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Journal of Organometallic Chemistry 689 (2004) 4803–4810
Journal of Organometallic Chemistry
www.elsevier.com/locate/jorganchem

Conjugates of vitamin B12 with N_ε-functionalized histidine for labeling with $[^{99m}Tc(OH_2)_3(CO)_3]^+$: synthesis and biodistribution studies in tumor bearing mice

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Received 6 September 2004; accepted 21 September 2004
Available online 28 October 2004

Abstract

In this work, histidine derivatives bearing an acetic acid or a propyl amine substituent on the N_ε-atom are conjugated to the *b*-acid, *c*-acid and *d*-acid moiety of cyanocobalamin (vitamin B12) via amide formation. Four different derivatives were prepared with different sites of conjugation (*b*-, *c*- or *d*-acid) and different spacer lengths between histidine and the acid moiety. These conjugates can be efficiently labeled with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ at yields higher than 95% under mild conditions (50 °C, 30 min, 10^{-4} M). The biodistribution of the $^{99m}Tc(CO)_3$ labeled conjugates is determined in mice bearing B16-F10 melanoma tumors. The organ distribution varies significantly for each of the derivatives with the percentage injected dose per gram of tumor tissue ranging from 4.4 ± 0.9 to 9.2 ± 2.0.

Keywords: Technetium; Radiopharmacy; Vitamin B12; Biodistribution; Histidine; Bioorganometallic chemistry

1. Introduction

The labeling of biologically active molecules with ^{99m}Tc is a field of intense research [1–4], as ^{99m}Tc is one of the most widely employed isotopes for imaging in nuclear medicine. This is due to the favorable properties of this isotope: the emission of a 140 keV γ-ray with an abundance of 89% and a half-life of 6.02 h. Beside the usual precursors which are based on the $[Tc{=}O]^{3+}$ moiety, the organometallic complex $[^{99m}Tc(OH_2)_3(CO)_3]^+$ has recently attracted much attention [5]. Several reasons account for this interest: – the robustness of complexes with the $[^{99m}Tc(CO)_3]^+$ core – its high affinity for a large variety of donor atoms and – the convenient preparation of $[^{99m}Tc(H_2O)_3(CO)_3]^+$. In fact, GMP-compliant kits for the preparation of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ are now commercially available (Isolink® Tyco–Mallinckrodt Med. B.V.). To avoid dissociation of the metal ion from the bioconjugate in biological systems, the ligand covalently attached to the biomolecule should bind as tight as possible to the "*fac*-$[^{99m}Tc(CO)_3]^+$" moiety. On the basis of thermodynamic considerations a tridentate ligand is therefore preferred.

One of the most efficient tridentate ligands for conjugation to biomolecules and labeling with $[^{99m}Tc(H_2O)_3$

Abbreviations: BOP, (Benzotriazol-1-yl-oxy)tris(dimethylamino)-phosphonium hexafluorophosphate; DIPEA, diisopropyl ethyl amine; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBT, *N*-hydroxybenzotriazole; TBTU, 2-(1*H*-Benzotriazol-1-yl)-1,-1,3,3-tetramethyluronium tetrafluoroborate.

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doi:10.1016/j.jorganchem.2004.09.050

$(CO)_3]^+$ is histidine [6–8] since the stability of the complex unit $Tc(His)(CO)_3$ is high and its formation can be accomplished in high yield under mild conditions. As a mono-anionic ligand, histidine forms a neutral complex, and thus, no additional charge is introduced on the biomolecule. Furthermore, the $Tc(His)(CO)_3$ moiety is rather hydrophilic and has a small size. Consequently, polarity and steric properties of the resulting biomolecule are not influenced to a large extent. Another virtue of histidine is its chirality, which implies that the formed $Tc(His\text{-}N_\varepsilon\text{-}R)(CO)_3$ complex moiety will be enantiomerically pure if enantiomerically pure histidine is used. In contrast, other favorable mono-anionic ligands with a NNO donor set such as ([pyridine-2-yl-methyl)-amino]-acetic acid, will always lead to a racemic complex [9]. In combination with chiral biomolecules, mixtures of diastereomers will result, which should be avoided for pharmaceutical applications in particular.

Very recently our group reported the synthesis of histidine derivatives with an acetic acid and a propyl amine chain attached to N_ε [10,11]. Herein, we employ this pair of complementary derivatives for conjugation with modified cyanocobalamin (vitamin B12) using standard peptide chemistry techniques. The obtained conjugates could be efficiently labeled with $[^{99m}Tc(H_2O)_3(CO)_3]^+$ under mild conditions. Because of the potential application of cobalamin derivatives for tumor targeting, we have undertaken a biodistribution study of the $^{99m}Tc(CO)_3$ conjugates in mice bearing B16-F10 melanoma.

