-BR-3[b]	SK-N-MC	B16-F1	SK-BR-3	SK-N-MC	B16-F1
colchicine[a]	16	31	65	6	19	67
2	64	30		11	17	80
6	99	43		60	23	302
9	20 000	76 000	90 000	18 000	61 000	66 000

[a] Average of 3 independent cell viability experiments [b] SK-BR-3 is a human breast adenocarcinoma cell line; SK-N-MC is a human neuroblastoma cell line; and B16-F1 is a mouse melanoma cell line.

in SK-N-MC neuroblastoma cells. For all agents, concentrations of 0.001 and 0.01 μM drug had no cytotoxic effect, whereas concentrations of 0.1 μM and higher resulted in a substantial decrease in SK-N-MC cell viability. In SK-BR-3 human breast adenocarcinoma cells, concentrations above 0.1 μM produced a significant decrease in viability. For this cell line, colchicine appears to be more toxic than **2** and **6**, showing a 4- and 6-fold decrease in toxicity, respectively, for the Luc. Assay. The MTT assay had a similar trend, but bioconjugate **6** showed a 10-fold decrease in toxicity relative to colchicine. In the final cell line tested, B16−F1 cells, analogue **2** had a slight decrease in toxicity, but **6** had a 5-fold decrease in toxicity. For all cell lines tested, a dramatic decrease in toxicity was observed for the stable bioconjugate **9**, in most cases greater than a three log decrease, thereby confirming the expectation that the drug does need to be released from cobalamin for efficacy.

Conclusions

Colchicine was functionalized at the C7 position with a *p*-alkoxyacetophenone linker to produce analogue **2**. Hydroxycobalamin was functionalized with a small hydrazide tether, and the two analogues were combined to form the hydrazone-containing cobalamin bioconjugate **6**. The bioconjugate showed excellent stability in cell media and in neutral pH buffer, while undergoing rapid and clean hydrolysis at slightly acidic pH as anticipated. The acid-labile hydrazone is expected to be hydrolyzed within lysosomes inside the cells and thereby release active drug. In vitro data show that colchicine analogue **2** exhibits only a minimal loss in toxicity compared to authentic colchicine, and the colchicine−cobalamin bioconjugate **6** shows a 10-fold maximum decrease in toxicity in the three cancer cell lines tested. The inherent advantage of this bioconjugate is to target cancer cells over healthy cells to decrease the side effects of current nonselective chemotherapeutic drugs. The cytotoxicity of this bioconjugate in vitro is comparable to current chemotherapeutic drugs such as paclitaxel and docetaxel, yet the bioconjugate is highly water-soluble and significantly less expensive (less than $50 per gram) than either paclitaxel or docetaxel. The stable control bioconjugate **9** shows a dramatic 3-log decrease in cytotoxicity, indicating that the release of colchicine from cobalamin is necessary to cause cell death. This decrease in toxicity also suggests that the acid-labile hydrazone undergoes hydrolysis within the cell, as indicated by the nM cytotoxicity of **6**, which was not seen in the stable cobalamin bioconjugate **9**.

Experimental Section

Materials. All chemicals were purchased from commercial suppliers and used without further purification. Mass spectrometric analysis of the analogues was carried out using an electrospray mass spectrometer in positive ion mode.

Standard NMR Conditions. All NMR samples were dissolved in 250−300 μL of DMSO to a final concentration of ~30 mmol in a 5-mm Shigemi NMR tube, and all NMR spectra were collected at 500 MHz unless otherwise noted. ^{13}C NMR data were collected using a 5-mm switchable broadband probe, and all ^{1}H and 2-D NMR data sets were collected using a 5-mm indirect pulse field gradient probe.

Standard HPLC Conditions. Analytical runs were performed using a C18 15 μm, 100 Å, 3.9 mm × 300 mm column with detection at 254 nm. Preparatory runs were carried out using a C18 15 μm, 100 Å, 25.0 × 200 mm column, monitored at 254 nm. The buffers used in all chromatography runs were as follows: Buffer A, 0.05 M phosphoric acid, pH = 3.0 (or pH = 7.0 for compound **6** only) with NH_4OH. Buffer B, 9:1 acetonitrile/ddH_2O.