2. Results and discussion

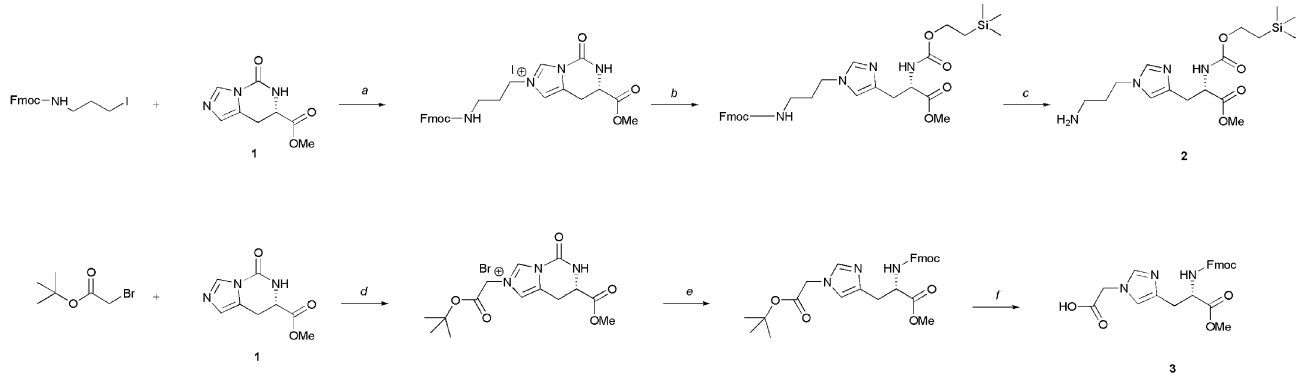
2.1. Synthesis of the cyanocobalamin conjugates

For coupling with cyanocobalamin we selected a pair of complementary histidine derivatives, having a propyl amine chain (**2**) or an acetic acid functionality (**3**) on the N_ε (see Scheme 1). These derivatives were prepared via an organic chemical synthetic sequence starting from protected histidine **1**, as reported very recently in our group (see Scheme 1) [10,11].

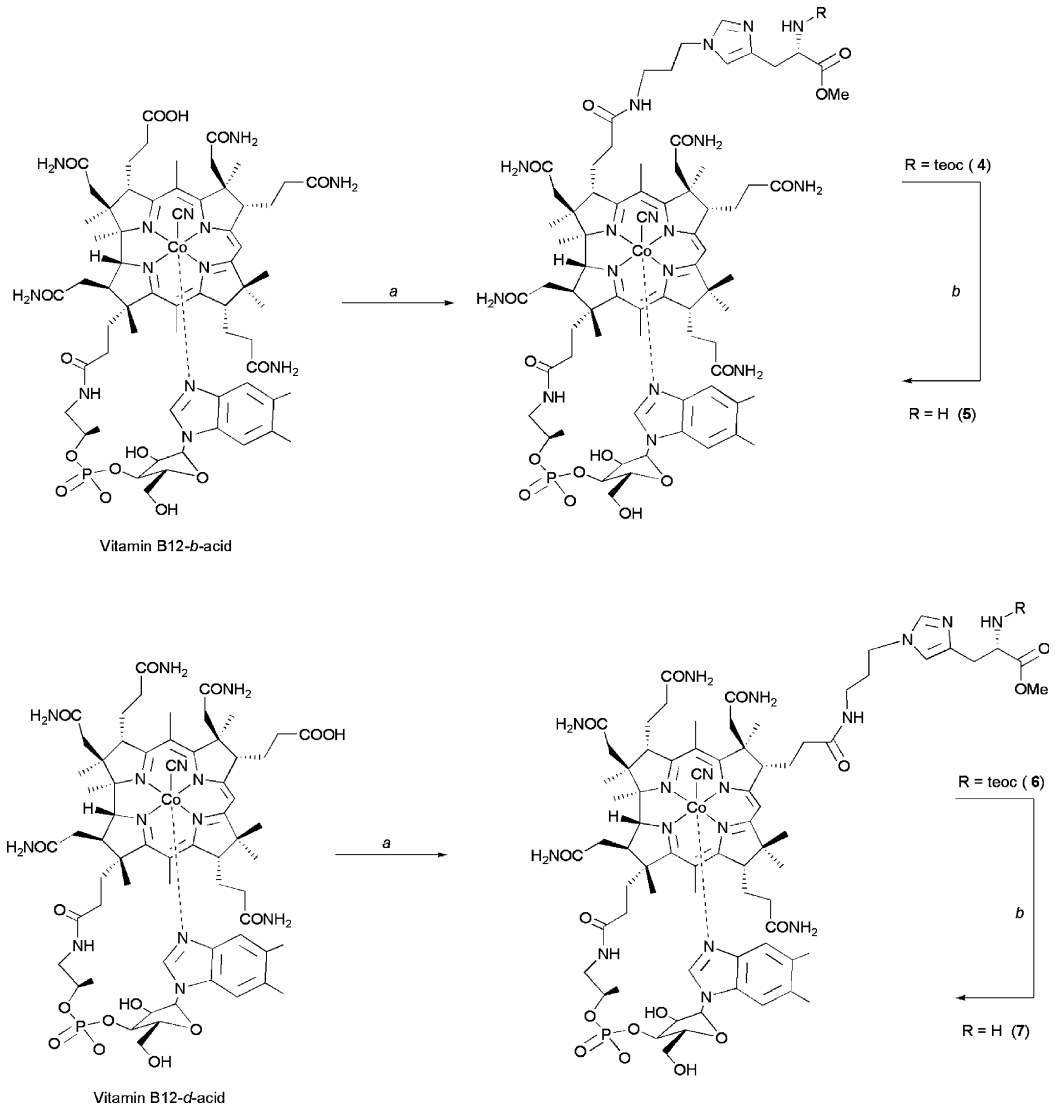
Coupling of functionalized histidine derivatives to cyanocobalamin requires a reactive group on B12. Controlled acid hydrolysis of the amide side chains in cyanocobalamin in 1 M HCl yields a mixture of acids, with the *b* and the *d* acids making up the largest portion [12–14]. These can be obtained in pure form via an elaborate purification method, involving ion-exchange chromatography and preparative HPLC. Derivatisation at the *c*-acid position is possible by formation of the *c*-lactone, followed by a ring opening reaction with a diamine [12]. For our labeling and biodistribution study, we selected and prepared in total four cyanocobalamin conjugates of **2** and **3** (see Schemes 2 and 3).

The synthesis of the cyanocobalamin conjugates with **2** was accomplished as shown in Scheme 2. Reaction of **2** with vitamin B12-*b*-acid or vitamin B12-*d*-acid in DMF in the presence of the coupling reagent 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) afforded the conjugates **4** and **6**, respectively, in pure form after preparative HPLC purification. In order to make the histidine accessible for labeling with $[^{99m}Tc(OH_2)_3(CO)_3]^+$, it was necessary to remove the trimethylsilylethoxycarbonyl (teoc) group. This was achieved by stirring **4** and **6** in a 4/1CH_2Cl_2/TFA mixture for 3 h at 0 °C to yield compounds **5** and **7**, respectively.

Vitamin B12 compounds with different linkers, having a spacer and an additional amide group between the histidine moiety and the corrin ring, attracted our interest as well (Scheme 3). We selected 1,4-diaminobutane as the spacer and the vitamin B12 starting materials were cyanocobalamin-*b*-acid and cyanocobalamin-*c*-lactone. The former was prepared via acid hydrolysis of cyanocobalamin. The latter on the other hand is easily accessible by reaction of cyanocobalamin with *N*-chlorosuccinimide and potassium iodide in aqueous acetic acid [12].



Scheme 1. Reagents and conditions: (a) MeCN, Δ, 4.5 days; (b) 2-trimethylsilyl ethanol, MeCN, DIPEA, RT, 14 h, 53% (over 2 steps); (c) $HNEt_2$, DMF, RT, 1 h; (d) MeCN, Δ, 24 h; (e) 9-fluorenyl methanol, MeCN, DIPEA, RT, 14 h, 55% (over 2 steps); (f) TFA, CH_2Cl_2, RT, 1 h.