Analytical HPLC Method. A flow rate of 2.0 mL/min was used; 0−2 min, with isocratic elution of 95:5 A:B; 2−10 min, linear gradient to 70:30 A:B; at 10−15 min, linear gradient to 0:100 A:B; isocratic elution of 0:100 A:B; and 18−27 min, linear gradient to 95:5 A:B.

Semipreparative HPLC Method. A flow rate of 40 mL/min was used; 0−2.7 min, isocratic elution of 95:5 A:B; 2.7−13.7 min, linear gradient to 70:30 A:B; 13.7−20.5 min, linear gradient to 5:95 A:B; 20.5−21.2 min, linear gradient to 0:100 A:B; 21.2−24.7 min, isocratic elution of 0:100 A:B; and at 24.7−37 min, linear gradient to 95:5 A:B.

General Desalting Procedure. All cobalamins were desalted using a C18 column by first rinsing the column with two column volumes of methanol followed by three column volumes of ddH_2O. The cobalamin analogue was applied to the column and rinsed with three column volumes of ddH_2O followed by elution with CH_3OH. The CH_3OH was removed via rotary evaporation, and the solid was dried over P_2O_5 at 50−100 mTorr.

Cell Culture. All cell lines were obtained from ATCC and incubated at 37° C with 5% CO_2. SK-N-MC cells were maintained in Minimum Essential Medium (MEM) with Earle's salts, with L-glutamine. SK-BR-3 and B16-F1 cells were maintained in Dulbecco's Modified Eagle Medium (D-MEM) with GlutaMAX, with high glucose, with pyridoxine hydrochloride, without sodium pyruvate. All media were supplemented with 10% heat-inactivated defined fetal bovine serum, penicillin-streptomycin to a final concentration of 10 units penicillin and 10 μg of streptomycin per mL and an additional 2 mM L-glutamine. For the viability assays, cells were plated in 96-well plates in 100 μL of growth media. Initial cell density varied from 1000 to 20000 cells/well depending on cell type and assay.

Determination of Bioconjugate Cytotoxicity. The effect of colchicine and the cobalamin−colchicine bioconjugates on cell viability was determined using two commercially available assays. The CellTiter-Glo luminescent cell viability assay (Luc. Assay) quantifies the ATP present in cells, which correlates with metabolically active cells. The CellTiter 96 non-radioactive cell proliferation assay (MTT Assay) is based on the reduction of a blue tetrazolium salt into a purple formazan product by living cells. These two assays measure different cellular activities and thus allow for independent confirmation of the effects on viability obtained in each assay.

Stock solutions of colchicine and compounds **2**, **6**, and **9** were prepared in 5% DMSO, stored at −20 °C, and protected from light. Each compound was tested at six concentrations covering 10-fold dilutions from 100 μM to 10 nM, except **6** for which

100 μM was not tested. Ten-fold stock solutions were prepared and 10 μL was added to each of three wells containing cells. The compounds were added 24 h after the cells were plated. Untreated control cells received 10 μL of 5% DMSO. Three wells containing media alone were included to determine background absorbance. The plates were incubated at 37 °C with 5% CO_2. The incubation times for the different cell lines were 48 h for SK-N-MC cells, 96 h for SK-BR-3, and either 72 or 96 h for B16-F1 cells.

At the end of incubation, cell viability was quantified as described. The CellTiter-Glo luminescent cell viability assay was carried out according to the manufacturers protocol. Luminescence was recorded over an integration time of 0.1 s. The CellTiter 96 non-radioactive cell proliferation assay protocol was used with the following changes: Incubation with the dye solution was reduced to 1 h at 37 °C. Following addition of the solubilization/stop solution, the plate was incubated at 37° C for 30 min. The contents of the wells were mixed, and the plate was returned to 37° C for 1 h to ensure complete solubilization of the formazan crystals. Absorbance of the solutions at 570 and 650 nm was measured, with 650 nm used as the reference wavelength.

Calculations and Data Representation. Data from both assays were processed in the same manner. The average value, either luminescence or absorbance, of the three wells containing media alone was subtracted from the raw data values to give corrected values. Measured absorbance values were to correct for the background absorbance imparted by the red color of high concentrations of **9** by subtraction of the background value for cells treated with 100 μM **9** or the luminescence of media + 100 μM **9**, instead of just medium alone. The three corrected values for wells dosed with the same concentration of compound were averaged and the standard deviation was calculated. These data were plotted as average absorbance (av abs ± SD) or average luminescence (av lum ± SD) versus concentration of compound (data not shown).