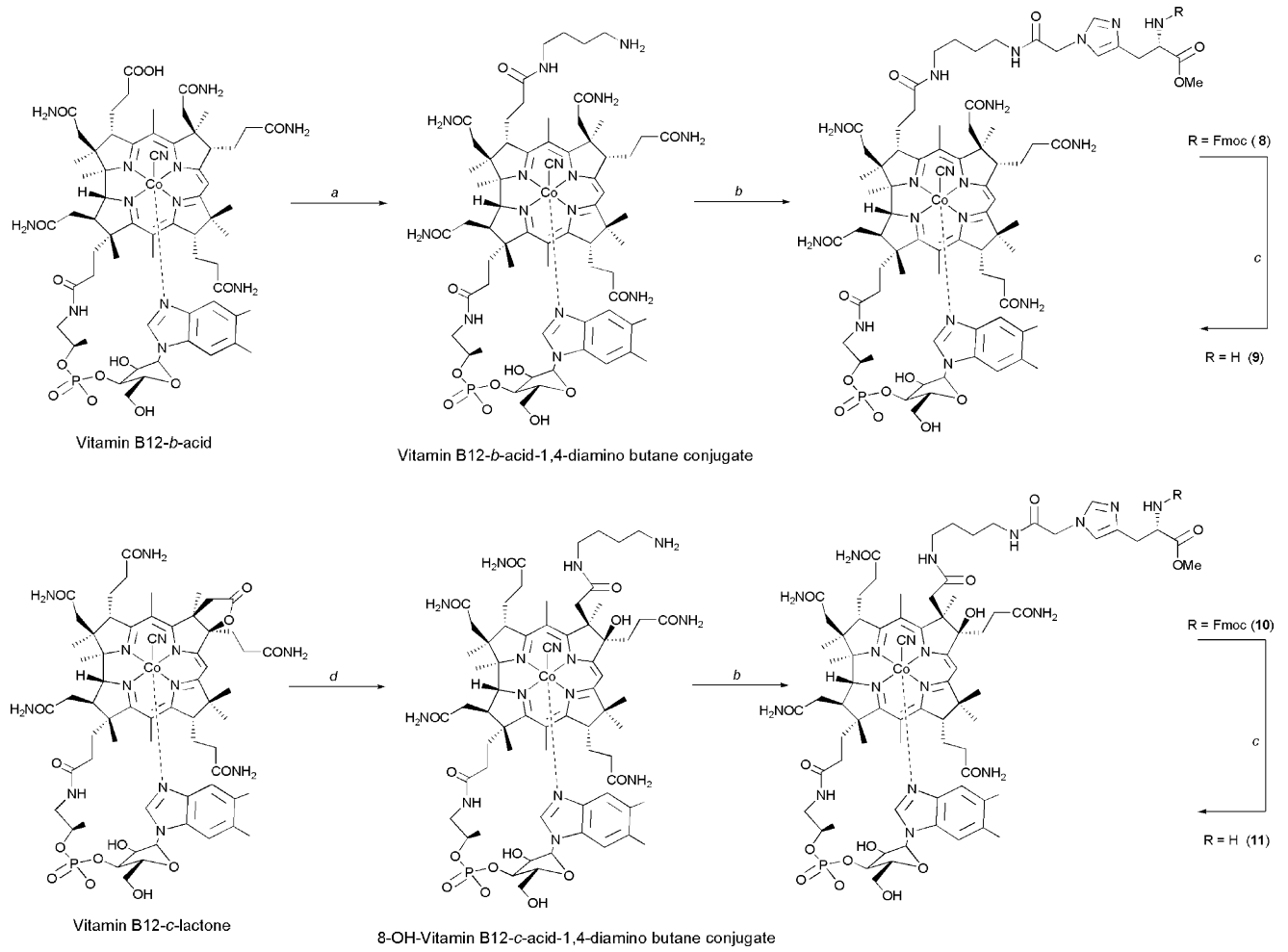


Scheme 2. Reagents and conditions: (a) **2**, TBTU, DMF, NEt_3, RT, 45 min; (b) TFA, CH_2Cl_2, 0 °C, 3 h.

In the first step of the synthesis of histidine conjugate **9**, cyanocobalamin-*b*-acid was conjugated with 1,4-diamino butane (Scheme 3) by employing 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxybenzotriazole (HOBT) as the coupling reagents according to a procedure employed by Pathare et al. [12] for the synthesis of the dodecane analogue. Subsequently the 1,4-diamino butane conjugate was reacted with **3**, employing benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the coupling reagent to afford **8**. This compound was not isolated but instead, the Fmoc protecting group was removed by the reaction with piperidine, followed by preparative HPLC purification to yield compound **9** in pure form. Conjugate **11** was prepared in three steps starting from cyanocobalamin *c*-lactone. First, the lactone was stirred in molten 1,4-diaminobutane to form 8-OH-cyanocobalamin-*c*-(4-aminobutyl)amide as described for the dodecane analogue [12]. The additional hydroxy group at the C8 position is a relict of the ring opening reaction at the lactone. In a second step, **3** was coupled to the free amino group to give compound **10** which was subsequently deprotected to give **11**.

2.2. Characterization of the cyanocobalamin conjugates

The B12-conjugates **4**–**7** and **9** were characterized by ESI-MS and by ^{1}H, ^{13}C and ^{31}P spectroscopy, **11** by ESI-MS and ^{1}H NMR. Clear evidence for the constitution of the compounds is obtained from the mass spectra. For each of the compounds the [M + H] peak as well as doubly charged ions at approximately half this value is clearly observable. Additional evidence for the proposed constitution is obtained from the ^{13}C NMR spectra. Although each compound has a large number of C-atoms, additional signals owing to the ligand frag-



Scheme 3. Reagents and conditions: (a) 1,4-diaminobutane, EDC, HOBT, DMF/H_2O, RT, 3 d, See [12]; (b) **3**, BOP, DIPEA, DMSO, RT, 16 h; (c) pipiridine, DMF, RT, 1.5 h; (d) 1,4 diaminobutane, 50 °C, 2.5 h.

ment in comparison to the starting acids can be clearly identified. The ^1H spectra are difficult to interpret since many signals overlap. However, information on the constitution of the conjugates can be extracted from the region between 8 and 6 ppm. The starting vitamin B12 derivatives only show five signals with intensity 1H in that region, whereas in the case of the histidine conjugates two additional signals, also with intensity 1H, are observed owing to the imidazole portion of histidine.

The ^1H and ^{13}C spectra of **6**, **7** and **9** and the ^1H NMR spectrum of **11** are given in the Supplementary material.

2.3. Labeling of the cyanocobalamin conjugates with $[^{99m}Tc(H_2O)_3(CO)_3]^+$

The labeling of **5**, **7** and **9** with $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was achieved under mild conditions. Reaction of a 10^{-4} M solution of these derivatives at 50 °C for 30 min afforded the $^{99m}Tc(CO)_3$ labeled conjugates at yields higher than 95%, as indicated by radiochromatographic analysis. As a representative example the labeling trace of **5** is shown in Fig. 1. The methyl ester does not affect the labeling yield. This is consistent with the results previously obtained for various other conjugates of histidine [11]. Presumably, the methyl ester is cleaved concomitantly during the labeling reaction as a result of the Lewis acidic character of the Tc(I) ion. Labeling of **11** with $[^{99m}Tc(H_2O)_3(CO)_3]^+$ proceeds in the same way but has to be performed carefully. In contrast to the other compounds, labeled **11** is not stable at elevated temperature and cleavage of the chelator from the cobalamin was observed at 70 °C. Labeling of a 10^{-4} M solution of **11** at 50 °C for 60 min, followed by HPLC purification, yielded a radioanalytically pure cobalamin conjugate suitable for animal testing.

2.4. Biodistribution studies in tumor bearing mice

To assess the potential of ^{99m}Tc labeled cobalamin derivatives for tumor targeting, we studied the biodistribution of the $^{99m}Tc(CO)_3$ conjugates of **5**, **7**, **9** and **11** in mice bearing B16-F10 melanoma tumors. ^{57}Co labeled cyanocobalamin was taken as a reference (Table 1).

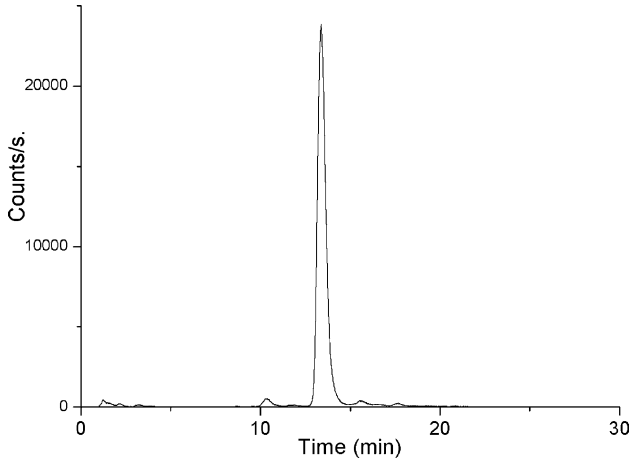


Fig. 1. Radiotrace of the labeling of **5** with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ (10^{-4} M of **5**, 30 min, 50 °C). The used gradient is defined in Section 4.

None of the derivatives shows an improved tumor uptake with respect to the parent cyanocobalamin. This does not come as a surprise, since modification of biomolecules is generally accompanied by decrease of affinity. Interesting differences in the distribution into other organs are present. The three most important organs/fluids from a radiopharmaceutical point of view are liver, kidney and blood. High liver and kidney uptake increases the dose burden to these organs and leads to a low target to non-target ratio. Therefore low kidney and liver values are crucial for a good imaging or therapeutic agent. Table 1 shows that $^{99m}Tc(CO)_3$ labeled **9** displays the lowest kidney and liver uptake, approximately half of those observed for the ^{57}Co-cyanocobalamin. Especially for imaging, for which Tc radiopharmaceuticals are used [1–5], low blood values are mandatory because high blood values will result in high background noise. It can be observed that all four conjugates display a significantly lower blood value than the parent cobalamin. Interestingly, the blood value for labeled **7** is about ten times lower than that of the other conjugates, but its values for kidney and liver uptake are much higher than that of the other.

From a radiopharmaceutical point of view, labeled **9** and **11** are the most promising candidates. However, the tumor-to-blood, tumor-to-kidney and tumor-to-liver ratios need to be improved further.

3. Conclusion

The two complementary histidine derivatives **2** and **3** are excellently suited for coupling to cyanocobalamin. The obtained conjugates can be labeled with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ under mild conditions in yields higher than 95%. The affinity of the $Tc(CO)_3$ moiety for histidine as a tripodal ligand is high and no coordination to other potential donor atoms in vitamin B12 could be observed. The biodistribution studies with tumor bearing mice showed tumor accumulation in a comparable range with parent cobalamin. At the same time relatively high kidney and liver uptake was observed as well. Currently, we are investigating other derivatives in order to reduce kidney and blood values.

4. Experimental

4.1. Materials and methods

Electrospray ionization mass spectra (ESI-MS) were recorded on a Merck–Hitachi M-8000 spectrometer in the positive ion mode using methanol as the solvent. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker DRX-500 (^{1}H at 500.25 MHz) spectrometer. ^{1}H and ^{13}C NMR spectra were referenced to TMS, using the ^{13}C or residual protio signals of the deuterated solvents as internal standards. ^{31}P NMR spectra were referenced against 0.1 M H_3PO_4 in H_2O. HPLC analyses were performed on a Merck–Hitachi L-7000 system equipped with a EG&G Berthold LB 508 radiometric detector, using Waters XTerra RP8 column (5 μm particle size, 1×100 mm) and a flow rate of either 0.5 or 1 ml/min.