Percent cell viability was also calculated by considering the values from untreated cells as representing 100% viability. The corrected absorbance or luminescence for each value was divided by the average corrected absorbance or luminescence of untreated cells and multiplied by 100 to give the percent cell viability. Average percent cell viability of the triplicate wells and the standard deviation were calculated. These data were plotted as average percent cell viability (av % viability ± SD) versus concentration of compound (data in Supporting Information).

Synthesis. *N*-Deacetylcolchicine (1). Colchicine (515.8 mg, 1.29 mmol) was dissolved in 5 mL of acetonitrile. To this were added 4-(dimethylamino)pyridine (157.5 mg, 1.29 mmol), triethylamine (350 μL, 2.52 mmol), and di-*tert*-butyl dicarbonate (678 mg, 3.11 mmol). The reaction flask was attached to a reflux condenser and placed into an oil bath at 100 °C. The reaction was monitored over time via HPLC (product $t_R = 16.7$ min). After 1 h the reaction stalled at 70% conversion, so additional di-*tert*-butyl dicarbonate (610 mg, 2.79 mmol) was added. The liquid chromatogram showed only one new peak at 16.7 min, with colchicine having a retention time of 14.0 min. The reaction was complete in 3 h. The reaction was quenched by the addition of 35 mL of $CHCl_3$ and was washed with 3 × 50 mL of saturated aqueous citric acid. The combined aqueous layers were back extracted with 30 mL of $CHCl_3$, and the organic layers combined. The organic layer was washed with 30 mL of saturated brine and then concentrated to a reddish brown solid. The crude compound was >95% pure via HPLC, and the only impurity was unreacted colchicine. The crude sample was carried on through the next step.

The crude *N*-Boc-colchicine was dissolved in 1 mL of CH_3OH. To this was added 2 M $NaOCH_3$ in CH_3OH (130 μL). The solution was stirred for 30 min at room temperature and monitored via HPLC (product $t_R = 16.1$ min). After 30 min the solution was transferred to 40 mL of brine, and the product was extracted with 3 × 30 mL of diethyl ether. The product was dried over Na_2SO_4, filtered, and concentrated to a crude solid (~90% purity via HPLC). The only other peak evident by HPLC was unreacted colchicine, and the crude sample was carried through the next reaction.

The crude *N*,*N*′-Boc-deacetylcolchicine was dissolved in 1 mL of neat trifluoroacetic acid (TFA) and stirred for 5 min. The TFA was removed via rotary evaporation to yield a crude sample of the desired product (>87% pure via HPLC, $t_R = 12.5$ min). Again, the only detected impurity was unreacted colchicine. The product was purified at this point by extraction of *N*-deacetylcolchicine into saturated aqueous citric acid, allowing for the isolation of the unreacted colchicine. Pure *N*-deacetylcolchicine was isolated by adjusting the aqueous layer pH to 10 with 1 M NaOH and extracting with several portions of CH_2Cl_2, resulting in a product with >95% purity (542 mg, 85% yield). ES^+ calcd for $C_{20}H_{24}NO_5$ ($M + H^+$) 358.17, found 358.2 and for $C_{20}H_{23}NO_5Na$ ($M + Na^+$) 380.17, found 380.2.