Table 1
Biodistribution of mice with syngeneic B16-F10 melanoma turmors at 24 h post-injection, % injected dose per gram tissue (average of $3 \pm \sigma$)

Tissue	Compound				
	5-$^{99m}Tc(CO)_3$	**7**-$^{99m}Tc(CO)_3$	**9**-$^{99m}Tc(CO)_3$	**11**-$^{99m}Tc(CO)_3$	^{57}Co-B12
Blood	2.10 ± 0.11	0.28 ± 0.02	2.43 ± 1.01	1.27 ± 0.21	6.31 ± 0.46
Heart	3.06 ± 0.13	2.91 ± 0.32	2.94 ± 1.06	3.41 ± 0.60	6.83 ± 0.62
Spleen	6.17 ± 0.33	4.56 ± 0.27	3.59 ± 1.47	5.68 ± 0.65	7.97 ± 1.58
Kidney	19.96 ± 0.71	50.09 ± 3.69	15.76 ± 0.91	17.06 ± 1.74	28.99 ± 1.79
Stomach	14.57 ± 1.03	9.20 ± 1.05	2.59 ± 1.09	5.21 ± 1.03	8.69 ± 3.42
Intestine	3.02 ± 2.08	4.21 ± 0.77	2.82 ± 1.02	2.49 ± 0.64	5.54 ± 0.59
Liver	21.06 ± 2.38	31.14 ± 3.48	7.42 ± 1.42	14.15 ± 2.65	12.44 ± 1.35
Muscle	1.20 ± 0.05	0.85 ± 0.06	1.20 ± 0.14	1.03 ± 0.13	2.45 ± 0.01
Bone	2.21 ± 0.21	1.39 ± 0.13	1.49 ± 0.69	1.77 ± 0.29	4.25 ± 0.21
Tumor	9.23 ± 2.03	4.35 ± 0.86	7.33 ± 1.68	6.46 ± 0.89	9.86 ± 0.28

Chromatograms were recorded at 250 and 360 nm. Sodium acetate buffer "a" was prepared by mixing 2.9 ml acetic acid and 4.55 ml sodium hydroxide 2M in 900 ml water and 100 ml methanol. Buffer "b" was always methanol. Preparative HPLC separations were performed on a Varian Prostar system equipped with two Prostar 215 pumps and a Prostar 320 UV/Vis detector, using a Waters XTerra Prep RP8 column (5 μm particle size, 30 × 100 mm). The flow rate was 30 ml/min. After the preparative HPLC purification, the cobalamin derivatives were desalted by applying an aqueous solution of the compound to a Chromafix RP18ce cartridge, followed by thoroughly rinsing with water. The desalted product was then eluated with methanol, the solvent was removed in vacuo, and the product was dried at high vacuum.

4.2. Synthesis

Cyanocobalamin *b*- and *d*-acid [11–14] as well as cyanocobalamin *c*-lactone [12] were prepared according to methods published in the literature. Histidine derivatives **1**, **2** and **3** as well as cobalamin derivatives **4** and **5** were prepared as published previously [10,11]. All other used chemicals were obtained from commercial sources.

4.2.1. Teoc-protected histidine conjugate 6

Compound **3** (44 mg, 0.07 mmol) was dissolved in DMF (1.5 ml) and $HNEt_2$ (1.5 ml). The mixture was stirred for 1 h at RT. Subsequently the mixture was evaporated to dryness in vacuo. In another flask, cyanocobalamin-*b*-acid (20.0 mg, 14.8 μmol) was dissolved in 4.5 ml of a DMSO/DMF (1/5 v/v) mixture. This mixture was transferred to the flask containing the deprotected histidine derivative. Subsequently NEt_3 (1.0 ml) and TBTU (32.1 mg, 0.1 mmol) were added. The mixture was stirred for 45 min at RT, after which period it was concentrated to dryness in vacuo. Purification by preparative HPLC (C8-column; acetate buffer; gradient: 2.0% per min, starting from buffer a) afforded 14 mg (59%) of a red solid.

$C_{79}H_{114}CoN_{17}O_{18}PSi$ = 1708.9 g/mol.

^{13}C NMR (CD_3OD; 125.8 MHz): 181.7, 180.3, 177.7, 177.6, 177.5, 175.8, 175.7, 175.5, 174.7, 174.4, 174.2, 174.1, 167.2, 167.1, 158.8, 143.6, 138.4, 135.6, 134.0, 131.6, 118.6, 118.0, 112.6, 108.8, 105.3, 95.7, 88.1, 86.6, 83.8, 76.5, 75.6, 73.7, 70.9, 64.3, 62.8, 60.5, 57.8, 56.8, 55.8, 55.2, 52.8, 52.6, 50.0, 48.5, 46.9, 45.7, 43.9, 43.2, 40.2, 37.4, 36.4, 35.5, 33.6, 33.1, 32.5, 32.4, 32.1, 31.2, 29.8, 27.6, 27.6, 21.1, 20.6, 20.5, 20.5, 20.3, 20.1, 18.7, 17.6, 17.2, 16.5, 16.2, −1.3; ^{31}P NMR (CD_3OD; 202.5 Hz) 1.29. *m/z* (MeOH; ESI-pos.): 1710.3 $[M + H]^+$, 854.8 $[M + 2H]^{2+}$, 866.8 $[M + Na + H]^{2+}$.

4.2.2. Histidine conjugate 7

To a suspension of **6** (19mg, 11.1 μmol) in CH_2Cl_2 (4 ml) at 0 °C under an atmosphere of N_2 was added CF_3COOH (1 ml), resulting in a clear red solution. The mixture was stirred for: 3 h at 0 °C followed by evaporation of the solvent in vacuo. Purification by preparative HPLC (RP8 30 × 100 mm column; acetate buffer; gradient: 1.0% per min, starting from buffer a), yielded 13 mg (75%) of a red solid.

$C_{73}H_{103}CoN_{17}O_{16}P$ = 1564.6 g/mol.

^{13}C NMR (CD_3OD; 125.8 MHz): 181.8, 180.3, 177.7, 177.7, 177.5, 174.8, 174.7, 174.4, 174.1, 167.2, 167.1, 143.6, 138.9, 138.5, 137.8, 135.6, 134.1, 131.6, 119.0, 118.1, 112.6, 108.8, 105.3, 95.8, 88.1, 86.6, 83.9 (d, J_{pc} = 5.7 Hz), 76.5, 75.6, 73.8 (d, J_{ph} = 6.1 Hz), 70.9, 62.7, 60.6, 57.8, 56.8, 55.2, 55.2, 53.1, 52.6, 47.0, 45.7, 43.9, 43.2, 40.3, 37.4, 36.4, 35.6, 33.7, 33.2, 32.8, 32.5, 32.5, 32.0, 29.8, 27.7, 27.6, 21.1, 20.6, 20.6, 20.5, 20.3 (d, J_{ph} = 2.8 Hz), 17.7, 17.3, 16.5, 16.3; ^{31}P NMR (CD_3OD; 202.5 Hz) 1.29. *m/z* (ESI-pos.; MeOH): 1565.3 $[M + H]^+$, 783.2 $[M + 2H]^{2+}$, 794.3 $[M + Na + H]^{2+}$.

4.2.3. Histidine conjugate 9

A solution of cyanocobalamin-*b*-butylamine (49.6 mg, 34.8 μmol) in dry DMSO (2 ml) was added to methyl 1-carboxymethyl-*N*-Fmoc-histidinate hydrochloride (**3** · HCl; 35.5 μmol) and BOP (46.2 mg, 104.4 μmol). Diisopropyl ethyl amine (DIPEA; 12 μl, 70.0 μmol) was added, and the solution was stirred at RT for 16 h. HPLC analysis confirmed full conversion of the cobalamin starting material into the Fmoc protected intermediate. The intermediate was precipitated by adding diethyl ether, and the suspension was centrifuged and decanted three times to give a fine powder. The intermediate was dissolved in DMF (5 ml), and piperidine (225 μl) was added. After stirring at RT for 1.5 h, the product was precipitated by adding diethyl ether, and the suspension was centrifuged and decanted three times to give a fine powder. Purification by preparative HPLC (acetate system, gradient: 1% min^{-1} starting from 100% buffer a) gave the pure product **9** in a yield of 17.1 mg (32%).

$C_{76}H_{108}CoN_{18}O_{17}P$ = 1635.7 g/mol.

^{13}C NMR (CD_3OD; 125.8 MHz): 181.6,180.1, 177.8, 177.6, 176.7, 175.7, 175.7, 175.6, 175.6, 174.8, 174.7, 174.4, 169.7, 167.1, 167.0, 143.5, 139.9, 138.3, 138.1, 135.7, 134.0, 131.5, 120.1, 117.9, 117.1, 112.7, 108.7, 105.2, 95.8, 88.0, 86.5, 83.7, 76.4, 75.4, 73.8, 70.8, 62.7, 60.5, 57.4, 56.7, 55.1, 55.0, 52.9, 52.7, 50.3, 46.8, 43.8, 43.2, 40.3, 36.8, 35.4, 33.5, 33.4, 33.1, 32.9, 32.6, 32.5, 29.8, 27.9, 27.8, 27.7, 27.4, 21.2, 20.7, 20.6, 20.3, 20.0, 17.7, 17.2, 16.5, 16.3. ^{31}P NMR (CD_3OD; 202.5 Hz) 2.38. ESI-MS: *m/z* = 1535.5 $[M - CH_2 - CH(NH_2) - COOCH_3 + 2]^+$, 778.0 $[M - CH_2 - CH(NH_2) - COOCH_3 + Na]^{2+}$. UV/Vis:

λ/nm (ε/mol l^{-1} cm^{-1}) = 279.1 (19200), 361.0 (24700), 521.0 (9600), 551.1 (10700).