2-(4-Acetylphenoxy)-*N*-acetamidocolchicine (2). *N*-Deacetylcolchicine **1** (504.6 mg, 1.1 mmol) was dissolved in 20 mL of $CHCl_3$. To this was added 4-methylmorpholine (400 L, 3.6 mmol) followed by addition of acetylphenoxyacetic acid (242 mg, 1.2 mmol) and BOP (580 mg, 3.0 mol). The reaction was complete after 90 min (product $t_R = 15.4$ min). The reaction was diluted with 10 mL of $CHCl_3$ and washed with 3 × 35 mL of saturated citric acid. The combined aqueous layers were back extracted with 2 × 15 mL of $CHCl_3$. The combined organic layers were washed with 2 × 40 mL of saturated $NaHCO_3$ and 30 mL of saturated brine. The crude sample was evaporated to a brown oil via rotary evaporation and dried to solid **2** with P_2O_5 under high vacuum (540 mg, 84% yield): mp 115 °C dec; ^{1}H NMR (500 MHz, DMSO) δ 8.90 (d, $J = 7.6$ Hz, 1H), 7.92 (d, $J = 8.6$ Hz, 1H), 7.23 (s, 1H), 7.12 (d, $J = 10.7$ Hz, 1H), 7.00−7.06 (m, 3H), 6.78(s, 1H), 4.68 (s, 2H), 4.36−4.44 (m, 1H), 3.87 (s, 3H), 3.83 (s, 3H), 3.78 (s, 3H), 3.52 (s, 3H), 2.60 (dd, $J = 5.8$, 13.2 Hz, 1 H), 2.51 (s, 3H), 2.21−2.30 (m, 1H), 1.95−2.10 (m, 2H); ^{13}C NMR (125 MHz, DMSO) δ 197.25, 177.91, 166.66, 163.56, 161.53, 152.95, 150.42, 150.15, 140.75, 135.02, 134.45, 134.12, 130.54, 130.36, 130.30, 125.34, 114.41, 112.11, 107.75, 66.58, 60.78, 60.65, 56.00, 55.82, 55.24, 35.45, 29.14, 26.35; ES^+ calcd for $C_{30}H_{32}NO_8$ ($M + H^+$) 534.20, found 534.2 and for $C_{30}H_{31}NO_8Na$ ($M + Na^+$) 556.20, found 556.2.

***N*-(4-Chloro-butanoyl)-hydrazinecarboxylic Acid *tert*-Butyl Ester (3).** In a 50 mL flask, *tert*-butyl-carbazate (2.5 g, 18.9 mmol) was dissolved in 20 mL of dry CH_2Cl_2, and 4-methylmorpholine was added (2.1 mL, 22.5 mmol). To the stirring solution at 0 °C was added 4-chlorobutylacid chloride (2.1 mL, 11.8 mmol) dropwise. The reaction was checked after 10 min via TLC (1:1 EtOAc/hexanes and visualized with ninhydrin stain). The reaction was worked up upon completion of the reaction ($R_f = 0.29$). The product was extracted with 40 mL of CH_2Cl_2 and was washed with 3 × 50 mL of 50% citric acid solution. The organic layer was dried over $MgSO_4$ and filtered, and the solvent was removed under vacuum. The crude oil was crystallized from hot EtOAc and hexanes yielding small needle crystals (2.68 g, 61% yield): mp 65 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.65 (br s, 1 H), 6.65 (br s, 1H), 3.62 (t, $J = 6.0$ Hz, 2H), 2.42 (t, $J = 7.0$ Hz, 2H), 2.15 (p, $J = 6.6$ Hz, 2H), 1.44 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 155.6, 82.0, 44.1, 30.6, 28.1, 27.7.

Cobalamin Hydrazide (5). Hydroxocobalamin (990 mg, 0.71 mmol) was placed into a 25-mL round-bottom flask and dissolved in 8 mL of ddH_2O. To this was added NH_4Cl (414 mg, 7.7 mmol). In a separate 50-mL round-bottom flask excess zinc dust was added (467 mg, 7.1 mmol). In a 10-mL round-bottom flask, **3** (344 mg, 1.5 mmol) was dissolved in 1 mL of MeOH. The three flasks were purged in series with nitrogen for 30 min. The dissolved hydroxocobalamin solution was transferred via cannula to the flask with zinc dust and allowed to react for 20 min to form reactive Co(I). The solution of **3** was then transferred via cannula to the Co(I) reaction and monitored via HPLC. Hydroxocobalamin has a retention time

of 8.3 min, and the product has a retention time of 12.9 min. After 30 min the reaction was complete and was worked up.

The excess zinc was filtered off using a Buchner funnel, and then the reaction was desalted on a 1 g C18 column as above to obtain a red solid **4** at >98% purity by HPLC. The Boc group was removed via treatment with 1 mL of neat TFA for 10 min. The reaction mixture was added to 100 mL of 2:1 Et_2O/CH_2Cl_2 and a red precipitate was filtered and triturated with 2 × 10 mL CH_2Cl_2 and 3 × 10 mL Et_2O. The red solid **5** was checked via HPLC, and one peak was evident at 10.8 min at >97% purity (1.05 g, 95% yield). ES^+ calcd for $C_{66}H_{98}CoN_{15}O_{15}P$ ($M + H^+$) 1430.64, found 1430.6 and for $C_{68}H_{97}CoN_{15}O_{15}PNa$ ($M + Na^+$)1452.64, found 1452.7.