4.2.4. Histidine conjugate **11**

Cyanocobalamin-*c*-lactone (304.4 mg, 224.8 μmol) was stirred in melted 1,4-diaminobutane (0.7 ml, 67 mmol) at 50 °C for 2.5 h. Addition of diethyl ether (20 ml) gave an oily precipitate which was collected by centrifugation and decanting. Re-dissolving the residue in methanol and repeating of the precipitation steps gave a powdery crude product which was purified by preparative HPLC using HCl (1 mM) as buffer "a", gradient: 0.5% min^{-1} starting from 100% buffer a. to give 8-OH-cyanocobalamin-*c*-(4-aminobutyl)amide in an yield of 210.9 mg (65.0%).

$C_{67}H_{97}CoN_{15}O_{15}P$ = 1442.5 g/mol.

ESI-MS: *m*/*z* = 721.8 $[M + 2]^{2+}$, 796 $[M + 2]^{2+}$, UV/Vis: λ/nm (ε/mol l^{-1} cm^{-1}) = 280.1 (11700), 360.9 (19700), 550.0 (6600).

A solution of 8-OH-cyanocobalamin-*c*-(4-aminobutyl)amide (9.8 mg, 6.8 μmol), **3** (6.9 μmol) and BOP (8.6 mg, 20.4 μmol) in DMSO (1 ml) was reacted as described in the synthesis of **9** to give **10**, and, after Fmoc deprotection, **11**. Purification was done as described in the synthesis of **9** with the exception that no methanol was added to the acetate buffer because the product elutes very early. The pure product **11** was isolated in an yield of 3.7 mg (33%).

$C_{76}H_{108}CoN_{18}O_{18}P$ = 1651.7 g/mol.

ESI-MS: *m*/*z* = 1651.1 $[M + 1]^{+}$, 826.0 $[M + 1]^{2+}$. UV/Vis: λ/nm (ε/mol l^{-1} cm^{-1}) = 279.0 (12400), 360.0 (23500), 523.0 (6700), 552.0 (7000).

4.2.5. General procedure for labeling with $[^{99m}Tc(OH_2)_3(CO)_3]^+$

A solution of the cyanocobalamin derivatives **5**, **7**, **9** or **11** (10^{-3} or 10^{-4} M in H_2O, 20 μl) was added to a vial with a septum, which was then sealed and degassed with a stream of nitrogen gas for 10 min. A solution of $[^{99m}Tc(OH_2)_3(CO)_3]^+$ buffered at pH 7.4 (180 μl) [15,16] was added to the vial via a syringe and the vial was heated to 50 °C for 30 min to yield the $[^{99m}Tc(CO)_3]$ labeled compounds, which was demonstrated by HPLC with radioactive detection. For the analysis of labeled **5**, **7**, **9** and **11** the NaOAc buffer (described above) was used as buffer "a" with the RP8 column. The following program was used: from 0% to 30% MeOH in 15 min, then the MeOH content increased to 100% over 10 min. For the animal studies, the labeled cobalamin derivatives were separated from their unlabeled analogues via HPLC. Fractions of about 200 μl were collected and the fraction with the highest intensity was used for the biodistribution studies.

4.3. Biodistribution studies

The biodistribution of $^{99m}Tc(CO)_3$ labeled **5**, **7**, **9** and **11** as well as ^{57}Co cyanocobalamin for reference purposes was studied in mice bearing B16-F10 melanoma tumors. Female balb/c mice (10–12 weeks old), which were kept on folate and vitamin B12 deficient food, were injected subcutaneously in the flank with 10^6 B16-F10 mouse melanoma tumor cells (ATCC CRL-6475). At 2 weeks post-inoculation mice bearing, B16-F10 tumors were injected with 0.5–1 ng of the ^{99m}Tc-labeled derivative (specific activity 10 mCi/μg) or 1 ng ^{57}Co labeled vitamin B12 as control (specific activity 0.2 mCi/μg) via the tail vain. Groups of three mice per compound were sacrificed and dissected at 24 h post-injection. Organs were weighed and counted in a gamma scintillation counter. Experiments were carried out in compliance with Swiss laws related to the conduct of animal experimentation.

Acknowledgments

Christine De Pasquale is acknowledged for performing the animal distribution studies. The authors are grateful to Solidago AG for financial support.

Appendix A. Supplementary data

^1H and ^{13} NMR spectra of **6**, **7** and **9** as well as the ^1H NMR spectrum of **11** are available as supplementary material. These can be obtained from Elsevier. Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.jorganchem.2004.09.050.

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