Cobalamin Bioconjugate (6). Cobalamin hydrazide **5** (176 mg, 0.11 mmol) was dissolved in 2 mL of MeOH. To this was added colchicine analogue **2** (60 mg, 0.11 mmol). The solution was stirred for 12 h at room temperature and monitored via HPLC (product t_R = 14.1 min). The sample was then transferred to 60 mL of a 2:1 mixture of Et_2O/CH_2Cl_2, resulting in the precipitation of the product. The crude sample was filtered using a medium glass fritted filter and triturated with 3 × 15 mL of Et_2O, yielding a sample of bioconjugate **6** at >75% purity. The sample was purified via preparatory HPLC, and pure fractions were desalted as above to yield pure bioconjugate **6** (88.8 mg, 40% isolated yield). ES^+ calcd for $C_{96}H_{127}CoN_{16}O_{22}P$ ($M + H^+$) 1945.83, found 1945.2 and for $C_{96}H_{126}CoN_{16}O_{22}PNa$ ($M + Na^+$) 1967.82, found 1967.2.

***N*,*N*′-Deacetyl-colchicine-hemiglutaramide-NHS (7).** *N*-Deacetylcolchicine (105 mg, 0.22 mmol) was dissolved in 500 μL DMSO. To this was added excess 4-methyl morpholine followed by glutaric anhydride (26 mg, 0.23 mmol). The reaction was complete in 15 min (as monitored by HPLC, product t_R = 14.1 min). To the stirring reaction was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (69 mg, 0.36 mmol) and *N*-hydroxysuccinimide (29 mg, 0.25 mmol), and the reaction was complete in 24 h (product t_R = 14.8 min). The reaction mixture was diluted with 30 mL of $CHCl_3$ and washed with 3 × 30 mL of saturated aqueous $NaHCO_3$ followed by 30 mL of saturated brine. The solvent was removed via rotary evaporation to yield crude product that was used without further purification (100 mg).

CNCbl-5′-[(6-Amino)-hexylcarbamate] (8). Cobalamin **8** was prepared as reported previously.[34] In brief, CNCbl was dissolved in DMSO at 30 °C followed by addition of carbonylditriazole (CDT). After 15 min the reaction was quenched by addition to Et_2O/EtOAc and filtration to removed excess CDT. The compound is redissolved in DMSO, and excess 1,6-diaminohexane was added. The progress of the reaction was monitored via HPLC (t_R = 12.7 min) and was completed in 15 min. ES^+ calcd for $C_{70}H_{103}CoN_{16}O_{15}P$ ($M + H^+$) 1497.68, found 1497.5 and for $C_{70}H_{102}CoN_{16}O_{15}PNa$ ($M+Na^+$) 1519.68, found 1519.6.

Cobalamin Bioconjugate (9). Cobalamin **8** (54 mg, 36.0 mol) was dissolved in 1.0 mL of DMSO. To this was added the crude compound **7** (50 mg, 88.0 mol). The reaction was monitored via HPLC, and a new peak was evident after 20 min (t_R = 13.7 min). The reaction was complete in 1 h and was transferred to 60 mL of 2:1 $Et_2O.CH_2Cl_2$. The resulting red precipitate was filtered using a medium frit and washed with 3 × 5 mL of Et_2O. The sample was purified via preparatory HPLC, and pure fractions were desalted as above to yield pure **9** (23 mg, 34% isolated yield). ES^+ calcd for $C_{95}H_{130}CoN_{17}O_{22}P$ ($M + H^+$) 1950.9, found 1950.7 and for $C_{95}H_{129}CoN_{17}O_{22}PNa$ ($M + Na^+$) 1972.9, found 1972.6.

Stability Studies. Bioconjugate **6** (2.0 mg, 1.0 mol) was dissolved in 100 μL of DMSO followed by dilution with 5.0 mL of the corresponding buffer (50 mM). Each sample was monitored over time for a least three half-lives via HPLC. All data were fitted to a first-order rate equation, and the calculated half-lives are tabulated in Table 2.

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Supporting Information Available: Cell viabilty versus concentration plots in three cell lines, as well as NMR spectra of compounds **2**, **3**, **5**, and **6** and mass spectra of compounds **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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